Molecular data extend Australian *Cricotopus* midge (Chironomidae) species diversity, and provide a phylogenetic hypothesis for biogeography and freshwater monitoring

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Resolving species relationships and confirming diagnostic morphological characters for insect clades that are highly plastic, and/or include morphologically cryptic species, is crucial for both academic and applied reasons. Within the true fly (Diptera) family Chironomidae, a most ubiquitous freshwater insect group, the genera *Cricotopus* Wulp, 1874 and *Paratrichocladius* Santos-Abreu, 1918 have long been taxonomically confusing. Indeed, until recently the Australian fauna had been examined in just two unpublished theses: most species were known by informal manuscript names only, with no concept of relationships. Understanding species limits, and the associated ecology and evolution, is essential to address taxonomic sufficiency in biomonitoring surveys. Immature stages are collected routinely, but tolerance is generalized at the genus level, despite marked variation among species. Here, we explored this issue using a multilocus molecular phylogenetic approach, including the standard mitochondrial barcode region, and tested explicitly for phylogenetic signal in ecological tolerance of species. Additionally, we addressed biogeographical patterns by conducting Bayesian divergence time estimation. We sampled all but one of the now recognized Australian *Cricotopus* species and tested monophyly using representatives from other austral and Asian locations. *Cricotopus* is revealed as paraphyletic by the inclusion of a nested monophyletic *Paratrichocladius*, with in-group diversification beginning in the Eocene. Previous morphological species concepts are largely corroborated, but some additional cryptic diversity is revealed. No significant relationship was observed between the phylogenetic position of a species and its ecology, implying either that tolerance to deleterious environmental impacts is a convergent trait among many *Cricotopus* species or that sensitive and restricted taxa have diversified into more narrow niches from a widely tolerant ancestor.

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INTRODUCTION

Although central to biodiversity and evolutionary studies, systematics is relevant to applied biology (Cracraft, 2002). Questions often focus on species relationships and diagnostics (Cracraft, 2000; Walter, 2003), because
closely related species may vary broadly in their ecologies. Across the globe, freshwater biomonitoring is undertaken to infer and monitor the ‘health’ of aquatic ecosystems, relying on the accurate taxonomy of macroinvertebrates for ecosystem-level inferences via changes in species composition across space and time. The method hinges on accurately resolved species-level systematics, as decisions regarding so-called ‘indicator’ species are underpinned by an understanding of the actual biological relationships among closely allied taxa (Carew & Hoffmann, 2015).

One group of freshwater macroinvertebrates widely incorporated in biomonitoring surveys are the immature stages of the widespread and abundant non-biting midges of the family Chironomidae (Insecta: Diptera). *Cricotopus* Wulp, 1874 is a diverse and speciose genus, currently with 180 species described and with near global distribution, albeit with the greatest diversity in the Palearctic and Nearctic (Ashe & O’Connor, 2012). Although a morphological framework exists for delimiting northern hemisphere *Cricotopus* species in all life stages, austral taxa are poorly understood. Indeed, only two Australian species were assigned formally to *Cricotopus*, namely *Cricotopus albitibia* (Walker, 1848) and *Cricotopus annuliventris* (Skuse, 1889) (Freeman, 1961). *Cricotopus albitibia*, reported originally from Sierra Leone (Freeman, 1956), is widespread in sub-Saharan Africa (e.g., Scott, 1958; Hughes, 1966; Dejoux, 1983; Beneberu et al., 2014). Specimens from New South Wales (NSW), Australian Capital Territory (ACT), and Western Australia were believed to be conspecific by Freeman (1961). *Cricotopus annuliventris*, described first from the Blue Mountains of New South Wales (Freeman, 1961), is now reported from throughout NSW, ACT, Victoria, South Australia, and Tasmania (Drayson et al., 2015). Two unpublished theses extended the species diversity of the Australian *Cricotopus* fauna. Hergstrom (1974) recognized four additional species from South Australia to which she gave the manuscript names *Cricotopus albitalis*, *Cricotopus parbicinctus*, *Cricotopus hirtellus*, and *Cricotopus phaesomatus*. Subsequently, Drayson (1992) informally described nine additional species under simple ‘codes’, sampled largely from southeastern Australia. The codes, converted to prospective names by Cranston (1996), were formalized recently in a parallel study (Drayson et al., 2015). Presently, 11 species are recognized from Australia based on morphology from all life stages. Hergstrom’s *C. hirtellus* and *C. phaesomatus* remain unrecognized.

Not only has the differentiation of Australian *Cricotopus* species proven difficult, but distinguishing *Cricotopus* from its close relative *Paratrichocladius* Santo-Abreu, 1918 (also most diverse in the Palearctic and Nearctic) has proven elusive. The two genera are differentiated only by a few characters in the adult, and the close relationship between these genera has been explored in relation to designing reliable identification keys for Australian members of these genera (Drayson et al., 2015; Cranston & Krosch, 2015). Larvae of *Paratrichocladius* are essentially inseparable from those of *Cricotopus draysoni* (Cranston & Krosch, 2015), and the purported differentiation in keys to Holarctic genera (Cranston, Oliver & Sæther, 1983; Andersen et al. 2013) is unreliable. Moreover, the Australian *Paratrichocladius* pupae sampled did not conform to the keys for the Holarctic genera of Coffman et al. (1986; see Cranston & Krosch, 2015). Molecular phylogenetic analysis across global Chironomidae suggested that *Paratrichocladius* nested within a paraphyletic *Cricotopus* (Cranston, Hardy & Morse, 2012). Molecular analysis of Australian taxa (Cranston & Krosch, 2015) inferred close affinity between two Australian *Paratrichocladius* species and a *C. albitibia* group. The implied genus-level paraphyly indicates systematic relationships between Australian members of both genera need resolution, with increased sampling depth and breadth.

