Non-destructive DNA extraction from Chironomidae, including of fragile pupal exuviae, extends analysable collections and enhances vouchering

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Introduction

It is well established that the traditional taxonomy and nomenclature of Chironomidae relies on adult males whose usually characteristic genitalia provide evidence of species distinction. In the early days some names were based on female adults of variable distinctiveness – but females are difficult to identify (Ekrem *et al.* 2010) and many of these names remain dubious. In Russia especially, a system based on larval morphology grew in parallel to the conventional adult-based system. The systems became reconciled with the studies that underlay the production of the Holarctic generic keys to Chironomidae, commencing notably with the larval volume (Wiederholm, 1983). Ever since Thienemann's pioneering studies, it has been evident that the pupa, notably the cast skins (exuviae) provide a wealth of features that can aid in identification (e.g. Wiederholm, 1986). Furthermore, the pupae can be readily associated with name-bearing adults when a pharate ('cloaked') adult stage is visible within the pupa. Association of larvae with the name-bearing later stages has been much more difficult, time-consuming and fraught with risk of failure. Yet it is identification of the larval stage that is needed by most applied researchers due to the value of the immature stages of the family in aquatic monitoring for water quality, although the pupal stage also has advocates (reviewed by Sinclair & Gresens, 2008). Few use the adult stage for such purposes as their provenance and association with the water body can be verified only by emergence trapping, and sampling of adults lies outside regular aquatic monitoring protocols.

An answer to this mismatch in demand and taxonomic expertise may lie with the actual and potential use of molecular data. In the past two decades researchers have used data derived from DNA to estimate phylogenies for their inherent interest (e.g. Guryev *et al.* 2001), in supplementing morphological characters (Cranston *et al.* 2012), in estimation of support for biogeographic hypotheses (e.g. Krosch *et al.* 2009; Cranston *et al.* 2010) and

increasingly to propose identification of any or all stages (Carew *et al.* 2007; Ekrem *et al.* 2007). In the realm of understanding midge diversity and community structure, cryptic diversity has been detected (Carew *et al.* 2005) including by use specifically of a 'DNA barcoding' approach (Pfenninger 2007; Sinclair & Gresens 2008; Ekrem *et al.* 2010). Phylogeographic molecular genetic studies have been undertaken to reveal the origin and maintenance of chironomid populations (e.g. Martin *et al.* 2002; Krosch *et al.* 2009; Kaiser *et al.* 2010) and fine-scale patterns of gene flow among adjacent streams have been assessed (Krosch *et al.* 2011a). Early on, adult stages were used in such studies with immature stages incorporated in some. Hence, many techniques for DNA extraction from single chironomid specimens were developed specifically for adult bodies and, thus, relatively larger amounts of tissue (e.g. Willassen 1999; Wang & Wang 2012). Although molecular techniques have been used to associate the sexes of adults (Willassen 2005), explicit attempts made to associate previously unknown immature stages with adult stages have been few. Species associations were either 'known' from cultivation (e.g. *Chironomus*, Guryev *et al.* 2001) or by rearing in the field (e.g. *Archaeochlus / Austrochlus,* Cranston *et al.* 2002) or in the laboratory (Sinclair & Gresens 2008). Targetted 'taxonomic' associations of life histories solely via DNA have been often incidental and remain rare in chironomid studies.

In seeking to maximize numbers and/or diversity of taxa for large-scale molecular phylogenetic and population genetics studies, we sought the aquatic immature stages, using conventional techniques with aquatic nets and kicking substrates or interception of drifting individuals (Cranston *et al.* 2010, 2012; Krosch *et al.* 2011a, b). In over 2 decades of working intermittently with molecular aspects of Chironomidae, between us we believe we have encountered most problems, solved many, and have unpublished insights into what can go wrong, what works well, and what can be im-

proved in our protocols. Largely in response to requests from interested colleagues, we describe here the simple technique that makes possible retrieval of PCR-quality DNA from chironomids including even from single pupal exuviae. Previous attempts to extract DNA from exuviae using a modified salting out protocol (Miller *et al.* 1988) failed, and we assumed such specimens lacked sufficient remaining tissue post-adult emergence for DNA extraction. However with careful handling of specimens and more sensitive modern extraction techniques described below, good DNA can be recovered, thereby allowing access to greater numbers of immature stages, and avoiding the need for laborious and often unrewarding rearing, making molecular associations between life stages simpler.

