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EFFECTIVE PROCEDURES FOR THE EXTRACTION, AMPLIFICATION AND SEQUENCING OF ODONATE DNA

P.T. CHIPPINDALE, D.H. WHITMORE, V.K. DAVÉ*, T.G. VALENCIA and J.V. ROBINSON
Department of Biology, Box 19498, University of Texas at Arlington, Arlington, TX 76019, United States

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The methods of specimen preservation, DNA extraction, DNA amplification, choice of primers, and DNA sequencing are described. These are primarily adaptations of those developed by other workers, but the Authors describe modifications that they have found to be optimal when working with odonates. It is likely that some of the described protocols will have more general applications to other arthropods as well.

INTRODUCTION

In recent years, there has been an explosive increase in the application of DNA sequence data to problems in population genetics and phylogeny reconstruction (see HILLIS et al., 1996b for the most comprehensive review). Introduction of the polymerase chain reaction (PCR) for amplification of specific regions of DNA has been key to this development (reviewed by PALUMBI, 1996), and methods for sequencing DNA have become increasingly refined and streamlined (e.g. HILLIS et al., 1996a). The current renaissance in molecular systematics has dramatically increased our understanding of both micro- and macroevolutionary phenomena in diverse taxonomic groups, and those working with insects have benefitted greatly from these technological advances. Initially, workers focused largely on mitochondrial sequences (for insects, see review by SIMON et al., 1994) or nuclear ribosomal genes (reviewed by BROWER & DeSALLE, 1994); more recently there has been substantial development of techniques for amplifying and sequencing other nuclear regions as well (e.g CHO et al., 1995; BROWER & DeSALLE, 1994 and references therein; FRIEDLANDER et al., 1994, 1996; BESANSKY & FAHEY,

* **Present address**: University of Texas Southwestern Medical Center, Dallas, TX 75235, United States

1997). Currently, we are conducting studies of phylogenetic relationships and biogeographic history of damselflies of the genus *Ischnura* and closely related genera (Zygoptera: Coenagrionidae) using both mitochondrial and nuclear sequences (DAVÉ, 1996 and work in progress). In doing so, we have refined techniques for extracting, amplifying, and sequencing DNA from coenagrionid damselflies. In this paper we describe these methods; we hope that we can facilitate other molecular studies of odonates and contribute to development of the growing community of odonate researchers who use nucleic acid data to study this group. The methods that we describe primarily are adaptations of those developed by others, but here we describe modifications that we have found to be optimal when working with odonates. It is likely that some of these protocols will have more general applications to other arthropods as well.

METHODS AND RESULTS

SPECIMEN PRESERVATION

We routinely extract DNA from damselflies in various states of preservation. However, the yield and quality of DNA vary tremendously, depending on the way in which the specimens have been preserved. Ideally, specimens are collected alive and either killed immediately prior to DNA extraction, or frozen at ultracold temperatures (below -60°C). Specimens treated in this way provide excellent, high molecular weight DNA that amplifies readily using PCR. Freezing at -20°C for short periods (days to weeks) also yields satisfactory results, although we suspect that DNA quality will diminish if specimens are stored at regular freezer temperatures for extended periods of time (we have not tested this quantitatively, however). Quality of DNA from dried specimens depends on how quick and thorough the drying was. High quality, high molecular weight DNA can be obtained from specimens air-dried rapidly at low humidity. C. Simon (pers. comm.) recommends use of silica gel (used, for example, in storing photographic equipment) to ensure quick and complete drying. Our experience with standard dried specimens (e.g. those simply killed and pinned or stored in glassine envelopes) is that these typically give low yields of sheared DNA. For example, we have obtained amplifiable DNA from dried specimens of *Zoniagrion exclamationis* (stored in glassine envelopes) that are at least 20 years old. However, PCR amplification of mitochondrial sequences and nuclear ribosomal regions from these samples is difficult and generally requires use of closely spaced PCR primers (less than about 200 bp apart). Specimens preserved in 70% isopropanol have yielded similar results: we have obtained amplifiable DNA from *Ischnura gemina*, *I. graellsii*, and *I. elegans* preserved in 70% isopropanol for approximately five years, but only closely spaced primers yield consistent, sequenceable amplifications. Use of more concentrated alcohol (95% or greater) may enhance DNA preservation (J. Brown, C. Simon, pers. comm.;

FRIEDLANDER et al., 1996), but we have not yet tested this and at present prefer other preservatives if possible (see below).

When specimens cannot be frozen or returned to the laboratory alive, we generally preserve them in screwcap microcentrifuge tubes in one of the two following solutions, both of which allow extraction of high quality DNA.