Given that the ecology, habitat preference, and enrichment tolerance of *Cricotopus* species vary widely across the genus, this group represents an ideal system for exploring the conservatism/convergence of such ecological traits. For example, some species inhabit inner-city fountains in Europe (Hamerlik & Brodersen, 2010), some are rice crop pests in mainland Asia (Ree & Kim, 1998), Europe (Ferrarese, 1992), and Australia (Stevens, Helliwell & Cranston, 2006), others are highly resistant to industrial run-off (Surber, 1959), and some have mutualistic relationships with the toxic cyanophyte *Nostoc* (Brock, 1960). Members of *Cricotopus* are known also from a wide variety of habitat types in Australia, ranging from highly degraded coastal streams and manmade urban drainages to pristine streams in upland tropical rainforest (Drayson, 1992; McKie, Pearson & Cranston, 2005; Haase & Nolte, 2008; Drayson et al., 2015). Interestingly, there is much variation among species such that some species are widespread and largely tolerant of impacted environments (e.g., *Cricotopus albitalis* Drayson, Cranston & Krosch, 2015; *Cricotopus parbicinctus* Drayson, Cranston & Krosch, 2015, *C. draysoni*), others are widespread, but apparently sensitive to deleterious impacts (e.g., *Cricotopus concornis* Drayson & Cranston, 2015; *Cricotopus hillmani* Drayson & Cranston, 2015; *Cricotopus annuliventris*, *Cricotopus tasmania* Drayson & Cranston, 2015), whereas others are both highly sensitive and geographically restricted (e.g., *Cricotopus acornis* Cranston & Krosch, 2015; *Cricotopus houensis* Cranston & Krosch, 2015; *Cricotopus varicornis* Cranston & Krosch, 2015; *Cricotopus wangi* Cranston & Krosch, 2015) (Nolte & Haase, 2001; Drayson et al., 2015). The in-stream microhabitat used by
Crictotopus also varies among species, from sand beds to submerged vegetation, and algal and cyanophyte colonies.

Despite varied life-history traits within *Crictotopus*, biomonitoring surveys frequently equate the presence of any *Crictotopus* as an indicator of negative impact (Dimitriadis & Cranston, 2007). Such generalizations that all members of the genus are highly tolerant of deleterious stream impact arises from our previously poor understanding of larval morphology among many Australian *Crictotopus* species (Carew, Pettigrove & Hoffmann, 2003), as is the case elsewhere in the global distribution of the genus (e.g. North America; Sinclair & Gresens, 2008; Gresens, Stur & Ekrem, 2012). From a biomonitoring perspective, important information about stream health may be overlooked because accurate identification is elusive, especially of larval taxa, and ‘lumping’ all taxa together potentially homogenizes important differences among species. Testing morphological hypotheses of species boundaries with molecular data is a crucial first step in resolving these issues (e.g. Brodin et al., 2013).

We aimed to resolve the identity and relationships among the Australian *Crictotopus* species by inferring a molecular phylogeny for the Australian *Crictotopus* and selected members of *Paratrichocladius*. Specifically, we assess the morphological species concepts of Drayson et al. (2015), and the monophyly of *Crictotopus* and *Paratrichocladius* (Cranston et al., 2012; Cranston & Krosch, 2015, in press). Representatives from other austral locations (South Africa, New Zealand, and Chile) were incorporated to test the monophyly of the Australian fauna. Molecular associations between immature and adult life stages complement individual rearings: we thus test diagnostic morphological characters from the larvae and pupae in species identification. Furthermore, we explore trends in the phylogeny associated with ecological tolerances of species to assess evolution of pollution tolerance. Such an approach allows us to assess the conservatism of ecological tolerances among species and determine the suitability for a predictive framework for biomonitoring programmes. Our wide taxonomic and geographical sampling allows for the exploration of biogeographical patterns that may have important implications for understanding the evolution of impact tolerance in the Australian *Crictotopus*. As such, a timeline for diversification within the Australian *Crictotopus* was estimated, allowing for the assessment of important events that may have influenced species divergence. Taken together, the resolution and clarification of the interspecific systematics for Australian *Crictotopus*, and the tolerance to adverse stream conditions of each species, may reveal pollution tolerance in macroinvertebrates in general. Our research may undermine broad generalizations regarding pollution tolerance in other taxa and improve our understanding of ecological responses in Australian freshwater taxa.

**MATERIAL AND METHODS**

**SPECIMEN COLLECTION AND HANDLING**

Members of *Crictotopus* are holometabolous, with aquatic immature stages. Although species in the Holarctic inhabit both lotic and lentic environments, the Australian fauna is known predominately from running water. Our sampling was thus limited to lotic habitats, and we sought any and all life-history stages of *Crictotopus* from across Australia to enable molecular associations among life stages without rearing. Mostly we used aquatic dip nets (mesh size 300 μm) enhanced by kick sampling, disturbing depositional areas, washing immersed logs, and sieving snagged leaves. Dip nets were sometimes located to intercept the natural downstream drift of larvae, pupae, and pupal skins in the flowing water. Specimens were obtained from colleagues across Australia to be included in the phylogenetic analysis, including from Victoria, southwestern Queensland, and South Australia, along with Singaporean representatives and South African *Paratrichocladius*. Our study included specimens taken from archived samples from governmental freshwater monitoring and from private collections, but DNA extraction for these was unsuccessful (presumably as a result of sample storage techniques). Ultimately, this has resulted in some sampling gaps, especially concerning Western Australia; however, we do not believe that these represent a significant shortcomings of this study. All species recorded from these regions are included from other localities, and we have no morphological evidence for additional unsampled diversity from these regions.