Methods

Field

We use all traditional and some less usual sampling when collecting material in the field, according to the purpose. For population genetics studies we sample intensively in a narrowly defined area containing the appropriate microhabitat(s), seeking known target taxa. For phylogenetic studies in which we seek also to maximise diversity, we sample all visible microhabitats. At all lotic sites we place a drift net downstream of other collecting activity for the duration of the visit, or longer, including overnight if feasible, for pupal including exuvial sampling. In contrast to most studies, we sort specimens from the substrate at or close to the sampling location using dissector microscopes. Live sorting is preferable with specimens being picked with fine forceps and placed immediately into isopropanol or molecular grade ethanol, and never into formalin, methanol or ethanol that has in any way been 'denatured'. Only if a sample cannot be sorted 'live' in timely manner do we preserve in alcohol, after removal of larger particulate matter and sieving for medium-sized organic removal. Isopropanol, although more expensive, is widely available for use in molecular labs, preserves DNA very well, is less volatile and less flammable (important if flights are involved in field work) and is not hygroscopic – your 100% alcohol remains 'absolute'. Most failures to extract DNA from specimens are associated with the age and storage of older material (which should be kept cold and dark) or unwitting use of certain denatured ethanols as supplied to many 'wet labs'. We suspect that some total DNA destruction is caused by customs irradiation of international mailed material but cannot verify this.

For some taxa, life history stage and location it is possible to know the specimen identity prior to laboratory processing for molecular work. For instance, reared monoculture such as species of *Chironomus* may be assumed to be that species, and those familiar with the fauna of a particular area can identify much without slide preparation. However as we know, accurate identification using morphology requires slide preparation, which is *prima facie* incompatible with DNA extraction. For larvae we amputate the head and prepare it as a temporary slide mount (in Hoyers mountant) for rapid identification prior to decision on DNA extraction: the same applies to male genitalia. If sequenced, these vouchers are remounted in Euparal for permanence. For pupae, identification often can be made without prior slide preparation.

Laboratory

We extract total genomic DNA from specimens using a Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) following manufacturer's protocol. Modifications to this protocol were as follows: (1) tissue was digested with proteinase K overnight at 37ºC; (2) after digestion, cuticles were removed carefully using fine-tipped forceps and transferred to 90% isopropanol before vouchering as slides in Euparal; (3) the final elution volume was 100 μL to increase the final DNA concentration of the eluate; and (4) the incubation time for elution was increased to 5 minutes. Furthermore, digestions were never vortexed while cuticles remained in solution to avoid damage to diagnostic morphological characters. At all stages, care with handling is essential and we found using the solution's surface tension, rather than direct pressure from forceps, to be sufficient for transferring specimens between micro-centrifuge tubes. We anticipate that extraction kits from other manufacturers may produce similar results under the same modification, but this remains untested currently.