SOLUTION 1 (from COCKBURN & FRITZ, 1996; this is their recommended solution for preservation of mosquitos for DNA extraction):

- 0.25 M EDTA
- 2.5% (w/v) SDS
- 0.5 M Tris-HCl pH 9.2.

SOLUTION 2 (based on the protocol of PHILLIPS & SIMON, 1995):

- 4.0 g DTAB (dodecyltrimethylammonium bromide; available from Aldrich or Sigma)
- 4.4 g NaCl
- 0.84 g EDTA (disodium)
- Bring to 50 mL with 100 mM Tris/HCl pH 8.6.

High quality DNA can be extracted from specimens stored in either solution for at least several weeks, and probably longer. As COCKBURN & FRITZ (1996) point out, solution 1 has the advantage that it is nontoxic, nonflammable, and nonvolatile; also, its ingredients are inexpensive and readily available in many laboratories. Solution 2 has the advantage that it is also used in our preferred method of DNA extraction, but it is caustic, and specimens stored in this solution for more than a few months begin to disintegrate. For long-term storage, it is probably safest to remove specimens from these solutions and freeze them, preferably at ultralow temperatures.

Most of our DNA amplification has been of mitochondrial (mt) regions (cytochrome *b*, cytochrome oxidase II, and 12S ribosomal RNA-encoding DNA) or tandemly repeated nuclear ribosomal DNA. Such multi-copy sequences are usually fairly easy to amplify by PCR. Single-copy nuclear genes may require especially high quality DNA; this has been our experience with the nuclear elongation factor 1 alpha gene with which we recently started to work, and for these sequences we use fresh or frozen specimens whenever possible.

DNA EXTRACTION

Many methods have been used for DNA extraction from insects and other organisms (e.g. see COCKBURN & FRITZ, 1996; PHILLIPS & SIMON, 1995; HILLIS et al., 1996a), and we have experimented with a wide variety of techniques with varying degrees of success. For coenagrionid damselflies, the following method has consistently yielded amplifiable DNA for specimens preserved in the buffers described above, alcohol, or dried. It represents a modification of the technique described by PHILLIPS & SIMON (1995). Whenever possible, we isolate thoracic flight muscle by dissection, as DNA extractions using tissue samples that include cuticle sometimes fail to amplify by PCR. We suspect that the cuticle of some

species may contain inhibitory substances.

PROTOCOL FOR DNA EXTRACTION FROM SMALL AMOUNTS OF ODONATE TISSUE:

(1) Grind tissue to a powder with liquid nitrogen, using a mortar and pestle. The amount of tissue varies; we usually use as much damselfly flight muscle as we can get from a single individual. Put powdered sample in 175.0 µL of the buffered DTAB solution described above (Solution 2). The sample may simply be minced and placed in the DTAB solution and refrigerated, but in our experience it may take days or weeks for sufficient DNA to leach out. PHILLIPS & SIMON (1995) were able to obtain DNA nondestructively from dried specimens of a variety of insect taxa simply by soaking them in DTAB solution. Unfortunately, we have had little success with this approach for damselflies, although the reasons are unclear.

(2) Heat at 55°C for about 30 min, then at 75-80°C 10 min with occasional agitation.

(3) Extract twice with 175.0 µL chloroform:isoamyl alcohol (24:1), each time saving the upper (aqueous) phase after spinning at full speed in a microcentrifuge for about 2 min.

(4) Add 175.0 µL double-distilled water to supernatant. Then add 21.0 µL of 5% CTAB (cetyltrimethylammonium bromide) in 0.4 M NaCl and leave about 5 min.

(5) Add 30.0 µL 3.0 M sodium acetate, 350.0 µL absolute isopropanol, and 2.0 µL Pellet Paint (Novagen). We usually place at -20°C for about 30 min to overnight, but this may not be necessary.

(6) Spin in microcentrifuge at full speed for 10 min. We use a refrigerated microcentrifuge, but this probably is not crucial.

(7) A bright pink pellet consisting of DNA + Pellet Paint should be visible. Carefully remove supernatant. It is best to pipet out the supernatant, as the pellet often comes loose.

(8) Add about 50 µL 70% ethanol to wash the pellet, let sit 5 min, and then remove alcohol by pipetting. Finally, add about 50 µL 95-100% ethanol and remove after a minute or two.

(9) Dry pellet (air dry or in vacuum concentrator), then resuspend in about 50-100 µL of 1X TE (or water; TE is safer for long-term storage; 1X TE consists of 0.001 M Tris/HCl pH 7.5 and 0.0001 M EDTA). For PCR, 0.5-2.0 µL of this damselfly DNA usually yields successful amplification for a 12.5 µL (total volume) PCR reaction.

