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# DNA preservation: a test of commonly used preservatives for insects

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**Abstract.** Understanding the impact of collection and storage preservatives is important for all specimen-based research, ranging from morphological studies to genetic- and genomic-based research. We evaluated the effectiveness of four commonly used preservatives for their ability to preserve insect DNA for several ant species as well as the DNA from host-associated microbes of one ant species. We made replicated collections of ant specimens of different sizes and from three different environmental climates into four different preservatives (95% ethanol, dimethyl sulfoxide (DMSO), propylene glycol and RNAlater), isolated DNA across two different time periods and performed PCR on all DNA extracts ($n = 180$ samples + 10 controls). Although ethanol returned the best overall results for DNA yield and PCR success, our analyses did not show a significant difference between specimens preserved in ethanol or propylene glycol on the timescales we investigated. We found that average DNA yield was significantly higher when specimens were originally collected in ethanol instead of DMSO, propylene glycol, or RNAlater™ (Applied Biosystems/Ambion). PCR results for both the insect and endosymbiotic bacteria showed a significant advantage for preserving ants in ethanol or propylene glycol over DMSO or RNAlater for room temperature storage. Our findings suggest that collection of insect specimens into ethanol is the preferred method for preserving host and host-associated bacterial DNA, but that propylene glycol is a suitable alternative when ethanol is not available or permitted.

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## Introduction

The use of molecular methods and DNA technologies has brought forward new uses for biological specimens that were not appreciated even a few decades ago. With these new scientific opportunities, biologists have come to realise that how specimens are preserved is an important consideration beyond being deposited in museum and university collections for morphological research. In the past, the manner in which researchers handled specimens and tissues – especially in the field – was often decided without consideration of options bolstered by controlled experiments.

Although there is little debate that ultracold freezing ($\leq -80°C$) (Catzeflis 1991; Post *et al*. 1993; Reiss *et al*. 1995; Dillon *et al*. 1996; Caterino *et al*. 2000; Frampton *et al*. 2008) and high percentage ethanol (Catzeflis 1991; Post *et al*. 1993; Reiss *et al*. 1995; Dillon *et al*. 1996; Huber 1998; Adams *et al*. 1999; Quicke *et al*. 1999; King and Porter 2004; Frampton *et al*. 2008) are effective preservatives for DNA and morphological research for animal and plant tissue alike, maintaining samples at ultracold temperatures in the field is often impractical, and obtaining and travelling with high-concentration ethanol can be difficult in some locations (for example, flying with high-concentration ethanol within the United States is currently prohibited; International Air Transport Association 2012). Further, scientists interested in using passive collecting techniques such as pitfall traps employing ethanol have to consider the issue of the volatile ethanol evaporating if left unattended for an extended amount of time. Dimethyl sulfoxide (DMSO) has been shown to be effective for DNA preservation in some cases; however, it can distort morphological characters useful for species identification (Dawson *et al*. 1998; Kilpatrick 2002; Williams 2007; Frampton *et al*. 2008). Propylene glycol has been suggested to potentially be effective at preserving DNA in concert with freezing the sample in the preservative (Leal-Klevezas *et al*. 2000) or transfer of the specimen from propylene glycol to ethanol for longer-term storage (Rubink *et al*. 2003). RNAlater™ (Applied Biosystems/Ambion, Austin, TX, USA) and propylene glycol have been shown to be significantly more effective preservatives of DNA in arachnids than ethanol (Vink *et al*. 2005), though relatively few studies have investigated RNAlater for DNA preservation, which may in part be due to the fact that this preservative is fairly new to the market and significantly more expensive than other buffers (Vink *et al*. 2005; Williams 2007).

In addition to concerns of preserving the specimen or specific tissue it is also of interest to investigate how collection and storage preservatives affect host-associated microbes. Through controlled studies of commonly used preservatives, we investigated the effect of collection and storage media for DNA-based research of both insects and their host-associated bacteria from several different biogeographical areas.

