Odonatologica 34(2): 183-187 *June 1, 2005*

EXUVIAE AS A RELIABLE SOURCE OF DNA FOR POPULATION-GENETIC ANALYSIS OF ODONATES

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Received October 5, 2004 / Revised and Accepted November 8, 2004

Genetic analyses are widely used for a variety of ecological research scenarios, especially to aid species' conservation programs. Where genetic material is required from rare or endangered spp. it is essential that the samples be collected non-destructively, the ultimate goal should be to develop reliable DNA extraction protocols that may be used with non--invasively collected samples. In this paper 3 methods of DNA extraction (DNeasy tissue kit, proteinase-K/TNES and Chelex-100) that use odonate (*Coenagrion mercuriale*) exuviae as a non-invasive source of genetic material are described and compared. DNA extracted from exuviae produced consistent genotypes at 5 polymorphic microsatellite loci for all of the samples processed using the DNeasy tissue kit and proteinase-K/TNES methods and 4 out of the 6 exuviae treated with Chelex-100. Exuviae offer an effective source of genetic material from endangered odonates and also highly mobile spp. that are too difficult to catch in significant numbers. As such, it is expected DNA extracted from exuviae to be widely applied to odonatological genetic research.

INTRODUCTION

DNA-based genetic analyses undoubtedly play an important role in many species' conservation plans (FRANKHAM et al., 2002; DESALLE & AMATO, 2004). Because of the inherent problems (i.e. time, expense and short-term bias) in directly tracking many species (SLATKIN, 1985), molecular markers offer a rapid and cost--effective method to indirectly estimate the extent of inter-population dispersal and thus help define management units. With increasing degradation and fragmentation of freshwater habitats, it is perhaps not surprising that many odonate populations/

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species are under threat from extinction (CORBET, 1999). For odonates, a molecular-genetic appraisal of population connectivity and response to fragmentation would benefit the development of appropriate habitat-restoration/management plans.

A reliable DNA extraction protocol is the foundation for any population-genetic investigation. For conservation purposes it is obviously essential that a non-destructive sampling regime is followed. Where possible, however, the ultimate goal for any conservation-genetic research should be the non-invasive isolation of genetic material. WATTS et al. (2001) and LODGE & FREELAND (2003) reported methods to obtain DNA from odonate legs. Although leg samples may be removed from odonates without measurably affecting their fitness (FINCKE & HADRYS, 2001; D.J.Thompson, unpublished data), this procedure is nonetheless invasive. In our experience, a rapid, large-scale genetic analysis of protected species can be hindered because of the need to acquire licences from several different government agencies. Rather than sampling adults directly, potential sources of genetic material are the shed exuviae that remain on aquatic vegetation after larval emergence.

DNA from butterfly and honey bee 'exuviae' has been recovered by FEINSTEIN (2004) and GREGORY & RINDERER (2004) respectively. The former study used a DNeasy Tissue Kit (Qiagen Inc.) to extract DNA while the latter authors followed a Chelex-100 protocol (see also WALSH et al., 1991). WATTS et al., (2001) and LODGE & FREELAND (2003) both employed Geneclean kits (Bio101) to extract DNA from odonate legs, although to reduce cost and increase sample throughput we now use a proteinase-K/TNES method (SAMBROOK & RUSSELL, 2001) to extract DNA from odonate leg samples. Here, we report the relative success of 3 simple methods - DNeasy, Chelex-100 and proteinase-K/TNES - of extracting DNA from odonate (*Coenagrion mercuriale*) exuviae.

METHODS AND RESULTS

Twenty four *Coenagrion mercuriale* exuviae were collected from emergent vegetation in the afternoon of 27 May 2004 during a preliminary habitat survey in the New Forest (Hampshire, UK). All exuviae were dry when collected and because *C. mercuriale* emerges during early morning likely to be at least 5 hours old. Exuviae were stored at room temperature in separate 1.5 ml microcentrifuge tubes containing approximately 1 ml of 100 % ethanol. DNA extraction was undertaken 4 months later.

All exuviae were blotted on tissue paper to remove ethanol and then ground in liquid nitrogen immediately before DNA extraction. For the first DNA extraction method we simply followed the manufacturer's protocol (available online at: http://www1.qiagen.com/literature/protocols/DNeasyTissue.aspx) for their DNeasy Tissue kit (Qiagen) with the modification that the final elution volume was reduced to 100 µl. The second extraction protocol is summarised as follows: (i) add 600 µl of TNES buffer (10 mM TRIS pH 7.5, 400 mM NaCl, 100 mM EDTA, 0.6 % SDS) and 20 µl proteinase-K (20 $mgml^{-1}$) to each crushed exuvia, mix and incubate overnight at 50°C, (ii) centrifuge the samples at 13,000 rpm for 6 min and transfer supernatants to new 1.5 ml tubes along with 140 µl saturated (6M) NaCl solution, (iii) shake samples for about 20 s, (iv) centrifuge for 6 min at 13,000 rpm, (v) transfer the supernatants to new 1.5 ml tubes, (vi) add 900 µl of absolute ethanol to each sample, (vii) precipitate DNA overnight at -20°C, (viii) centrifuge samples at 13,000 rpm for 30 min at 4°C, (ix) wash samples in -20°C, 70 % ethanol, (x) leave samples to air dry, and finally (xi) re-suspend DNA in 100 µl 1 x TE. For the third DNA ex-

traction protocol we (i) added 1 ml 5% Chelex-100 (BioRad) and 20 μl proteinase-K (20 $mgml^{-1}$) to the crushed sample, (ii) incubated overnight at 50°C, (iii) heated samples at 95°C for 2 min and then (iv) ice-quenched the samples. We extracted DNA from 8, 8 and 6 exuviae for the DNeasy, proteinase-K/TNES and Chelex-100 protocols respectively. All samples were stored at -20°C until PCR.