Notes. – (1) Pellet Paint (a DNA co-precipitant) is available from Novagen (PO Box 88641, Milwaukee WI USA 53288-0641; web site: http://www.novagen.com). Its use is not essential (and it is quite expensive), but it seems to increase DNA yields and renders the pellet easily visible. This is particularly helpful when extracting DNA from specimens preserved in suboptimal ways (i.e. when yields are low and the DNA is in poor condition). If DNA yield is to be quantified by spectrophotometry, a correction must be made for the absorbance of the Pellet Paint; information on how to do this is

provided with the product. – (2) Resuspended DNA gradually deteriorates if stored in the refrigerator, even if it is in TE. It is safest to freeze most of the resuspended DNA in aliquots, ideally at ultracold temperatures, and keep refrigerated only the portion that will be used for time blocks of about 1-2 months.

If amplification is poor following this extraction protocol (especially if the cuticle was ground along with muscle tissue), we sometimes carry out a subsequent purification step in which we use the solutions and columns in the blood DNA purification kit from Qiagen (9600 De Soto Ave., Chatsworth CA 91311; phone 800-426-8157). In doing so, we follow the kit directions except that we bypass the initial protease treatment step and start with our resuspended DNA sample, and when we elute the DNA from the column we use a maximum of 100 µL of 70°C 1X TE.

DNA AMPLIFICATION

Many authors have presented protocols for DNA amplification by PCR. Here we present our standard protocol, and offer brief suggestions for methods that we have found useful in amplification of DNA from odonates. For a much more detailed review of the general subject, we recommend PALUMBI (1996) and references therein.

Most published PCR protocols involve reaction volumes of 50-100 µL. For most applications, we find this wasteful, and we almost always use total volumes of 12.5 µL. When optimizing PCR conditions or experimenting with new primers, 6.25 µL reactions can be performed to further minimize use of reagents. At these low volumes, use of an overlay is recommended to minimize evaporation, even if using a thermal cycler with a heated lid (we use wax overlays; see below). Our protocols were developed using thermal cyclers from MJ Research (PTC-100 and MiniCycler); there are numerous other thermal cyclers on the market, and most perform very well.

Our standard "hot start" PCR reactions are performed as follows:

(1) Add 15 µL of PCR wax (we use "Chill-Out 14" from MJ Research, 149 Grove St., Watertown MA 02172, 800-729-2165) to a 0.5 or 0.2 µL PCR tube. Then add 0.5-1 µL of resuspended DNA (see above). Place on ice. The DNA template solution forms a "bead" in the wax, and the wax hardens when cooled. Thus, when the reaction master mix (see below) is added, it is separated from the template until heated, which minimizes artifacts due to polymerase activity at suboptimal temperatures. We always include a negative control (water instead of DNA template solution) to ensure that there is no contamination of reagents by foreign DNA.

(2) To make a reaction master mix, add together the following in the order that they are listed (these volumes are for each 12.5 µL reaction that is to be performed):
   (a) Double-distilled water: Use enough that the final reaction volume (including DNA template) will be 12.5 µL.
   (b) PCR buffer (use the standard buffer that comes with the Taq polymerase that you are using): 1.25 µL.

(c) Primers: Use 0.2 µL of each of the two primers, each at a concentration of 10 µM prior to addition to the master mix.

(d) Taq polymerase (other thermostable polymerases may be used, but Taq is the most widely available and generally applicable): 0.5 unit.

(e) Other components: We usually adjust the MgCl2 concentration so that it is 2.5 mM, but optimal magnesium ion concentration depends on the particular template/primer combination and may require experimentation. We often add 0.1-0.5 µL of dimethylsulfoxide (DMSO) to 12.5 µL PCR reactions; this seems to enhance amplification (at best) or have no effect (at worst). PALUMBI (1996) discusses use of DMSO and other PCR enhancers; however, his recommended upper limit of 1% for DMSO concentration is too low in our experience, and concentrations of up to 5% may prove useful .

(3) Add enough reaction master mix on top of the tubes containing wax and DNA template that the final volume will be 12.5 µL. Keep on ice until ready to place in thermal cycler. Do not place in thermal cycler until its temperature exceeds 90°C.

Cycling conditions: These vary, but typically are as follows:

- 1 X 95°C, 2 min/42-55°C 30 s/72°C 30 s-2 min
- 35 X 94°C 30 s/42-55°C 30 s/72°C 30 s-2 min
- 1 X 72°C 5 min
- 1 X 4°C indefinitely.