## Materials and methods

### *Specimen collections*

Specimens were collected from three locations directly into four different preservation media. These geographic locations were chosen to represent significantly different habitats and external environments (including temperature and humidity) and all sites were visited within two months of one another: (1) rainforest habitat in Queensland, Australia; (2) desert habitat in Arizona, USA; and (3) subtropical hammock in the Florida Keys, USA. In each location, four to six ant colonies were targeted and 12–40 individuals (whole, not ruptured or broken) from each colony were collected into 8–10 mL of the following four preservatives: (1) E = ethanol (95% EtOH); (2) D = DMSO (20% DMSO saturated with 5M NaCl); (3) P = propylene glycol (100% food grade); or (4) R = RNAlater. The samples were kept in their original collection medium at room temperature until time of DNA extraction. In addition to the initial collections, we subdivided the collected samples after 6 months and moved a portion of the individuals from the original media into 95% EtOH after a single wash in sterile water (EE, DE, PE, RE). Again all samples, regardless of preservation medium, were stored at room temperature until time of DNA extraction.

Species were selected to cover both taxonomic and size breadth. Representatives from seven of the 21 ant subfamilies (15 species) were included and encompassed a wide size range for ants (Table 1). The dry weight of specimens was measured using a Voyager™ V12140 analytical scale (Ohaus, Parsippany, NJ, USA). This scale has a readability of 0.1 mg, which unfortunately was not low enough to accurately weigh some of the smallest ant species. The head width of all specimens was measured using a Leica M205 C microscope with micrometer. When more than one size class of workers was present in a single colony, the same subcaste (major worker) or size class was used for all analyses. Voucher specimens for all collections have been deposited in the scientific collections at the Field Museum of Natural History, Chicago, IL, USA.

### *DNA extraction*

DNA extractions were completed 6 months (E, D, P, R) and 10 months after initial collection (E2, D2, P2, R2) into the original preservation media using the DNeasy™ Tissue Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocols. In addition, the samples that were transferred from the initial collection media into 95% EtOH after 6 months were also extracted at 10 months after initial collection (EE, DE, PE, RE). One entire individual ant was used for genomic DNA extractions for all 15 species across all four initial collection media after six months ($n = 60$) and across all original and 95% EtOH transferred subsamples after 10 months ($n = 120$). In total, 180 DNA extractions were completed from 15 species of ants of varying sizes and from diverse environmental habitats.

### *DNA quantification*

DNA quantity and quality were measured within 1 day of DNA extraction using a NanoDrop™ ND-2000 (Thermo Fisher Scientific, Wilmington, DE, USA) microvolume spectrophotometer. For this method, 1 μL of DNA extraction (without dilution) was loaded directly on the optical surface for nucleic acid analysis.

**Table 1. Specimen collection information**

Location, collection code, and taxonomy are included. Dry weight of whole ant measured in grams (g). Head width is measured in millimetres (mm). Collection preservative codes are as follows: E = 95% ethanol; D = DMSO (20% DMSO saturated with 5M NaCl); P = propylene glycol (100% food grade); R = RNAlater™; * = control (collected into 95% ethanol and kept at −20°C or live specimen)

| Collection location | Collection code | Formicidae subfamily | Genus | Species | Dry weight (g) | Head width (mm) | Collection preservative |
|---|---|---|---|---|---|---|---|
| Australia (rainforest) | CSM0954 | Ponerinae | *Odontomachus* | *cephalotes* | 0.0027 | 3.7 | E,D,P,R |
| | CSM1006 | Formicinae | *Oecophylla* | *smaragdina* | 0.0023 | 3.15 | E,D,P,R,* |
| | CSM1071 | Myrmicinae | *Pheidole* | sp. | 0.0017 | 3.3 | E,D,P,R |
| | CSM1118 | Ectatomminae | *Rhytidoponera* | *metallica* | 0.0044 | 3.75 | E,D,P,R |
| Arizona (desert) | CSM1125 | Myrmicinae | *Pheidole* | *hyatti* | 0.0009 | 2.7 | E,D,P,R,* |
| | CSM1131 | Myrmicinae | *Aphaenogaster* | *cockerelli* | 0.0093 | 3.8 | E,D,P,R |
| | CSM1141 | Formicinae | *Myrmecocystus* | *mendax* | 0.0018 | 2.95 | E,D,P,R |
| | CSM1147 | Pseudomyrmecinae | *Pseudomyrmex* | *apache* | 0.0006 | 1.9 | E,D,P,R |
| | CSM1150 | Ecitoninae | *Neivamyrmex* | *opacithorax* | 0.0004 | 1.45 | E,D,P,R |
| | CSM1151 | Dolichoderinae | *Dorymyrmex* | *insanus* | 0.0004 | 1.55 | E,D,P,R |
| Florida Keys (subtropical) | CSM1213 | Formicinae | *Camponotus* | *tortuganus* | 0.001 | 2.1 | E,D,P,R |
| | CSM1237 | Myrmicinae | *Cephalotes* | *varians* | 0.0007 | 2.95 | E,D,P,R,* |
| | CSM1250 | Formicinae | *Paratrechina* | *longicornis* | 0.0 | 0.95 | E,D,P,R |
| | CSM1269 | Myrmicinae | *Wasmannia* | *auropunctata* | 0.0 | 0.9 | E,D,P,R |
| | CSM1315 | Myrmicinae | *Solenopsis* | *invicta* | 0.0 | 1.3 | E,D,P,R |