All freshly collected bulk samples were preserved in isopropanol and sorted following the procedures described by Krosch & Cranston (2013), with specimens preserved in fresh, high-quality 100% isopropanol. Pupal exuviae (cast skins) were preserved in isopropanol both for subsequent morphological taxonomic studies and for DNA extraction using recently developed techniques that retain a morphological voucher for each extracted exuviae (Krosch & Cranston, 2012). The recognition of larval morphological features requires microscope slide preparation to examine head capsule morphology. All individual dissected heads were placed in Hoyer’s mountant, used for ease of preparation and for its excellent optical qualities for digital photography, despite issues of impermanence. All dissected heads were photographed at Queensland University of Technology (QUT) by M.N.K. prior to the validation of identity by M.N.K. and P.S.C. Larval bodies, related individually and uniquely to each vouched larval head,
were prepared for molecular study as described below. Subsequently, taxonomically critical vouchers were soaked from the Hoyer's mounts in water, desiccated in isopropanol, and remounted into Euparal mountant for permanent storage. All molecular voucher material is deposited in the Australian National Insect Collection (ANIC), CSIRO Ecosystem Sciences, Canberra, Australia.

We included representatives of five out-group taxa: Parapsectrocladius acuminatus (Edwards 1931), Orthocladius/Euorthocladius luteipes Goetghebuer 1938, and Synorthocladius sp. as distant out-group taxa; and two representatives of Rheocricotopus sp. Sequence data for all out-group taxa derive from Cranston et al. (2012), except for a single Australian Rheocricotopus larva that was collected and sequenced here. Members of the purported sister taxon of Cricotopus, Paratrichocladius [specifically Paratrichocladius micans (Kieffer, 1918)], were acquired from South Africa via colleagues, and Cricotopus specimens from outside Australia were sourced as fresh material from colleagues (Cricotopus flavozonatus Freeman, 1953 and C. albithorax) or from existing collections held by M.N.K. (New Zealand), and as DNA sequences from Cranston et al. (2012) (North America and Africa) and Krosch et al. (2011) (Chile).

**DNA EXTRACTION, POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION, AND SEQUENCING**

Total genomic DNA was extracted using the ISOLATE II Genomic DNA Kit (Bioline, Alexandria, Australia) following the protocol recommended by the manufacturer. Modifications to the protocol to enhance extraction from individual pupal exuviae follow Krosch & Cranston (2012). One region of the nuclear rRNA gene 28S, two regions of the nuclear protein-coding gene CAD (CAD1 and CAD3), and two non-overlapping regions of the mitochondrial protein-coding gene cytochrome c oxidase subunit I (COI and FolCOI) were amplified. These three genes, which evolve at different rates, providing different levels of information across the tree, have become standard molecular markers for much of Dipteran systematics. Primers, reaction protocols, and cycle programmes used for amplification of 28S, CAD1, CAD3, and COI can be found in Krosch et al. (2011), and for FolCOI in Krosch et al. (2009). Amplification products were purified using an ISOLATE PCR and Gel Kit® (Bioline), following the manufacturer’s instructions. The direct sequencing of PCR products was performed using ABI Big Dye® Terminator 3.1, and was carried out in an ABI 3500 Capillary Electrophoresis Genetic Analyser at the Molecular Genetics Research Facility (QUT, Brisbane, Australia). All sequences were lodged with GenBank (accession numbers KP954743–KP955399).

**DATA ANALYSES**

Potential heterozygous sites in nuclear sequence data were recognized by double peaks in sequence chromatograms and were coded as ambiguous bases according to IUPAC codes. COI and CAD sequences were compiled and edited by eye using BioEdit 3.0.9 (Hall, 1999), whereas 28S sequences were aligned initially by eye and refined using MUSCLE 3.7 (Edgar, 2004). Fifteen alignment gaps were recognized among 28S sequences and ten of these were excised after analysis using GBlocks 0.91b (Castresana, 2000) with default settings, and the remaining alignment gaps were treated as missing data.

Sequences were concatenated and individual partitions assigned for each locus, thus resulting in three partitions. Phylogenies were inferred for single-locus data sets and for two concatenated partitioned data sets: one with maximized taxon sampling (referred to hereafter as ‘total’); and one reduced to remove the redundancy of multiple specimens of species from the same location, and with molecular coverage maximized (referred to hereafter as ‘reduced’). Bayesian phylogenetic inference was performed in MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) under the general time-reversible (GTR) model of sequence evolution applied to each partition individually, and incorporating a gamma distribution of nucleotide frequencies. Two simultaneous runs of four chains (two cold and two hot) were performed for 5–50 million generations, depending on the data set. Runs were sampled every 1000 generations, with 25% of the total samples removed as burn-in, and convergence was maximized by ensuring the standard deviation of split frequencies fell below 0.01 and potential scale reduction factors approached 1.0 for all parameters. Maximum-likelihood (1000 bootstraps) reconstruction was performed using RAXML 8.0.24 (Stamatakis, 2006) under the GTR+Γ model of sequence evolution. All analyses were conducted on the CIPRES Science Gateway High Performance Computing platform (http://www.phylo.org; Miller, Pfeiffer & Schwartz, 2010).