Specific details of PCR temperature cycling depend on the following:

(1) Annealing temperature (temperature at which primers bind to template, listed above as 42-55°C) depends on how well matched the primers are to the template. When starting out — particularly if using "universal primers" (those designed to work in a wide range of organisms) — an annealing temperature of 45-48°C is usually a good place to begin. If amplification occurs but non-specific bands are present (i.e. bands that are a different size than expected, usually smaller), annealing temperature can be increased until (ideally) this ceases to occur. In general, the highest annealing temperature that gives reasonable amplification is desirable.

(2) Extension time (period during which the polymerase copies the sequence, typically at 72°C) depends on the length of the sequence to be amplified. A general rule of thumb is to allow one minute of extension time for every kilobase to be amplified.

(3) Number of cycles: Fewer cycles generally give fewer artifacts, but it may be necessary to increase cycle number to obtain sufficient DNA for sequencing. We rarely exceed 50 cycles, and often 30 is sufficient.

(4) Amount of template DNA: 0.5-2.0 µL of template DNA extracted as described above is normally appropriate for a 12.5 µL PCR reaction. However, more or less may be necessary, depending on the particular template and the region to be amplified. If amplification is poor, or if the sample is being used

for PCR for the first time, it is best to try several amounts of template DNA, typically ranging from 0.2-2.0 μL.

Following PCR, we electrophorese 2.0 μL of the product on a 1.0-1.5% agarose minigel, stain with ethidium bromide, and view on an ultraviolet transilluminator to check quality and yield. If a single, well defined band of the expected size is visible it should be sequenceable using the protocol described below, even if the fluorescence is very weak.

CHOICE OF PRIMERS

Many potential primers that have proven successful for amplification of insect mtDNA are listed by SIMON et al. (1994), and are available in the insect mtDNA primer kit described below (see Discussion). The insect nuclear primer kit described below (see Discussion) provides a good starting point for experimentation with nuclear sequences, and the accompanying documentation cites relevant references. In general, the best strategy when working with a new group of organisms for which specific primers are not available is to start with "universal" primers (those designed to work in a wide range of organisms), obtain initial sequence, then design taxon-specific primers nested inside the original primers for further PCR and sequencing (see PALUMBI, 1996 for detailed recommendations on primer design).

DNA SEQUENCING

Our approach to sequencing is manual, and involves use of radioisotopes. We have used a wide variety of DNA sequencing methods, including Amersham/USB's Sequenase kits with ^{35}S-labelled dATP, and various cycle sequencing kits using internal labelling with ^{35}S or end-labelling of the sequencing primer with ^{32}P. However, we have obtained the most consistent and easily interpretable results using the recently introduced Thermosequenase cycle sequencing kit with ^{33}P-labelled terminators (Amersham/USB catalog # 188403; includes sequencing reagents and labelled ddNTPs; P.O. Box 22400, Watertown MA, 800-323-9750). This approach is based on the original SANGER et al. (1977) enzymatic sequencing method, but the dideoxynucleotides that are used to terminate growing DNA chains synthesized by the polymerase contain the radioactive label. Thus, only DNA sequences that terminate correctly appear on the sequencing autoradiograph, eliminating many of the artifacts associated with other sequencing methods. Using this kit, we obtain clear sequence, can read very close to the primer, and can run sequencing gels for various lengths of time (see below) that enable us to obtain as much as 400+ bases of unambiguous sequence. As with all sequencing kits, we invariably cut the recommended volumes of all reagents in half, as this generates ample amounts of sequencing reaction and allows twice the number of sequencing reactions to be

conducted as stated in the directions.

Prior to sequencing PCR products, we treat 0.5-1.0 μL of PCR product with 5.0 units of exonuclease I and 0.5 units of shrimp alkaline phosphatase (both available from Amersham/USB). Exonuclease I degrades leftover primers, and the phosphatase renders leftover dNTPs unusable so that they do not interfere with subsequent sequencing reactions. We treat the PCR product with these enzymes for 15 min at 37°C (both enzymes are active in the PCR buffer), then heat to 80°C to denature the enzymes. No further treatment of PCR products is necessary. We then carry out sequencing reactions as per kit directions, with the following modifications:

(1) We use half volumes of all reaction components as described above.

(2) We mix together the termination master mix provided with the kit and the appropriate labelled ddNTP, add these to the termination tubes, and overlay these with 8 μL of the PCR wax described above. Then we chill the termination tubes, allowing the wax to harden, and mix the remaining reaction components (including treated template DNA and sequencing primer) separately; the appropriate amount is then added to each of the termination tubes. The termination tubes are then transferred directly from ice to thermal cycler when the temperature of the cycler is over 90°C. Normally we use the dGTP termination master mix provided with the kit (but see below for an alternate protocol using dITP). With the dGTP mix we use the following cycling parameters for most template/primer combinations:
   - 1 X 96°C 2 min/55°C 30 s/72°C 1 min
   - 34 X 94°C 30 s/55°C 30 s/72°C 1 min
   - 1 X 4°C indefinitely.