We report extraction success from pupal exuviae only here and do so simply in terms of reporting successful amplification and sequencing of the barcode region of the mitochondrial *COI* gene and the slower evolving nuclear *28S* rRNA region. We acknowledge that using more stringent quality control measures (e.g. NanoDrop or BioAnalyzer technologies) may provide more information about actual DNA concentration and quality. However, we envisage that the majority of end-users of extracted DNA from pupal exuviae will be interested in utilising DNA for traditional PCR and Sanger sequencing and thus we believe our qualitative assessment valid in this case. Reactions were carried out in a total volume of 25 μL, containing 2-5 μL
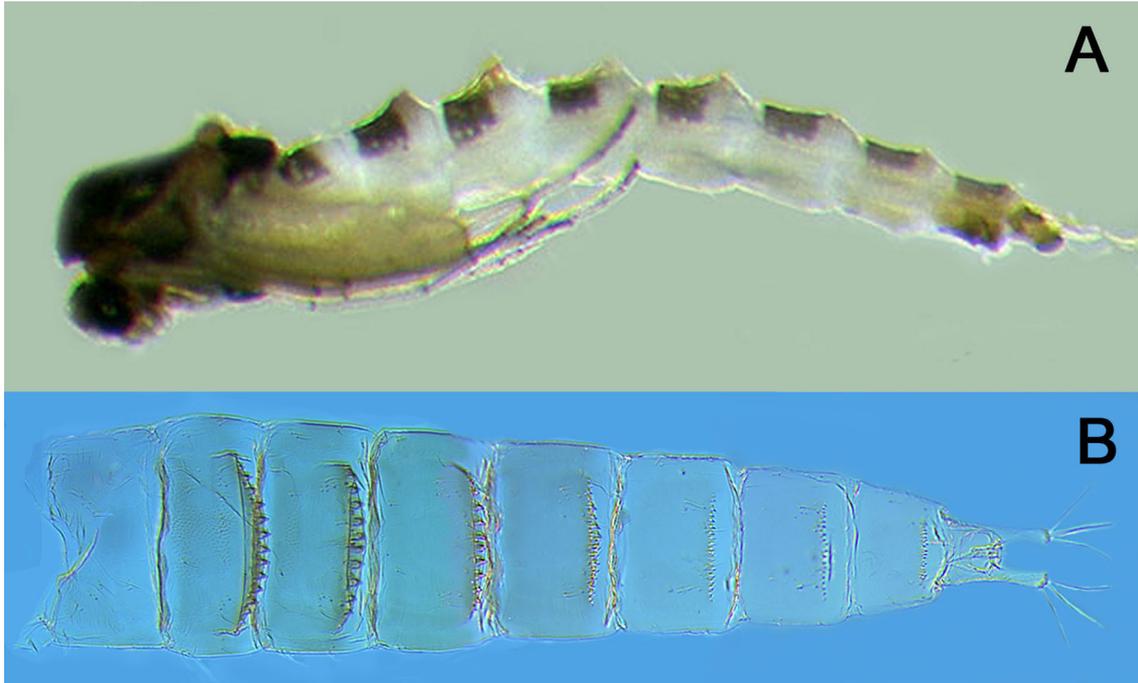
A. Pharate adult pupa of *Stictocladius lacuniferus* Freeman, from New Zealand. Note the leg sheaths are all extended and none recurved under wing sheath. B. Pupal exuviae: abdomen of *Stictocladius uniserialis* Freeman, from Australia. The genus *Stictocladius*, following morphological review by Cranston & Sæther (2010) and Sæther & Cranston (2012) is now the subject of molecular study by Matt Krosch and Peter Cranston. The pupal stage not only provides good morphological features, but has provided valuable DNA even from exuviae, as outlined in the article.

of template DNA, 0.6 μL of each primer (10 pmol/μL – manufactured by Geneworks, Adelaide, Australia), 1.75-3.5 μL of of 5X MyTaq Red Reaction Buffer (Bioline, London, UK) and 0.2 μL of MyTaq DNA Polymerase (Bioline). Primer sequences and PCR protocols for both genes can be found in Krosch *et al.* (2011b). Amplified products were purified using an ISOLATE PCR and Gel Kit (Bioline) according to manufacturer's protocols, but using only 13 μL of elution buffer and with a longer incubation time following addition of elution buffer to increase final concentration of amplified fragments. As with DNA extraction, choice of manufacturer of kits for the purification of amplified products is likely not to influence success rates greatly. Purified fragments were sequenced using ABI BigDye® Terminator v3.1 chemistry, precipitated using a standard ethanol-based protocol and analysed on an ABI3500 sequencing platform at the Molecular Genetics Research Facility (QUT, Brisbane). For more detailed information on methods and success of 'routine' DNA extraction from larvae or pupae we refer the reader to our existing publications cited below that use molecular data.

Vouchering specimens

In the early days, the few people working with chironomid DNA had a rather casual approach to vouchering material needed to verify that sequenc-es obtained actually derived from a named and retained specimen (Jon Martin U. of Melbourne, pers. comm, Peter Cranston, pers. obs.). This arose partially in the belief that DNA extraction from small insects involved grinding up (destroying) at least a complete specimen, sometimes more than one, because of the demands of the salting-out protocol (Miller *et al.* 1988). Maintenance of reliable vouchers followed with: 1. growing awareness that not all published sequences were what they were purported to be; 2. recognition that 'inessential' parts of a single specimen could provide enough DNA by newer extraction protocols, and the rest be retained as a voucher; 3. recognition that the cuticle alone, after gentle extraction of DNA, provides a complete voucher; and 4. multiple specimens from natural populations should and need never be co-extracted. However, the latter point does remain viable for multiple specimens from laboratory culture lines or egg masses for use in downstream applications that require large quantities of DNA, such as optimisation of DNA systems or PCR primers.

Procedures are a little time-consuming and need accurate recording – we give each potential molecular specimen a unique 'site (year date) plus sequential numerical code' at the outset – whether or not DNA is obtained. This unique identifier is as-

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sociated with the DNA throughout processing, and with the cuticile obtained after extraction, or the slide-mounted larval head capsule if it is only the body that is taken through extraction. These slide mounted materials are treated with the same care in preparation, curation and specified repository as type material in a taxonomic study. The unique specifier code and associated data remains linked to the data submitted to Genbank (http://www. ncbi.nlm.nih.gov/genbank/).