The molecular data set was trimmed to single representatives of each species to allow for the estimation of time to most recent common ancestor (tmrca) in BEAST 1.8.1 (Drummond et al., 2012). A soft calibration was placed on the root height of the tree according to estimated divergence times for the node that connected all members of the tribe ‘Orthocladiini’, as reported in the molecular analysis of Cranston et al. (2012): we used a normal prior with a mean of 80 Myr and a standard deviation of 15, such that the 97.5% highest posterior density (HPD) encompassed the error range of the age estimate of Cranston et al. (2012). An additional internal calibration was applied to the node connecting all sampled Cricotopus specimens to...
Orthocladius/Euorthocladius luteipes, which corresponded to the oldest known stem fossil Cricotopus from Baltic amber (Meunier, 1904), using the following parameters: a lognormal distribution with a zero offset of 33 Myr that corresponded to the lower age estimate of the fossil, a mean of 20 Myr, and a standard deviation of 0.5 Myr, such that the area of greatest probability covered the age range reported in Meunier (1904) and the 97.5% HPD provided sufficient uncertainty at the tail of the distribution. We used a mixed Hasegawa, Kishino, and Yano (HKY) model of evolution to minimize issues of over-parameterization, the ‘Speciation: Yule Process’ tree prior, and allowed substitution rates to vary across branches in accordance with an uncorrelated relaxed lognormal molecular clock prior (Drummond et al., 2006). Separate BEAST analyses were performed using only the fossil-derived internal calibration; these produced similar results to analyses with both calibration priors, but with greater error around the tmrca estimates. Thus, the inclusion of the molecular-derived internal calibration appeared not to have influenced divergence time estimates adversely. The convergence of runs was assessed by viewing log files in TRACER 1.5 (Rambaut & Drummond, 2009) and ensuring that estimated sample sizes (ESSs) were greater than 200 (Drummond & Rambaut, 2007). Four runs of 50 million generations were performed, sampled every 1000 generations, from which 5 million generations were removed as burn-in from each run prior to combining log files in LogCombiner 1.8.1 (Drummond et al., 2012), producing a total run of 180 million generations. Chronograms were produced from the stationary distribution by removing 10% of trees from each run as burn-in prior to combining tree files from each run in LogCombiner, and annotating in TreeAnnotator 1.8.1 (Drummond et al., 2012).

We used the BEAST chronogram to test for phylogenetic signal in ecological traits of species. Terminals were pruned to single representatives of the Australian Cricotopus species and discrete traits coded for impact tolerance (‘tolerant’ or ‘sensitive’), distributional range (‘widespread’ or ‘restricted’), and the two traits combined (‘sensitive + restricted’, ‘sensitive + widespread’, or ‘tolerant + widespread’). To account for uncertainty in coding traits for observed divergent forms of some species (Drayson et al., 2015), we ran the analysis in three different ways: (1) traits for divergent forms coded as for the parent taxon; (2) with traits for divergent forms coded based on assumptions about ecology and distribution made from their current sampled locations; and (3) with divergent forms removed altogether. We used Pagel’s $\lambda$ (Pagel, 1999) to assess phylogenetic signal in the original tree and compared this with the null hypothesis of a tree transformed to a basal polytomy by conducting likelihood ratio tests under a chi-squared distribution. All analyses were conducted in RStudio (http://www.rstudio.com) and used functions from the APE (Paradis, Claude & Strimmer, 2004) and GEIGER (Harmon et al., 2008) packages.

RESULTS

We vouchered and identified Cricotopus representatives from approximately 133 locations across Australia (except Western Australia), including Tasmania and Lord Howe Island. In total, in excess of 200 Cricotopus individuals were sequenced for COI, FolCOI, and 28S (but not always with all three fragments for each individual), along with 63 for CAD1 and 31 for CAD3. Individual data sets were condensed and concatenated to a ‘total’ data set comprising 200 individuals from 75 sites and a ‘reduced’ data set of 150 individuals (see Table S1 for loci sequenced per individual). We maximized sequencing coverage taxonomically and geographically whilst ensuring every included specimen possessed at least COI + 28S data, such that all major clades possess some individuals for which all five loci were sequenced. Details of all specimens, codes, collection locations, dates, collectors, life stage, and GenBank accession numbers are listed in Table S1. Many specimens were larvae unconnected to a mature specimen by live rearing, but some pupae and adults were included. The exclusion of hypervariable regions resulted in a final multilocus data set of 3463 characters. Lengths by locus were: COI, 1359 bp; 28S, 577 bp; CAD, 1527 bp.

Topologies were roughly concordant among methods of reconstruction, with no notable discrepancies in taxon groupings. Single locus trees produced good tip resolution, grouping members of each major clade, but deeper nodes were unresolved (data not shown). The concatenated partitioned data set produced topologies that were generally well supported across the tree for both the reduced (Figs 1, S1) and total (Fig. S2) data sets. We refer only to the topology estimated for the reduced dataset hereafter, except where stated explicitly otherwise.