Following temperature cycling, we add 3.0 μL 95% formamide/ 25 mM EDTA/ 0.05% bromophenol blue/ 0.05% xylene cyanole stop to each termination tube, yielding a final volume of about 6.5 μL per termination tube.

An option with the use of this sequencing kit is use of dITP (deoxyinosine triphosphate) in place of dGTP in the termination master mix. This is useful for sequences that are prone to artifacts such as "compressions" (in which bands appear across multiple lanes of the sequencing autoradiogram and/or band spacing is locally distorted). Such artifacts are often due to G-C pairing that causes secondary structure; substituting inosine, which does not bond with other bases, reduces or eliminates these problems. The only drawback to using dITP is that long extensions at temperatures below the optimum for Taq polymerase are necessary. We have had good success with dITP termination reactions using the cycling parameters described above for dGTP termination reactions, with the following modifications: (1) extension temperatures are reduced from 72 to 60°C, and (2) each extension is conducted for 10 min instead of 1 min.

Sequencing is conducted on 35 X 43 cm, 8%, 19:1 acrylamide:bisacrylamide gels using the glycerol tolerant buffer described in the instructions for the sequencing kit (a 20X stock consists of 1.78 M Tris, 0.58 M taurine, and 0.01 M Na_2 EDTA).

Gels are generally run at 32 mA, and 1-2 μL of each sequencing reaction is loaded per lane; normally the same samples are run separately three times for different lengths of time to obtain clear resolution of sequence regions different distances from the primer. "Short" gels are run for about 3 h and yield sequence up to about 200 bp; "long"gels are run for 7-8 h and yield sequence from about 150-300 bp; and "extra-long" gels are run for about 13 h and yield sequence from about 250--400+ bp from the primer. After running the gel, it is fixed for about 15 min in 20% ethanol, dried into Whatman 3M filter paper at 80°C, and exposed to X-ray film. We have obtained excellent results using Kodak Biomax MS film; it is relatively expensive compared to some other X-ray films, but allows short exposure times (usually about 24-48 h). Exposure time can be further minimized through use of Kodak's Transcreen LE, an intensifying screen designed for use with low-energy isotopes such as ^{33}P (note that exposures using these screens must be conducted at ultralow temperatures). Using the combination of the above film and intensifying screen, typical exposure times range from overnight to 24 h.

DISCUSSION

In the above protocols, we describe our approach to extraction, amplification, and sequencing of DNA from coenagrionid damselflies. As we have indicated, there are numerous methods available to accomplish these steps, but through trial and error we have concluded that this approach works very well for odonates, and probably would also work well for many other groups of arthropods. We have focused on amplification of DNA by standard PCR methods. Some workers use reverse transcriptase (RT) PCR (see KAWASAKI, 1990) to generate cDNA from RNA templates (e.g. FRIEDLANDER et al., 1996). Although we have not yet used this technique, the experience of one of us (PTC) in RNA manipulations suggests that great care will be necessary in preserving RNA for extraction, as it is much more prone to degradation than DNA. Given the increasing interest in use of nuclear protein coding sequences, many of which are single copy and have extensive introns, we expect that RT-PCR will become a widely used technique in insect molecular systematics, and that the need for fresh or flash-frozen specimens will grow.

There is an active community of workers using nucleic acid methods to investigate insect systematics at various levels. The Internet list "Bug-net" provides an excellent forum for communication among members of this group, and we encourage prospective and beginning insect molecular systematists to subscribe (this is accomplished by contacting Dr B. Crespi at crespi@sfu.ca). Two very useful insect primer kits are available from the Nucleic Acid-Protein Service Unit at the University of British Columbia, one for mitochondrial DNA and the other for nuclear DNA. These kits include a variety of primer pairs that have proven suitable for PCR amplification of diverse mitochondrial and nuclear regions. They can be obtained by contacting Dr J. Hobbs (Nucleic Acid-Protein Service Unit, Biotechnology Laboratory, Room 237 Wesbrook Bldg, 6174 University Blvd, University of British Columbia, Vancouver, B.C., Canada V6T 1Z3; e-mail hobbs@unixg.ubc.ca). As indicated above, we would be happy to share our experiences with amplification of odonate DNA with others, and will

provide primer sequences and details of amplification conditions on request.

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