### *PCR amplification*

PCR amplification was attempted for two genes (mitochondrial cytochrome oxidase I – cox1 and nuclear long-wavelength rhodopsin – LR) for each sample, and one bacterial gene (*Rhizobiales* rRNA 16S – 16S) was attempted for each *Cephalotes varians* (Smith) sample (only one *C. varians* was available from each preservation method). For cox1, the primers used were LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al*. 1994); for LR the primers used were LR143f (5′-GACAAAGTKCCACCRGARATGCT-3′) and LR639ER (5′-YTTACCGRTTCCATCCRAACA-3′) (Ward and Downie 2005); and for 16S, the primers used were tet199f (5′-GGGGAAAGATTTATTGGTT-3′) and 1513r (5′-TACIGITACCTTGTTACGACTT-3′) (Russell *et al*. 2009). The PCR reactions for the ant genes were all 25 µL with the following recipe: 13.98 µL DI water, 2.5 µL 10× PCR buffer, 1.5 µL $MgCl_2$ (25 mM), 2.5 µL dNTPs (8 mM), 1.2 µL of each primer (10 µM), 1.0 µL BSA, 0.125 µL Roche taq (5 U $\mu L^{-1}$), and 1 µL of DNA template. The 25 µL PCR recipe for 16S consisted of 15.4 µL DI water, 2.5 µL 10× PCR buffer (including 1.5 mM $MgCl_2$), 2.5 µL dNTPs (8 mM), 1 µL DMSO, 1.2 µL each primer (10 µM each) and 0.2 µL Roche taq (5 U $\mu L^{-1}$). In the thermalcycler, after the initial denaturation at 94°C for 2 min, there were 30 (for ant genes) or 35 (for bacterial 16S) cycles of 94°C for 1 min, 48°C, 54°C, or 55°C (cox1, LR, 16S, respectively) for 1 min and 72°C for 2 min, with a final extension at 72°C for 10 min. After PCR, 5.0 µL of sample was run out using electrophoresis on a 1% agarose gel and stained with ethidium bromide for visualisation. Amplification was considered successful when a single band was visualised at the correct size for the individual fragment. A qualitative determination was made for bands that were faint, suggesting that they may be difficult to sequence.

### *Statistical analysis*

We ran statistical tests using the DNA concentration data as well as the PCR success data. First we calculated the mean DNA concentration yield across all colonies for all ethanol (EX = E + E2 + EE), propylene glycol (PX = P + P2 + PE), RNAlater (RX = R + R2 + RE) and DMSO (DX = D + D2 + DE) treatments and used repeated-measures ANOVA on these groups, pairing extractions from the same colonies. We then ran *t*-tests on all pairs of treatments and applied Bonferroni correction for multiple testing. We also performed repeated-measures ANOVA and paired *t*-tests on all combinations of the 12 individual treatments. In order to assess the effect of the change into fresh ethanol after 6 months, we used repeated-measures ANOVA comparing ten-month treatments for each initial preservative. For the PCR data, we first ranked the values (0 = no amplification of either coxI or LR, 1 = one successful amplification of either coxI or LR, and 2 = successful amplification of coxI and LR, all scores counting faint bands as successful) and ran a Kruskal–Wallis rank sum test on both the combined treatments and the twelve individual treatments. We also performed individual paired Mann–Whitney tests on all pairs of the combined treatments.