PHYLOGENY OF CRICOTOPUS

Cricotopus was recovered as paraphyletic (Fig. 1: node A; posterior probability, PP 1.00; bootstrap, BS 81), with a monophyletic Paratrichocladius (node K; PP 1.00; BS 100) nested within it and sister to the C. albitibia group (C. albitibia + C. wangi + extra-Australian C. albitibia: node N; PP 1.00; BS 100), as described by node C (PP 1.00; BS 69). All other Cricotopus species, including representatives from non-Australian locations, formed a clade that was reciprocally monophyletic to what we term the C. albitibia group + Paratrichocladius clade (node B; PP 1.00; BS 79). Within the C. albitibia group,
Figure 1. Schematic phylogeny based on a majority rule consensus maximum-likelihood (ML) topology for the reduced data set. Relevant nodal support values are shown, which correspond to Bayesian posterior probabilities and ML bootstrap support, respectively; –, nodes unresolved by ML; *, posterior probabilities of 1.00 or bootstrap support of 100. Thick branches denote Australian clades; thin branches are non-Australian taxa. Geographical distributions and tolerances to ecosystem impact for Australian Cricotopus species are shown according to the inset legend, with the forms of divergent species coded based on assumptions made from the current sampled localities.
South African *C. albitibia* is sister to the remainder of the group, with the Singaporean species a poorly supported sister to Australian *C. albitarsis* + *C. wangi*. A divergent genetic form of *C. albitarsis* represented by a single larva from a site in the Northern Territory (NT), referred to hereafter as divergent NT *C. albitarsis*, was supported strongly as sister to remaining *C. albitarsis* + *C. wangi* (node V; PP 1.00; BS 94). Although extra-Australian members of the *C. albitibia* group subtended *C. albitarsis* + *C. wangi*, non-Australian *Cricotopus* placed in the remaining clade of solely *Cricotopus* taxa were scattered throughout. Within this clade, the sister pair of an undetermined North American species (CAM04) + South African *C. flavozonatus* were sister group to the remaining taxa, with *C. draysoni* subsequently defined as sister taxon to the remainder, although this relationship was poorly supported using both methods and data sets (node D; PP 0.75; BS –). *Cricotopus parbicinctus* and a genetically and morphologically divergent larval form of this species (Drayson *et al.*, 2015) clustered with strong support (node H; PP 1.00; BS 99), and these were sister to the remainder of the broader clade. The New Zealand representative was sister to the rest of this group (node F; PP 0.94; BS 69), and the remaining taxa split into two reciprocally monophyletic groups at node G (PP 1.00; BS 90). *Cricotopus tasmania*, *C. annuliventris*, and its divergent genetic form made up one of these monophyletic groups (node O; PP 1.00; BS 100), with the Chilean representative (CH6-3), *C. conicornis*, *C. varicornis* (both *varicornis* and *cooki* forms), *C. houvensis*, and *C. hillmani* forming the other clade, although with low support using the reduced data set (Fig. 1: node I; PP 0.71; BS 73), relative to the total data set (Fig. S1: PP 0.93; BS 78).

Within this clade, the Chilean taxon was sister to a monophyletic clade of Australian species, with *C. houvensis* and *C. hillmani* being well-supported sister taxa (node W; PP 1.00; BS 100). *Cricotopus hillmani* exhibited deep structure across Bass Strait, with mainland and Tasmanian representatives forming reciprocally monophyletic clades. Deep subdivisions were apparent within *C. conicornis*, apparently associated with geographical distance, with two subclades comprising individuals from far north Queensland and Tasmania, respectively (node U; PP 1.00; BS 100), along with structuring among locations within Tasmania. The two morphological forms of *C. varicornis*, *varicornis* and *cooki* (Drayson *et al.*, 2015), were weakly supported as sister taxa (node T; PP 0.67; BS 66), and appeared genetically divergent; however, the *varicornis* form was represented by a single specimen. *Cricotopus varicornis* was sampled from just two locations: the *varicornis* form from a pristine montane stream in Kosciuszko National Park in New South Wales, and the *cooki* form from a lowland, sandy, open river in the Australian Capital Territory.

**Phylogenetic correlation of ecological tolerances among species**

Visually, little correlation is apparent between a species phylogenetic position and its ecological tolerance: species sharing ecological affinities did not form reciprocally monophyletic groups to the exclusion of species with different affinities (Fig. 1). Formal tests of phylogenetic signal in ecological traits supported this interpretation and were non-significant across all tests (Table 1). *Cricotopus wangi*, known only from pristine waterfall faces and some fast-flowing riffles in conservation areas, was nested within the *C. albitarsis* group of otherwise highly tolerant and widespread species, including the Australian *C. albitarsis* sampled from urban drainages (e.g. Dandenong Creek, Victoria, Australia) and less impacted coastal streams (e.g. Nerang River, Queensland, Australia). *Cricotopus draysoni*, another apparently tolerant and widespread species that was sampled from sites of varying degrees of impact, was placed within a clade comprising species of varying tolerances and distributional ranges. *Cricotopus parbicinctus* was collected from a similarly wide range of sites and is considered tolerant to many forms of deleterious stream impact, but its divergent sister form was recovered largely from pristine upland sites. In contrast, the clade described by node G comprises many species of similar ecological tolerances. The sister taxa *C. annuliventris* and *C. tasmania* are both fairly widely distributed and apparently sensitive to disturbance. Equally, the geographically widespread but sensitive species *C. conicornis* and *C. hillmani* were nested within a clade that also contained the geographically localized and ecologically sensitive taxa *C. varicornis* and *C. houvensis*.

**Tempo of diversification of Cricotopus**

Estimates of tmrca suggest that the diversification of the sampled in-group taxa was initiated in the early Eocene, around 52 Mya (36–70 Mya; Fig. 2; Table 2). The sampled *Paratrichocladius* diverged from the *C. albitibia* group in the mid-Eocene (45 Mya, 30–61 Mya), near simultaneously with the diversification of the broader *Cricotopus* clade (47 Mya, 32–64 Mya). *Paratrichocladius* shared a common ancestor around 25 Mya (15–36 Mya), and African *P. micans* of the genus diverged from the eastern Australian *Paratrichocladius* species around 13 Mya (6–20 Mya). The *C. albitibia* group did not begin diversifying until around 18 Mya (10–28 Mya), with the divergence of the African and then the Singaporean members (around 14 Mya, 7–22 Mya) from the Australian taxa. *Cricotopus albitarsis* and *C. wangi* diverged around 6 Mya (3–10 Mya). The divergence of all other Australian species proceeded from around 38 Mya (26–52 Mya), with the divergence of the ancestor of *C. draysoni* followed closely.
by the divergence of ancestral *C. parbicinctus* at 33 Mya (22–46 Mya). The divergence of New Zealand and Chilean representatives from Australian taxa occurred from approximately 28 Mya (19–39 Mya). Events involving Tasmanian and mainland representatives varied in age: largely mainland *C. annuliventris* diverged from predominately Tasmanian *C. tasmania* around 14 Mya (7–22 Mya), Tasmanian and north Queensland members of *C. conicornis* diverged approximately 9 Mya (3–16 Mya), and Tasmanian and mainland *C. parbicinctus* and *C. hillmani* diverged around 4–5 Mya (2–8 Mya). The Lord Howe Island endemic *C. howensis* shared a common ancestor with all *C. hillmani* around 6 Mya (3–11 Mya). The two divergent forms of *C. varicornis* diverged approximately 11 Mya (5–18 Mya).