Results

This technique, which already has worked well across other life stages from many subfamilies of Chironomidae, was trialed on exuvial specimens across a range of Orthocladiinae taxa. Of 58 exuvial specimens derived from separate field collections made at various sites across Australia during 2011 and 2012, extracted as described above, 27 have been successfully amplified and sequenced for *COI* and 17 for *28S*. Sequence quality always was acceptable under standard QC metrics (e.g. signal strength) applied by the sequencing software and sequencing success was repeatable via both multiple attempts using the same purified PCR product and multiple initial PCR reactions. Moreover, vouchers made from cuticles post-digestion showed that diagnostic characters remained intact and morphology - and molecular-based identifications converged for all specimens. A number of specimens, in addition to those successfully sequenced for either locus, amplified successfully (as assessed using 1.5% agarose check gels) but did not produce clean or useable sequence. This suggests that further optimisation of protocols may be necessary, especially with regard to the ratio of DNA template to primer in sequencing reactions.

It is not surprising that even this technique can fail to recover usable DNA from some specimens. Extraction of DNA from pupal exuviae relies on the presence of muscle tissue that is left behind on the inner surface of the cuticle by the emerging adult. DNA cannot be extracted from the cuticle itself. Thus, we suspect that the success of this technique is highly correlated with how long exuviae have been exposed to the aquatic environment after adult emergence. The longer the exposure time prior to collection, the greater the likelihood of any remaining muscle tissue being scoured or degraded. The same principle applies to the duration that specimens spend in alcohol collections. Furthermore, we note also problems encountered with degraded ethanol for preservation and quarantine of shipped specimens using X-rays (particularly to the USA). These factors act to shear DNA

strands and greatly reduce efficiency of extraction and amplification, often to the point of complete failure for entire collections. Moreover, given the much higher copy number of mitochondrial genomes within each cell, we anticipated that DNA extracted from exuviae would perform better for amplifying mtDNA loci than nuclear. Although we did not observe a dramatic difference in total numbers sequenced for each locus here, *28S* amplification required more troubleshooting than *COI*. The use of intermediate primers for COI, which often produce sequence data from older more degraded specimens (Jon Martin, pers. comm.) may allow even sub-optimal specimens to be used.

Discussion

The addition of PCR-quality DNA sequences from 'wild caught' pupal exuviae described here expands the number of sequenceable specimens for chironomid researchers who collect in aquatic environments. The greatest benefit of this technique will likely be in aiding the development of molecular associations between life stages of species by negating the need for rearing of larvae through to adults. This technique also has potential importance for those investigating population genetic structure, gene flow and dispersal where sample sizes must be maximised from each study site. We encourage other researchers to retain, and attempt to extract DNA from exuviae and not consider these important specimens valueless for molecular purposes.

However the addition of a 'new' life history stage, the pupal exuviae, to the repertoire of those engaged in molecular studies of Chironomidae should not obscure that even this suite of techniques do not provide the perfect answer to all questions. We note that there are sporadic and difficult to diagnose difficulties with extraction for some specimens or collection conditions in addition to the unknown age and history of collected exuviae, or interceptions of larvae, pupae and adults by netting. There are sporadic problems with amplification of DNA from mermithids rather than the midge host, apparently restricted to 18S rRNA and probably due to primer degeneracy.

The procedures and protocols associated with specimen tracking, vouchering and truly geocoded and permanently labelled specimens are particularly important as we recognise increased cryptic diversity. Unique vouchers allow us to revisit actual specimens (and locations) of 'known' molecular identity and seek to validate perhaps obscure or overlooked morphology that will allow non-molecular identification. Furthermore, GenBank

is not prescriptive concerning vouchering and thus the database is error-ridden and requires validation and correction for many anomalous taxa. To date we have no complete and reliable library to test our species identifications (Ekrem *et al.* 2007) and thus morphological vouchers for DNA sequences remain as important as morphological types. Webb *et al.* (2012) illustrate a salutary case of difficulties in tracking down morphological vouchers for molecular databased N. American Ephemeroptera, despite the DNA barcode initiative's long-standing instructions concerning appropriate vouchering. DNA taxonomy does not over-ride the common sense understanding of how to go about systematic science, regarding repeatability and documentation of evidence.

Acknowledgments

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