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THE USE OF ODONATA MUSEUM SPECIMENS IN QUESTIONS OF MOLECULAR EVOLUTION

R.J. LODGE and J.R. FREELAND*
Department of Biological Sciences, Open University, Walton Hall, Milton Keynes, MK7 6AA, United Kingdom

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Studies of population genetics and phylogenetics require samples from individuals representing a variety of spp. and populations. Collecting the necessary individuals may be problematic, particularly for seasonal, rare, or geographically remote organisms. Museum collections therefore provide a potentially valuable resource, and the widespread use of polymerase chain reactions (PCR) means that target regions of DNA can be amplified from very small amounts of tissue. Here modifications to DNA extraction techniques are described that have allowed the authors to extract, amplify, and sequence a portion of mitochondrial DNA from parts of single dragonfly legs taken from museum specimens up to 80 yrs old. It is anticipated that in future these techniques will be applied to a range of odon. studies, including questions of conservation genetics.

INTRODUCTION

Successful extraction of DNA from museum samples can be of great benefit to a wide range of studies. First, museums often house samples from much broader geographical ranges than can be visited by individual researchers on grants of limited duration, and they may therefore allow biogeographical studies to cover much larger areas than would otherwise be investigated. Second, access to samples collected over varying time scales will add an important dimension to studies that are concerned with altered species distributions, conservation genetics, and other questions that may involve changes over time. Third, because museum samples already exist, they may provide an alternative to capturing and sampling live individuals, and therefore in some circumstances provide a more humane alternative to de novo sampling.

A number of studies on birds and mammals (e.g. ROY et al., 1994; TAYLOR et al., 1994; MUNDY et al., 1997) have used genetic information from museum samples to

*Author for correspondence: J.R.Freeland@open.ac.uk

address questions of conservation genetics (see also LANDWEBER, 1999 for review). However, insect museum samples have not yet been used in a comparable manner (CATERINO et al., 2000). Here we report on our successful extraction and amplification of DNA from dragonfly museum samples up to 80 years old, taken from *Anax junius* and *Aeshna mixta*.

METHODS AND RESULTS

In developing these protocols, we used *Anax junius* and *Aeshna mixta* samples that were provided by a number of museums and other collections (see acknowledgements). The samples included air or acetone dried, adult samples and small, up to 80 years old, (<10 mm long) larval samples stored in ethanol. In order to minimise contamination risk, extractions from tissue with a relatively low DNA yield (e.g. partial legs) were done in a laminar flow hood in a small, enclosed room on a different floor to the lab in which fresh material was processed. In addition, dedicated pipettes were used, and other equipment such as the microcentrifuge and vortex were specific to this separate 'clean' area.

Two methods of extraction were used. The first method was applied to samples up to ten years old, and is a modification of the single leg extraction protocol by WATTS et al. (2001), which is a salting-out extraction method using DTAB and CTAB buffers and Geneclean (Bio101) kit components (see WATTS et al., 2001 for details). The modifications to this existing protocol were necessary because museum samples are generally dried, and are seldom if ever stored in DTAB buffer. Single legs were placed in 1.5ml eppendorf tubes and crushed with forceps, then ground using a motorised pellet pestle with multiple additions of liquid nitrogen, until at least 50% of the sample had formed a fine powder. DTAB (prepared as for WATTS et al. (2001) short term storage buffer), a cationic detergent, was then added to the homogenate to lyse the cell components and denature proteins, thereby releasing the DNA. The second cationic detergent CTAB had the added advantage that it selectively binds DNA in high salt concentrations aiding in the separation of other cell components (SAUNDERS, 1999). When required, a proteinase K digestion was included (for details see later in this section), with incubation in DTAB which was extended from 20 to 48 hours if proteinase K was added. The additional proteinase K digestion step was required for small legs, where only small quantities of muscle may have been preserved. Proteinase K removes nucleases from DNA, aids in the digestion of adhering proteins and thus the separation of DNA from other cellular components (SAUNDERS, 1999), thereby increasing the DNA yield from these samples. This proved to be more effective than the overnight incubation used by WATTS et al. (2001) on newer samples.

