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Effect of Propylene Glycol Concentration on Mid-Term DNA Preservation of Coleoptera

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Abstract

The variety of arthropod specimen preservation protocols has expanded greatly with the increased interest in preservation of molecular traits such as DNA sequences. While "best practices" for DNA preservation exist, practical limitations often preclude their use. To test the efficacy of propylene glycol as a DNA preservative agent, adult specimens of *Cylindera lemniscata* (LeConte) (Carabidae: Cicindelinae) and an Athetini sp. (Staphylinidae: Aleocharinae) were stored in 20%, 40%, 60%, 80%, and 100% propylene glycol preservative at room temperature for up to six months. With the exception of the Athetini sp. preserved in 20% propylene glycol, all other treatments yielded the targeted COI gene sequences (*ca.* 800 base pairs). Propylene glycol appears to be a good preservative for DNA, even at low concentrations and ambient temperatures.

Key Words: molecular studies, preservative, flight intercept trap, emergence chamber, pitfall trap

Until recently, most researchers were primarily interested in preservation of external morphology and sclerotized internal anatomy of arthropod specimens, with little thought given to preservation of internal tissues or genetic material. However, with the utilization of DNA for phylogenetic and other research becoming more common, conscientious collectors and researchers have worked to develop better ways to preserve specimens for molecular applications (*sensu* Szinwelski *et al.* 2012).

The variety of arthropod preservation protocols can be divided into three broad categories, those optimized for preserving: 1) "hard" morphology such as sclerotized external and internal anatomy; 2) "soft" morphology such as internal tissues and weakly sclerotized external anatomy; and 3) "molecular" traits, including DNA sequences (from the specimen itself or from associated organisms or other biological materials), protein, heavy metals, etc. "Hard" and "soft" morphology are generally visualized through magnification and require that the specimen remain intact. "Molecular" traits are generally indirectly visualized and can be extracted from intact or macerated specimens or from a small part of the body, such as a leg. The three categories also apply to preservation of incidental organisms found with arthropod specimens such as endosymbionts, phoretic mites, pollen, and fungi (*e.g.*, Laboulbeniales: Ascomycota).

The preservation protocol for each category is impacted by several factors. The factors that are considered most important include 1) manner of death for all or part of the specimen (*e.g.*, leg), 2) preservative, 3) temperature, 4) taxon, 5) mechanical manipulation (maceration, injection), and 6) time since death (Dillon *et al.* 1996; Frampton *et al.* 2008). Each factor has one to several discrete or continuous dimensions. For example, type of preservative is discrete (ethanol, acetone, propylene glycol), but concentration and pH are continuous. Additionally, some factors may be altered or changed while a specimen is retained, such as preservative type or temperature.

Therefore, a protocol for a given preservation category consists of selecting dimensions associated with preservation factors and a schedule of alterations. For example, when preserving a butterfly for display only, the protocol category is "hard" parts, manner of death can be ethyl acetate but should not be immersion in alcohol, preservative should be "dry", and manipulation should not include maceration, while temperature and time are not issues. But a butterfly preserved for DNA should be killed with ethanol, not ethyl acetate (Dillon *et al.* 1996); it should be preserved in 95–100% ethanol, large-bodied specimens should be injected or macerated, the temperature should be low (*e.g.*, −80°C), and DNA should be extracted within a few years.

Dimensions of factors may need to be altered if multiple preservation categories are applied to the same specimen. A protocol to preserve both the external morphology and DNA of a particular butterfly specimen might include death by freezing or ethanol injection, followed by rapid preservation

through drying, and DNA extraction within a few years. Or the specimen may be disarticulated, with one piece following a "molecular" preservation protocol, while the remainder follows a "hard" parts protocol. Disarticulation is generally not an option for small specimens, such as mites, Ptiliidae, or leptotyphline staphylinids.

The great number of permutations of categories, factors, dimensions, and alterations (>100,000) preclude any single study from investigating all or even a large portion of possible protocols. Most studies of preservation are relatively specific and investigate techniques associated with a particular set of circumstances (see examples below). While many "best practice" protocols exist (Dillon *et al.* 1996; Quicke *et al*. 1999; Tayutivutikul *et al*. 2003), circumstances generally require researchers to compromise when faced with realities of collection technique, availability of resources, shipping regulations, etc.

Standard adult Coleoptera collection techniques include long-term active and passive trapping, such as pitfalls, bait traps, emergence traps, and various designs of flight intercept traps (Schauff 2001; Ferro and Carlton 2011). Specimens collected using these techniques are often killed in the field and left at ambient temperatures for days or weeks in preservatives that can become diluted over time. An increasingly important killing and preservation agent used for long-term trapping is propylene glycol due to its low toxicity, absence of flammability, low evaporation rate, and antibacterial properties that preserve external morphology even at low concentrations (approximately 13.5%) (Thomas 2008).