## Results

### *DNA quantification*

The average DNA yield for each preservation method ($n = 12$) is presented in Table 2 with all individual measurements ($n = 180$

**Table 2. Average concentration of DNA extractions in ng $\mu L^{-1}$ and success (pass/fail and percentage) of PCR for the mitochondrial cytochrome oxidase I (cox1) and nuclear long-wavelength rhodopsin (LR) genes**

The bacterial *Rhizobiales* rRNA 16S (16S) gene has only pass/fail/faint, as this gene was only tested in the *Cephalotes varians* samples. DNA extractions were completed six months after initial collection (E, D, P, R), 10 months after initial collection (E2, D2, P2, R2), and after samples were transferred from the initial collection media into 95% EtOH after six months and then extracted at 10 months after initial collection (EE, DE, PE, RE). For both the pass/fail and percentage numbers for PCR success of the two ant genes, faint bands were counted as successful

| Time in DNA preservative | DNA preservative | Average concentration of DNA extractions (ng/µL) | PCR success Insect Cox1 | % | Insect LR | % | Bacteria 16S |
|---|---|---|---|---|---|---|---|
| 6 months | | | | | | | |
| | Ethanol (E) | 15.9 | 16/16(1 faint) | 100 | 14/16(0 faint) | 88 | Pass |
| | DMSO (D) | 6.7 | 16/16(2 faint) | 100 | 10/16(1 faint) | 63 | Faint |
| | Propylene glycol (P) | 12.0 | 16/16(0 faint) | 100 | 14/16(1 faint) | 88 | Faint |
| | RNAlater (R) | 10.6 | 15/16(2 faint) | 94 | 11/16(5 faint) | 69 | Fail |
| 10 months | | | | | | | |
| | Ethanol (E2) | 15.0 | 15/16(0 faint) | 94 | 14/16(1 faint) | 88 | Pass |
| | DMSO (D2) | 6.4 | 14/16(2 faint) | 88 | 9/16(1 faint) | 56 | Fail |
| | Propylene glycol (P2) | 13.0 | 16/16(2 faint) | 100 | 11/16(3 faint) | 69 | Pass |
| | RNAlater (R2) | 11.6 | 14/16(2 faint) | 88 | 11/16(5 faint) | 69 | Pass |
| | Ethanol (Ethanol) (EE) | 17.2 | 15/16(1 faint) | 94 | 13/16(1 faint) | 81 | Pass |
| | DMSO (Ethanol) (DE) | 6.7 | 13/16(1 faint) | 81 | 9/16(3 faint) | 56 | Fail |
| | Propylene glycol (Ethanol) (PE) | 15.0 | 16/16(1 faint) | 100 | 13/16(5 faint) | 81 | Faint |
| | RNAlater (Ethanol) (RE) | 13.0 | 14/16(2 faint) | 88 | 10/16(5 faint) | 63 | Faint |

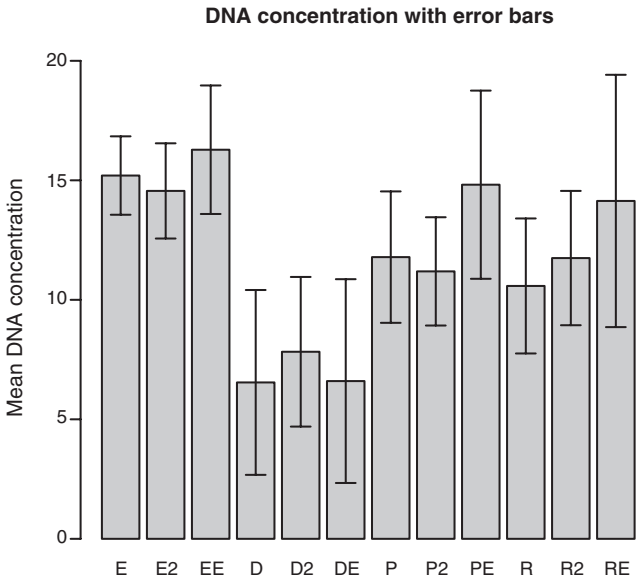


**Fig. 1.** Average DNA yield as measured by Nanodrop (ng $\mu L^{-1}$; Thermo Fisher Scientific, Wilmington, DE, USA) from different collection preservatives. Preservative types include E = 95% ethanol, D = DMSO, P = propylene glycol, and R = RNAlater™. DNA extraction and quantification was completed six months after initial collection (E, D, P, R), 10 months after initial collection (E2, D2, P2, R2), and after samples were transferred from the initial collection media into 95% EtOH after six months and then extracted at 10 months after initial collection (EE, DE, PE, RE). Each sample (total samples = 160) was measured twice and then the average for each preservative was calculated across all samples for each of the four preservatives.