**DISCUSSION**

Species concepts and relationships in the Australian *Cricotopus* fauna have proven troublesome ever since Freeman's (1961) revision of *C. albitibia* and *C. annuliventris*, and the unpublished theses of Hergstrom (1974) and Drayson (1992). In the decades since, *Cricotopus* has been sampled widely as part of freshwater biomonitoring programmes and other aquatic surveys, many of which have recognized additional putative morphospecies not covered by the keys available at the time (e.g. Nolte & Haase, 2001; M. Carew, pers. comm. 2013). Furthermore, the lack of formal descriptions for most of the morphospecies recognized by Drayson (1992) meant that many programmes did not or could not identify taxa below genus level. This project has largely confirmed the morphological species concepts of Drayson et al. (2015) by analysing molecular sequence data from all life stages in a phylogenetic context, and resolved evolutionary relationships among Australian *Cricotopus* and *Paratrichocladius* species.

**SYSTEMATICS OF CRICOTOPUS AND PARATRICHOCLADIUS**

The molecular phylogeny presented here and the morphological assignments of individuals to species were in close agreement, a trend that is well supported in chironomid research (e.g. Cranston et al., 2010; Krosch et al., 2011). Only three genetically divergent taxa were observed that did not fit the previous keys, represented by nine individuals within the total molecular data set of 200 individuals (although three additional individuals from the divergent form of *C. annuliventris* were sequenced for COI, but could not be included in the phylogeny). This is despite much broader geographical sampling in this study relative to
Figure 2. BEAST chronogram from a data set of single representatives per species/clade that corresponds with Table 1. Lettered nodes are those for which time to most recent common ancestor (tmrca) was estimated and correspond with Table 1; black stars indicate nodes to which prior calibrations were applied. The time scale is in millions of years before present.
Moreover, close examination of the divergent forms of assessed morphological diagnostics are largely reliable. Previous work, and demonstrates that recently reasessed morphological diagnostics are largely reliable. Moreover, close examination of the divergent forms of both *C. parbicinctus* and *C. annuliventris* have revealed subtle morphological differences that have since been integrated into a revised key (Drayson et al., 2015). Although we accept that these taxa may represent additional ‘cryptic’ species of *Cricotopus*, too many lacunae (e.g. all were represented by larvae only) currently exist to justify formal description.

Table 2. Times to most recent common ancestor, estimated using BEAST

<table>
<thead>
<tr>
<th>Node</th>
<th>Mean (Myr)</th>
<th>95% HPD range (Myr)</th>
<th>Effective sample size (ESS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>52</td>
<td>36–70</td>
<td>8165</td>
</tr>
<tr>
<td>B</td>
<td>47</td>
<td>32–64</td>
<td>7853</td>
</tr>
<tr>
<td>C</td>
<td>45</td>
<td>30–61</td>
<td>7951</td>
</tr>
<tr>
<td>D</td>
<td>43</td>
<td>30–59</td>
<td>7633</td>
</tr>
<tr>
<td>E</td>
<td>38</td>
<td>26–52</td>
<td>5986</td>
</tr>
<tr>
<td>F</td>
<td>33</td>
<td>22–46</td>
<td>4896</td>
</tr>
<tr>
<td>G</td>
<td>28</td>
<td>19–39</td>
<td>4169</td>
</tr>
<tr>
<td>H</td>
<td>27</td>
<td>13–41</td>
<td>4872</td>
</tr>
<tr>
<td>I</td>
<td>26</td>
<td>16–36</td>
<td>3401</td>
</tr>
<tr>
<td>J</td>
<td>26</td>
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<td>5471</td>
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<tr>
<td>K</td>
<td>25</td>
<td>15–36</td>
<td>3139</td>
</tr>
<tr>
<td>L</td>
<td>21</td>
<td>12–31</td>
<td>2714</td>
</tr>
<tr>
<td>M</td>
<td>19</td>
<td>11–28</td>
<td>1948</td>
</tr>
<tr>
<td>N</td>
<td>18</td>
<td>10–28</td>
<td>1931</td>
</tr>
<tr>
<td>O</td>
<td>18</td>
<td>10–27</td>
<td>2455</td>
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<tr>
<td>P</td>
<td>14</td>
<td>7–22</td>
<td>2413</td>
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<tr>
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<td>7–22</td>
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<td>13</td>
<td>8–20</td>
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<td>13</td>
<td>6–20</td>
<td>1774</td>
</tr>
<tr>
<td>T</td>
<td>11</td>
<td>5–18</td>
<td>1528</td>
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<tr>
<td>U</td>
<td>9</td>
<td>3–16</td>
<td>1203</td>
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<tr>
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<td>2171</td>
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<td>3–11</td>
<td>1222</td>
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<td>5</td>
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</tr>
<tr>
<td>Z</td>
<td>4</td>
<td>2–7</td>
<td>2700</td>
</tr>
</tbody>
</table>

HPD, highest posterior density.