Initial studies have shown that propylene glycol preserves DNA. Rubink *et al.* (2003) conducted experiments on DNA preservation protocols in honey bee (*Apis mellifera* L., Hymenoptera: Apidae) specimens. All specimens were initially killed and preserved in 92% propylene glycol. Time, temperature, and preservative were altered to create several trials; in the most extreme, specimens were left in propylene glycol for 90 days at 40°C before being transferred to 95% ethanol and held at 4–6°C. Amplifiable DNA was recovered from all samples.

Vink *et al.* (2005) tested numerous DNA preservation protocols using spider and scorpion specimens. One protocol stored specimens in "95.5%+" propylene glycol preservative for 42 days at room temperature before transferring specimens to 95% ethanol at 4°C for one day. "High quality DNA" was recovered from the propylene glycol-preserved specimens and propylene glycol was recommended when refrigeration is not available.

Stevens *et al.* (2011) tested numerous specimen preservation protocols using *Tribolium castaneum* (Herbst) (Tenebrionidae) and *Rhyzopertha dominica* (F.) (Bostrichidae). Their "Experiment 3" was conducted to test the effect of water content on the efficacy of propylene glycol as a DNA preservative. The killing/preservative agents of the two treatments were 100% propylene glycol and 80% propylene glycol/20% deionized water (vol/vol). In both treatments *T. castaneum* were killed in the preservative and held at 30°C for 3, 7, and 14 days. Specimens were removed, washed, placed in absolute alcohol and stored at −80°C. Specimens stored in 80% propylene glycol yielded slightly, but statistically significant, lower amounts of PCR product compared to specimens stored in 100% propylene glycol.

Sokolova *et al.* (2010) recovered useable DNA from microsporidian parasites of Psocoptera preserved in propylene glycol. Specimens were collected in traps deployed for 42 days (late June–mid August) and killed/preserved in *ca.* 45% propylene glycol. After retrieval, specimens were transferred to 100% ethanol and stored at room temperature. Not only was useable DNA recovered, but robust morphological characters of the microsporidian spores were suitable for limited analysis by electron microscopy.

Moreau *et al.* (2013) tested numerous specimen preservation protocols using several ant species. Two of their protocols consisted of using 100% propylene glycol as a killing and preservative agent. Specimens were held at room temperature for six months, then half were washed in sterile water and placed in 95% ethanol, while the others were undisturbed. All samples were held at room temperature until DNA extraction at six and 10 months. Amplification of mitochondrial, nuclear, and host-associated bacterial markers was most successful when ethanol or propylene glycol were used as preservatives.

With the exception of research by Sokolova *et al.* (2010), the effect of propylene glycol at concentrations below 80% has not been tested. Testing lower concentrations is critical since field conditions, such as rain, can easily dilute propylene glycol used in long-term traps to below 50% (personal observation) and specimens may be held in suboptimal conditions for months, especially during and after international expeditions. The purpose of this research is to explore the effect of low concentrations of propylene glycol on DNA preservation at room temperature over a six-month period.

Material and Methods

Two species, *Cylindera lemniscata* (LeConte) (Carabidae: Cicindelinae) and an Athetini species (genus undetermined, but all were the same

species) (Staphylinidae: Aleocharinae), were used in this study to represent relatively large (~10 mm) and small (~3 mm) specimens, respectively. Specimens were collected on 8 September 2012 using ultraviolet/mercury vapor light in Hidalgo County, NM, at the Painted Pony Resort (N31.904° W109.009°). Specimens were killed in 100% ethanol and kept at ambient temperature. Specimens were transported to the laboratory in Baton Rouge, Louisiana for a period of two days and were then transferred into vials containing 20%, 40%, 60%, 80%, or 100% propylene glycol (Neogen Corporation, Item No. 79231), or 80% or 100% ethanol (seven treatments total). Total volume of each vial was 100 ml, all dilutions were vol/vol and were made with distilled water. All vials were sealed and kept at room temperature (21°C ±1) for the remainder of the research.

DNA extractions were performed two weeks, three months, and six months after collection on specimens from each of the seven treatments. Each extraction was taken from a leg (femur to tarsus) of *C. lemniscata* and the head and thorax of Athetini sp. A different specimen from each treatment was used for each extraction (3 dates × 7 treatments × 2 taxa = 42 specimens total).