Suitability of the DNA extractions for PCR was tested by genotyping all samples at 5 microsatellite loci (LIST4-002, LIST4-060, LIST4-063, LIST4-066, LIST4-067) developed by WATTS et al. (2004a, b). Microsatellite alleles were amplified by PCR in a 10 μl final reaction volume using ReddyMix PCR Mix (ABgene) on a Dyad DNA engine (MJ Research Inc.). PCR conditions were: (i) 1 min at 95°C, (ii) 6 cycles of 30 s at 95°C, 30 s at T_a°C and 45 s at 72°C, (iii) 26 cycles of 30 s at 92°C, 30 s at T_a°C and 55 s at 72°C, and (iv) 72°C for 30 min. T_a is the locus-specific annealing temperature (see WATTS et al. 2004a, 2004b for details). Each PCR contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20®, 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 10 μg BSA, 1 μl DNA extract (DNeasy & proteinase-K/TNES stock DNAs were diluted 1:10 for PCR), 20 pmol forward primer, 30 pmol reverse primer and 0.25 units Taq polymerase (ABgene). The forward primers were 5' labelled with either 6-FAM, NED, PET or VIC fluorescent dyes (Applied Biosystems). PCR products were pooled with a 500 bp (LIZ) size standard (Applied Biosystems) and separated by capillary electrophoresis through a denaturing acrylamide gel matrix on an ABI3100 automated sequencer (Applied Biosystems). Genotyping was repeated twice for all samples extracted by DNeasy and proteinase-K/TNES to confirm repeatability of PCR profiles and sometimes a third time in the event of PCR failure or if an allelic profile was not consistent between successive PCRs.

All eight DNA samples extracted using the DNeasy kit and proteinase-K/TNES methods amplified alleles for all five microsatellite loci after PCR, while only 4 out of the 6 samples extracted using the Chelex-100 method amplified PCR products. Negative controls confirmed that neither DNA extracts nor PCR reagents were contaminated. DNA extracted using the DNeasy kit had the lowest PCR failure rate (1/80 PCRs), followed by proteinase-K/TNES (9/80 PCRs) during 2 rounds of genotyping. PCR failures from samples extracted using the proteinase-K/TNES DNA are likely to be a consequence of varying amounts of salt in the DNA extract. Altering the DNA concentration (i.e. reducing the amount of salt) lead to successful PCRs. Subsequent rounds of genotyping confirmed the repeatability of genotypes at all 5 microsatellite loci.

DISCUSSION

One potential limitation of sampling exuviae from the field is that the age of the samples is not known with the consequence that the DNA from older exuviae may be more degraded and therefore less amenable to PCR. Without further experimental work we cannot address this possibility but note that exuviae are relatively fragile and likely to persist only for a few days. If, on the other hand, exuviae do persist for longer than we believe then by chance we would have sampled exuviae over a reasonable time period. Sample age, therefore, may account for some PCR failures, but our results imply that genotyping success was qualitatively correlated with the extraction technique itself. Nevertheless, it is important to note that much DNA degradation occurs when samples are hydrated (enzyme action) and/or exposed to sunlight (uv-damage), so it may be preferable to collect exuviae from shaded sites

during warm weather when the samples will be rapidly dried. We stored our exuviae in ethanol after collection but given that DNA may be extracted from dry, historical specimens (LODGE & FREELAND, 2003) this may be an unnecessary precaution (especially if the samples are processed soon after collection). It perhaps would have been desirable to concurrently genotype individuals and their shed exuviae, however, we argue that the repeatability of the different genotype profiles generated at 5 polymorphic microsatellite loci is sufficient to demonstrate that these results are not an artefact of PCR contamination. To conclude, therefore, our results demonstrate that odonate exuviae are a reliable source of DNA that may be used for genotyping applications involving short fragments (ca. <400 bp) such as PCR amplification of microsatellite alleles. Given results from other work (e.g. FEINSTEIN, 2004) we expect also that genetic material extracted from odonate exuviae will be useful for amplifying regions of mtDNA.

The appropriate choice of extraction method will depend upon laboratory preferences. For example, the DNeasy kit appears to yield clean DNAs that may be used for PCR without having to vary the DNA concentrations for a significant minority of the samples. Using kits is, however, more expensive than preparing the reagents required for either the proteinase-K/TNES or Chelex-100 protocols. Using Chelex-100 is the quickest and cheapest protocol, however, this method does lead to significant percentage of PCR failures (varying DNA concentrations in the PCRs still further may overcome this). It should be noted that we have found (with other DNA samples) that Chelex-extracted DNA is not suitable for long-term storage.

Clearly exuviae offer an effective source of genetic material from endangered odonates where obtaining licences may limit or even prevent sampling of larvae or adults. In addition to aiding conservation research *per se*, sampling exuviae also facilitate genetic analyses of highly mobile odonates that would normally be too difficult to catch in significant numbers in their adult or larval stages. As such, we expect DNA extracted from exuviae to be widely applied to odonatological genetic research.

ACKNOWLEDGEMENTS

Coenagrion mercuriale is a protected species under Schedule 5 of the Wildlife & Countryside Act (1981). All work was carried out under licence from English Nature. We are grateful to the NERC (grant no. NER/A/S/2000/01322) for provision of funds that enabled the work to proceed and for the comments of an anonymous reviewer that helped improve this manuscript.

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