The final modification that improved yield was to rotate with glassmilk at room temperature for two hours, not 30 minutes as outlined in the original protocol. The incubation time was extended here to increase the yield of DNA by providing more opportunities for DNA molecules to collide and bind with the suspended silica matrix (Bio101, 2001).The selective binding of DNA to silica in high concentrations of a chaotrophic salt allows the separation of DNA from other proteins, most forms of RNA, and substances inhibitory to PCR, such as chitin and other polysaccharides

(SAUNDERS, 1999), which may form a substantial proportion of these samples.

The second method of extraction was used for samples more than ten years old. For these samples (often as small as half a single leg) we used the Geneclean kit for Ancient DNA (Bio101), which is designed for isolation of DNA from samples of bone, preserved tissue, or animal by-products. This kit contains instructions for DNA extraction using ultra-pure, ultra-clean solutions (to further minimise contamination risk), but nevertheless required some experimentation with the protocol. First, in addition to incubation with chemical denaturants in the kit's dehybernation solutions, the manufacturers suggested a pre-incubation of samples with proteinase K to aid in the separation of DNA from cell components and adhering proteins. However, we had much greater success when we did a 48 hour proteinase K digestion (as opposed to the maximum of 15 hours recommended by the manufacturer) in a soaking solution consisting of 2 µl 0.5M EDTA, 20 µl 10% SDS, 10 µl 20 mg/ml proteinase K and 168 µl distilled deionised water. EDTA was added to the soaking solution to inhibit DNases by chelating divalent cations such as calcium and magnesium and SDS, an anionic detergent, to solubilize cell membranes and denature proteins (MILLIGAN, 1998). Our second modification, echoing an alteration of the above protocol for younger museum samples, was an extension of the incubation with glassmilk to 1-2 hours at room temperature, as this improved the likelihood of DNA binding. The third modification was towards the end

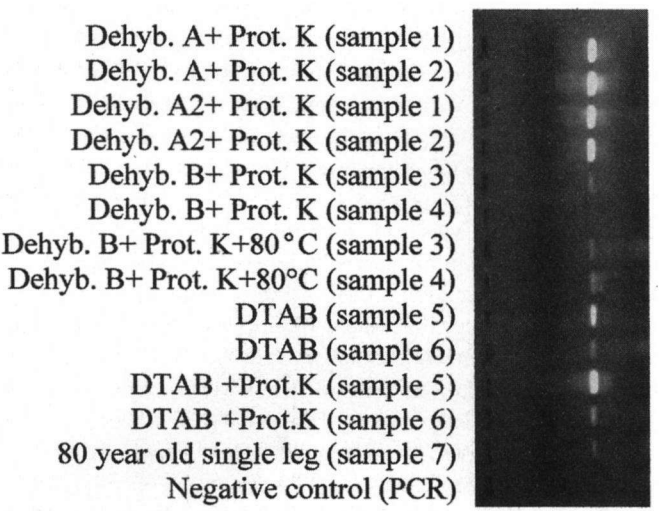

Fig.1. Cytochrome oxidase I mtDNA PCR products (354 base pairs long) from *Aeshna mixta*, run on a 1% agarose gel. Lane labels show the extraction buffer used (dehybernation A, A2, B or DTAB) and additions to standard protocols, which included proteinase K digestion (prot. K.), and/or an additional 10 min. heating step after initial incubation (80°C). Sample 7 (using protocol as for sample 1, with dehybernation A and proteinase K) represents the oldest *Aeshna mixta* sample (80 years old) for which mtDNA sequence was obtained. Samples 1-6 were 1 year old dried *A. mixta*.

of the protocol. At this stage, manufacturers suggest resuspending the DNA pellet in 50-100 µl elution solution by vortexing for 1-2 seconds, but we had higher DNA yields when we used only 35 µl of elution solution, and allowed this solution to soak into the glassmilk and then mixed by gentle pipetting.