After removal from the treatment solution, the portions of all specimens were washed in 100% ethanol immediately before DNA extraction. Extraction was performed using the Qiagen Blood & Tissue Kit (Qiagen, Hilden, Germany) based on the manufacturer's instructions. Approximately 800 base pairs (bp) of the mitochondrial gene cytochrome c oxidase subunit I (COI) were amplified using the primers Jerry_F (5′-CAACATT TATTTTGATTTTTTGG-3′) and Pat_R (5′-ATC CATTACATATAATCTGCCATA-3′) (Simon *et al.* 1994). Polymerase chain reactions (PCRs) were performed using AccuPower PCR Premix (Bioneer, Daejeon, Korea), consisting of 1 Unit of Taq DNA polymerase, 250 μM of dNTP, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5 mM of $MgCl_2$, and premixed stabilizer and tracking dye. For each reaction, 1 μl of template DNA, 1 μl of each primer, and 18 μl of distilled water were added into each premixed 0.2-ml tube, resulting in a final concentration of 5 pmole. The amplification procedure was five minutes of activation at 95°C, 35 cycles of one minute of denaturing at 95°C, one minute of annealing at 50°C, one minute and 30 seconds of extension at 72°C, and five minutes for final elongation at 72°C. PCR products were then visualized using gel electrophoresis techniques.

Successfully amplified products from the six-month trial were purified using MEGAquick-spinTM Total Fragment DNA Purification Kit (iNtRON Biotechnology, Sungnam-city, Korea). Amplified and purified samples were sent to Eurofins MWG Operon (Huntsville, Alabama, USA) for sequencing. The raw sequence data were read and edited using Chromas Lite 2.1.1 (Technelysium Pty Ltd., Australia) and BioEdit (Hall 1999). Alignments of sequences were performed using ClustalX 2.1 (Larkin *et al.* 2007) and confirmed manually. Sequences were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank/) and compared using NCBI Blast™ with sequences provided by other researchers.

Results

DNA product was recovered from all treatments at all time intervals (Fig. 1), with the exception of Athetini sp. preserved in 20% propylene glycol, which yielded no useable product at any date. Additional attempts to recover DNA from specimens of Athetini sp. preserved two weeks in 20% propylene glycol also failed (data not shown). Some Athetini sp. showed weak ~200 bp bands (*e.g.*, 100% EtOH at six months), but these were not recovered after purification and had no effect on sequencing. Bands associated with DNA product from *C. lemniscata* were weak for 20% and 80% propylene glycol at six months, but DNA was successfully purified and sequenced.

DNA extracted from different concentrations of propylene glycol from specimens stored for six months was of sufficient quality to amplify the targeted COI sequence. Thirteen sequences of approximately 800 bp each were amplified; seven of *C. lemniscata* and six of Athetini sp. (Table 1). Comparison of sequences from *C. lemniscata* with other sequences in the BLAST® database showed greatest alignment with the COI gene from *Cylindera elisae* (Motschulsky). Only one sequence of COI from *C. lemniscata* was previously in the database (accession # KC963869), but, since it was a much smaller (633 bp) fragment, the alignment score was low.

The same comparison in BLAST® using our sequences from Athetini sp. showed greatest sequence identity with COI genes of several genera, all within the tribe Athetini. Athetini is a large, taxonomically difficult tribe that currently contains 64 genera in North America (Newton *et al.* 2001). A complete revision is needed. Gusarov (2002a–e, 2003a–e, 2004a–b) has greatly contributed to our knowledge of many genera, and Elven *et al*. (2010) provided the first molecular phylogeny of the tribe.

Discussion

The results of this exploratory study show that useable DNA can be obtained from specimens preserved at room temperature in low concentrations