The strongly supported sister relationship between *Paratrichocladius* and the *C. albittibia* group was somewhat unexpected, given the larval morphology; however, given the inclusion of African *Paratrichocladius* and both African and Asian *C. albittibia* we believe the result is sound. Future studies would benefit from the inclusion of Holarctic members of both genera, as this is widely acknowledged as the centre of diversity for both genera.

**Biogeography and ecology of Australian Cricotopus**

The non-monophyly of the Australian *Cricotopus* fauna with regard to species from elsewhere in Asia and the austral region clearly implies a history of multiple colonization events into and possibly out of Australia. The recognized centre of extant diversity for both *Cricotopus* and *Paratrichocladius* is in the Palaeartic, and this is assumed to represent a centre of origin for both groups. Given this assumption, the nested and scattered phylogenetic positions of select specimens from Singapore, Chile, New Zealand, USA, and South Africa within clades of Australian taxa suggests that Australian *Cricotopus* are not likely to share a single common origin on the continent. Instead, this pattern suggests a history characterized by waves of colonization into and dispersal out of Australia. Determining the directionality for these migrations is fraught with difficulty under the current sampling strategy, requiring instead intensive and targeted sampling from non-Australian locations, and so lies outside the scope of this discussion. Nevertheless, divergence events involving other austral landmasses do not largely accord with the classic geological timeline of continental breakup, especially for African *C. flavozonatus* (47 Mya) and *C. albittibia* (18 Mya); however, the estimated divergence of the Chilean species at around 26 Mya falls at the lower end of the age estimates for trans-Antarctic connections between Australia and South America via the Drake Passage (Barker & Burrell, 1977), and divergence of New Zealand taxa around 28 Mya accords with much recent evidence for the post-Gondwanan mid–late Palaeogene divergence of many New Zealand groups (e.g. Krosch & Cranston, 2013; Buckley, Krosch & Leschen, 2014). Discordance between austral divergence events and Gondwanan fragmentation is not surprising, given this genus is considered to have originated in the Eurasian region and is not considered a Gondwanan relic (as opposed to true austral chironomid groups that have received attention in recent years; Cranston et al., 2010; Krosch et al.,
and diverged in allopatry across Bass Strait is also
ential dispersal among congeners that were isolated
vergences of Late Miocene–Pliocene age. This suggests
nian populations as reciprocally monophyletic, with di-
C. parbicinctus

Within the Australian Cricotopus, our sampling stra-
yeg allowed us to estimate divergence times of taxa
on certain Australian islands, namely Tasmania and
Lord Howe Island. Both islands were proposed to possess
endemic species not found on the mainland (C. tas-
mania and C. howensis, respectively; Drayson, 1992;
Cranston, 1996); however, we have shown clearly that
C. tasmania occurs also on mainland Australia, albeit
in apparently lower abundance. The non-monophy-
of island versus mainland specimens within C. tas-
mania suggests that this taxon may well have diver-
sified in isolation on the island prior to colonizing the
mainland. Likewise, its sister taxon, C. annuliventris,
is known mostly from the mainland but also from a
small number of locations in Tasmania, and exhibits
a similar phylogenetic pattern to C. tasmania, which
suggests that this species may have colonized Tasma-
nia following divergence. The divergence of the two
species around 14 Mya obviously pre-dates the most
recent closure of Bass Strait at the end of the last glacial
cycle (~18 000 years ago; Lambeck & Chappell, 2001),
along with Pleistocene divergences reported for some
other taxonomic groups (e.g. Toon et al., 2007; Gongora
et al., 2012; Martin & Zuccarello, 2012), but accords
with other molecular phylogenetic studies that report
Miocene–Pliocene divergences between mainland and
Tasmanian taxa (e.g. Waters & White, 1997; Symula,
Keogh & Cannatella, 2008). Thus, we argue that these
taxa diverged in allopatry, separated by the proto-
Bass Strait, and have both since dispersed across an
intermittent sea barrier to colonize the opposite land-
mass. Whether this occurred via direct transoceanic
dispersal, by island-hopping across the Bass Strait, or
during periods of exposure of the Bassian Isthmus
landbridge remains speculative. Nevertheless, there are
apparent precedents for dispersal between geographi-
cally distant austral landmasses in other chironomid
groups (Krosch et al., 2011; Krosch & Cranston, 2013).
Somewhat in contrast, phylogenetic relationships within
other Cricotopus species for which we have Tasma-
nian representatives (C. concornis, C. hillmani, and
C. parbicinctus) all supported mainland and Tasman-
ian populations as reciprocally monophyletic, with di-
vergences of Late Miocene–Pliocene age. This suggests
that populations of these three species were isolated
across the proto-Bass Strait and diverged in allopatry,
but have not dispersed across the strait since. Differ-
etial dispersal among congeners that were isolated
and diverged in allopatry across Bass Strait is also
known from terrestrial mammals (Antechinus; T.
Mutton, pers. comm., 2014), and highlights the bio-
geographical complexity of the region.