The other point worth noting about the Geneclean kit for Ancient DNA is that the manufacturers include two dehybernation solutions for the initial lysis/denaturation step. One of these, dehybernation solution A, is based on guanidine which is a strong chemical denaturant of proteins, capable of dissolving cytoplasmic and nuclear membranes and inhibiting nuclease activity (SAUNDERS, 1999). Dehybernation solution B is an aqueous EDTA based solution, which will inhibit DNases, and in the case of specific samples demineralise bone tissue, combined with a detergent to lyse cell membranes and other components (MILLIGAN, 1998; Bio101, 2001). The efficacy of these two solutions varies between samples, particularly with respect to the method of sample preservation and the environment in which samples were kept. For the dragonfly legs, we obtained the best results from dehybernation solution A (Fig. 1), without the suggested additional detergent (A2) provided (Bio101, 2001).

Success of DNA extractions was confirmed by amplifying a region of mitochondrial (mtDNA) cytochrome oxidase I using 'universal' COI primers C1-J-1751 (5'GGATCACCTGATATAGCATTCCC3') and C1-N-2191 (5'CCCGGTAAAAT-TAAAATATAAACTTC3') (PALUMBI et al., 1991). Amplification reactions included 2-6 µl DNA, 1X PCR buffer with 1.5 mM $MgCl_2$ (Promega), 200 µM dNTPs, 0.5 µM of each primer, and 1U Taq DNA polymerase (Promega) in a 50 µl reaction. The amplification program proceeded with 2 minutes at 95°C, followed by 40 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds, and a final step of 72°C for 5 minutes. An aeshnid-specific primer pair, internal to C1-J-1751 and C1-N--2191, was also used in nested PCR on difficult/ low yielding samples (J.R. Freeland et al., unpublished data). A large proportion of these were subsequently sequenced in order to verify that the DNA was from *A. junius* or *A. mixta*. An alignment of 92 *Anax junius* individuals plus 5 confamilials (including *Aeshna subpupilla* from the study of Wishart et al., GenBank accession no. AF429284) verified the sequences as aeshnid COI. These sequences have been submitted to GenBank (accession numbers AF550515 to AF550581). The maximum sequence divergence was 3.65 % for *A. junius*, increasing to 14% when the 5 confamilials were included. It is worth noting that the fragments we amplified were 354-490 bp long, and are therefore a somewhat conservative estimate of our success because relatively small fragments are generally easier to amplify from old, degraded DNA than are relatively large fragments.

To date, we have had reasonably high levels of success, but this varied with the age of the samples e.g. only 42% of 7 samples aged 80-40 years old, 75% of the 8 samples aged 20-39 years old, 100% of the 6 samples aged 10-19 years and 74% of 65 samples aged 2-9 years old. The drop in success rate for the last age category may be due to the lack of muscle tissue in very small larval samples (especially compared to the toughened exoskeleton), although as most of the recent samples were preserved in ethanol as

opposed to air dried, preservation technique may also affect the quality of the sample. Of the remaining dried adult samples the exact method of preservation and storage had no effect on the percentage of successful amplifications, with either the CTAB or Ancient DNA kit methods, although the addition of the proteinase K digestion did yield brighter bands upon PCR with samples over 20 years old (Fig. 1). Some samples from all age ranges did not yield any DNA upon PCR amplification, for reasons that remained largely unclear, although these were in the minority. For example, a 144bp fragment may amplify from a sample that did not generate a 345bp fragment (HANDT et al., 1994).

DISCUSSION

These results suggest that museum samples are a viable source of DNA for future research on odonates, and should be particularly useful for studies in which current sampling is highly restricted. Although there is no guarantee that any particular sample will yield DNA, by following the protocols outlined above, this likelihood can be kept reasonably high. Museum curators can be assured that drying samples (with acetone) or keeping samples in envelopes should not preclude possible DNA recovery. The ease with which useful sequence was obtained from very small quantities of sample e.g. parts of a single insect leg, allows the retention of a large proportion of samples at the respective museums for further study (CATERINO et al., 2000). Researchers should be aware that, although they may have to factor in the likelihood that not all samples will yield DNA, by obtaining approximately a 35% excess of samples (in the 10-80 year sample age range) they should be able to reach their target number of desired sequences from museum specimens. Degradation of DNA over time regardless of preservation method may limit the molecular techniques available, but as many studies have shown, the information obtained can be invaluable to studies of conservation genetics (ROY et al., 1994, CATERINO et al., 2000).

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