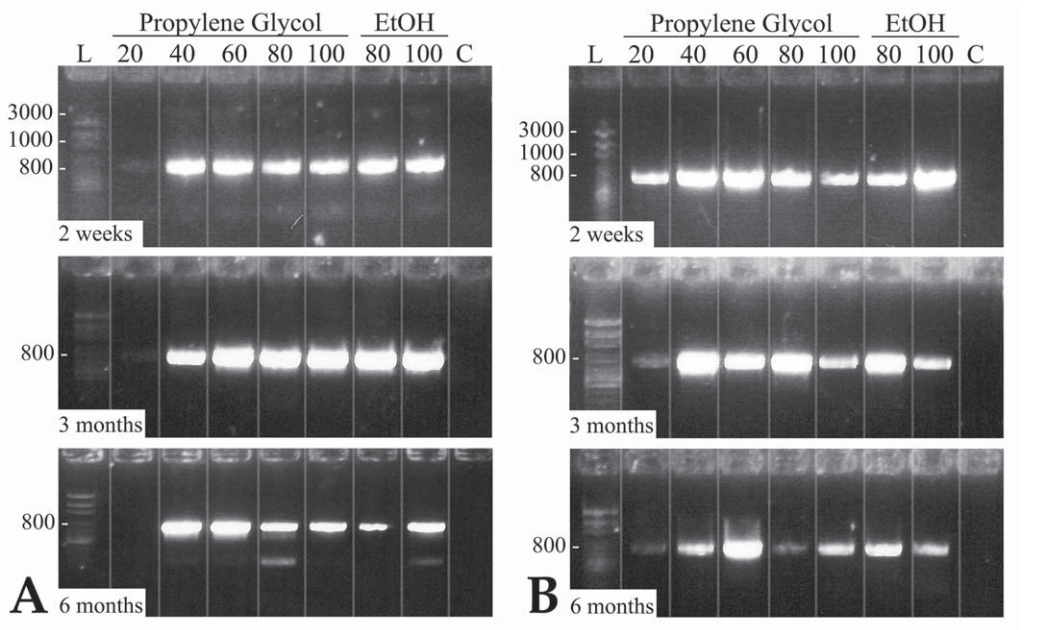

Fig. 1. Gel electrophoresis results showing amplification of mitochondrial COI, ~800 bp, at two weeks, three months, and six months for A) Athetini sp. and B) *Cylindera lemniscata*. The lane marked L denotes the molecular weight standard, quantities represent percentage of preservative agent (20 = 20% propylene glycol, etc.), and C denotes the negative control.

of propylene glycol (~20–40%) for mid-term periods of time (up to six months). These results agree with the findings of Sokolova *et al.* (2010), but expand preservation time up to six months. Based on these results, when propylene glycol is used as a preservative, "stressed" specimens, such as those from flooded traps or specimens kept at ambient temperature in the field for several weeks or months, may still have recoverable DNA and should be curated as molecular grade specimens as soon as appropriate resources are available. A particularly relevant aspect of this work is further illustration that propylene glycol is an appropriate alternative preservative when shipping specimens or in other situations when use of toxic/flammable preservatives is not allowed. Replacement of ethanol with small amounts of propylene glycol has come into wide use by the systematics community, so knowledge about the potential effect of this treatment on preservation of genetic material is desirable.

The preservation protocols we used were designed to simulate conditions of specimens killed in the

Table 1. List of species, treatment, and GenBank accession numbers for COI sequences.

Species	Treatment	GenBank accession #
Athetini sp.	40% propylene glycol	KF272921
Athetini sp.	60% propylene glycol	KF272922
Athetini sp.	80% propylene glycol	KF272923
Athetini sp.	100% propylene glycol	KF272924
Athetini sp.	80% ethanol	KF272925
Athetini sp.	100% ethanol	KF272926
Cylindera lemniscata	20% propylene glycol	KF272927
Cylindera lemniscata	40% propylene glycol	KF272928
Cylindera lemniscata	60% propylene glycol	KF272929
Cylindera lemniscata	80% propylene glycol	KF272930
Cylindera lemniscata	100% propylene glycol	KF272931
Cylindera lemniscata	80% ethanol	KF272932
Cylindera lemniscata	100% ethanol	KF272933

field and left at ambient temperature in various concentrations of propylene glycol. However, specimens used in this research were initially killed and preserved in 100% ethanol. Stevens *et al.* (2011) found reduced quality of DNA in specimens killed and preserved in propylene glycol compared to ethanol and speculated that slow penetration of the cells by propylene glycol was the reason. However, Stevens *et al.* (2011) also found that the rate of reduction of quality of DNA preserved in propylene glycol slowed with time. Again, they speculated this was because longer times allowed for greater penetration of cells and consequently reduced DNA degradation.

Therefore, use of 100% ethanol as an initial killing and preserving agent may have resulted in better initial preservation of specimens than if the specimens had been killed in their respective treatment concentrations. However, the specimens studied by Sokolova *et al.* (2010) were killed and preserved in *ca*. 45% propylene glycol and provided recoverable DNA. In our study, after only two weeks no DNA product could be obtained from specimens of Athetini sp. preserved in 20% propylene glycol. For those specimens, at least, any benefits of initial preservation in 100% ethanol appear to have been lost very quickly.

Future studies that include specimen death and initial preservation in 100% propylene glycol with subsequent concentration reduction ("flooded" model) or initial death and preservation in reduced concentration propylene glycol ("economy" model) will help illustrate how initial concentrations of preservative affect DNA preservation.

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