In contrast, the estimated divergence time for the
Lord Howe Island endemic C. howensis from its sister
mainland species C. hillmani of around 6 Mya accords
closely with the geological time frame for the emer-
gence of Lord Howe Island (6.4–6.9 Mya; McDougall,
Embleton & Stone, 1981) as a result of volcanism along
the Lord Howe Rise. This accords with molecular
phylogenetic information from other freshwater inver-
tebrates (Page et al., 2005), but contrasts with other
much older Lord Howe Island endemic insect taxa (e.g.
Buckley, Attanayake & Bradler, 2009). No informa-
tion exists concerning the Cricotopus faunas of other
nearby South Pacific Islands (e.g. Norfolk Island), so
it remains unknown whether the arrival of Cricotopus
on Lord Howe Island was facilitated by island hopping
via extant or now-submerged land areas or by transo-
ceanic dispersal, possibly mediated by West Wind Drift
(Cook & Crisp, 2005). Interestingly, there does not seem
to be a close relationship between C. howensis and our
New Zealand representative, given the many in-
stances of apparent colonization of New Zealand via
the Lord Howe Rise during the Eocene–Oligocene,
around the time at which we estimate the New Zealand
taxon to have diverged (node F); however, greater sam-
ping of New Zealand’s Cricotopus diversity is needed
to resolve this question fully.

The C. albitibia group also presents an interesting
biogeographical and ecological comundrum. Cricotopus
albitarsis and C. wangi are clearly close genetically (al-
though the equivocal position of the genetically diver-
gent form of C. albitarsis renders the actual sister
grouping of C. albitarsis + C. wangi uncertain), but they
exhibit divergent larval morphology and ecology
(Drayson et al., 2015). Larvae of C. wangi live in the
hygropetric zone of waterfalls and shallow but rapid
riffles in only a few locations in northern Australia.
Cricotopus albitarsis, on the other hand, is wide-
spread and abundant across mainland Australia, in both
lotic and some lentic habitats. Moreover, C. albitibia
group species from outside Australia possess broad tol-
erances to impact (e.g. Beneberu et al., 2014). Pos-
sibly the common ancestor of C. albitarsis + C. wangi
was tolerant to ecosystem impact, and the subse-
quent specialization of C. wangi represents diversi-
fication into a vacant niche. Interestingly, the ‘divergent
NT C. albitarsis’ taxon possessed an almost identical
28S sequence to putative C. albitibia from Papua New
Guinea (PNG; data not shown, but available on request).
The PNG specimens were not successfully sequenced
for the other loci used, and thus the relationships
between these three taxa remain somewhat enigmat-
ic. Several possibilities exist: (1) all three taxa repre-
sent a single species, with C. wangi an ecologically and
morphologically divergent variant and ‘divergent NT C. albitarsis’ representing highly divergent population-level structure (NT + PNG versus eastern Australia); (2) C. albitarsis and ‘divergent NT C. albitarsis’ form a single species, with genetic divergence related to population-level structure, and with C. wangi as an incipient species diverging from within C. albitarsis; (3) the equivocal placement of the ‘divergent NT C. albitarsis’ is misleading and this form is actually sister to eastern Australian C. albitarsis, with C. wangi as sister taxon to this clade; or (4) the ‘divergent NT C. albitarsis’ represents a third species distributed on both sides of the Torres Strait for which morphological differences have either not developed yet, are not apparent in the life stages (pupae and larvae) or specimens sampled currently, or exist in characters other than those covered by current identification keys. The first two scenarios do not fit the data adequately given the quite distinct morphological differences between C. albitarsis and C. wangi at all life stages (Drayson et al., 2015), and the placement of C. wangi as sister taxon to all eastern Australian C. albitarsis, rather than emerging from within the clade. Furthermore, no C. albitarsis from the NT were placed within the clade of eastern Australian specimens. The latter two scenarios emerge as the more likely; however, determining these relationships definitively will require additional sampling in northern Australia and PNG.

CONCLUSION

We demonstrate that the morphological species proposed by Drayson (1992) are largely supported by molecular data, with few major discordances and some added cryptic diversity revealed within some taxa. Our molecular phylogeny clearly indicates that Paratrichocladius renders Cricotopus paraphyletic, and thus the generic concepts that underpin their purported delimitation require revision. The estimated tempo of diversification reported here accords with other genus-level studies of the chironomids, and some divergence events match closely with well-known biogeographical events. Furthermore, we find no clear relationship between the phylogenetic position of a species and its ecological affinities, implying either that tolerance to impact is a convergent trait among many Cricotopus species or that sensitive and restricted taxa have diversified into more narrow niches from a widely tolerant ancestor. The applied outcome is that not only are generalizations about ecological tolerance at the genus level inappropriate, but also that no Australian species appear to indicate pollution on a broad geographical scale. Specifically, no species is sufficiently widespread and ecologically sensitive to be considered a useful indicator across the country (or even the eastern seaboard), and nor are there any species that are exclusively associated with polluted environments (tolerant species were also sampled from unimpacted sites). At a local scale, however, the loss of any of the sensitive species from sites that they are known to inhabit may be a good proxy for detrimental stream impact, and thus we recommend future biomonitoring surveys to identify taxa to species level, so as to incorporate this information. More broadly, many freshwater macroinvertebrate groups exhibit similar species-level ecological variation, yet their sensitivity to impact is generalized at higher taxonomic levels, and thus further eco-evolutionary research remains crucial for the appropriate assessment and management of freshwater ecosystems. In conclusion, this study has resolved species boundaries within the Australian Cricotopus and, in concert with parallel morphological work, provides a concrete foundation for applied biologists to identify and understand evolutionarily this important group of freshwater insects.

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REFERENCES


Krosch MN, Cranston PS. 2012. Non-destructive DNA extraction from Chironomidae, including fragile pupal exuviae, extends analysable collections and enhances vouchering. *Chironomus* 25: 22–27.


**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Majority rule consensus maximum - likelihood topology for the reduced data set.

**Figure S2.** Majority rule consensus maximum - likelihood topology for the total data set.

**Table S1.** Specimen details including molecular voucher code, geographical location of sample sites, life stage, sample provenance and gene regions sequenced per individual.