samples + 10 controls) presented in Supplementary Table S1. The average yield was the greatest for samples collected in ethanol and extracted 10 months after collection, and the lowest yield was for samples collected in DMSO and extracted 6 months after collection (Fig. 1). Repeated-measures ANOVA of combined preservative treatments are significant ($F = 7.987$, df = 3, $P = 0.0002518$). The paired *t*-tests of the mean values of combined preservatives yielded significant differences between three of the six pairs after Bonferroni correction (for each pair, the first preservative listed had the higher yield): EX-DX ($t = 5.569$, df = 44, $P = 1.361 \times 10^{-6}$), EX-PX ($t = 2.9808$, df = 44, $P = 0.004669$), and PX-DX ($t = -4.476$, df = 44, $P = 5.323 \times 10^{-5}$). Before correcting for multiple tests, there were two additional significant differences: EX-RX ($t = 2.432$, df = 44, $P = 0.01915$) and RX-DX ($t = -2.6427$, df = 76.672, $P = 0.009967$). PX and RX were not significantly different ($t = 0.3033$, df = 44, $P = 0.7631$). Repeated-measures ANOVA of the 12 individual treatments are significant ($F = 3.504$, df = 11, $P = 0.002147$). However, no paired *t*-tests between these treatments are significant after Bonferroni correction. None of the four ANOVAs for each of the combined treatments showed significant differences in DNA concentration between 10-month treatments (E: $F = 0.423$, df = 2, $P = 0.6592$; D: $F = 0.2724$, df = 2, $P = 0.7635$; P: $F = 1.6946$, df = 2, $P = 0.202$; R: $F = 0.6331$, df = 2, $P = 0.5383$).

### *PCR amplification*

PCR of the mitochondrial cox1 marker was fairly successful regardless of preservation medium. At the extreme of the range, three samples failed to be amplified for DNA collected from ants that were originally preserved in DMSO and later transferred to ethanol. Of the 60 DNA samples extracted from ants 6 months after collection into their original preservative (15 samples each for E, D, P and R), only one DNA sample failed to yield a band for the mitochondrial cox1 gene. Propylene glycol was the only preservative that yielded a cox1 band regardless of time and transfer to ethanol (P, P2 and PE) (see Table 2). Amplification of the nuclear LR marker was considerably more problematic than was amplification of cox1. Ethanol preservation (E, E2 or EE) was always the most successful in terms of allowing for amplification of LR when compared with the other preservatives. RNAlater was consistently the least successful preservative for LR amplification. The sample size we used in order to test for the preservation of DNA from ant microbes was small, including just a single ant species since the *Rhizobiales* bacteria is not widely known to be associated with most ant species included here; however, the results from this investigation are consistent with the findings for the preservation of ant DNA with ethanol (E, E2 and EE) producing the most successful PCR results, followed by propylene glycol, then RNAlater and finally DMSO (Table 2).

The Kruskal–Wallis rank sum test was significant for the combined treatments (EX, DX, PX, RX) ($\chi^2 = 11.8551$, df = 3, $P = 0.007896$) but not for the individual treatments ($\chi^2 = 15.2511$, df = 11, $P = 0.1713$). To understand the long-term differences between preservatives, we performed Mann–Whitney tests on all pairs of combined treatments, and the only significant differences we found after Bonferroni corrections were between EX-DX (V = 8, $P = 0.0006436$) and PX-DX (V = 130.5, $P = 0.006668$). Before multiple testing corrections, EX-RX (V = 18, $P = 0.01334$) and PX-RX (V = 88.5, $P = 0.01974$) were also significantly different.

## Discussion

There have been several studies on different organisms to try and determine the best taxon-specific preservative for preservation of DNA from specimens collected in the field, and although high-concentration ethanol has been shown to be a generally effective DNA preservation medium, there are exceptions to that rule (Fukatsu 1999; Vink *et al*. 2005). For DNA preservation of ant specimens and their associated microbes stored at room temperature, we have found that 95% ethanol consistently yields the highest concentration of DNA (Fig. 1), and that both ethanol and propylene glycol allow for the highest success of amplification of the mitochondrial, nuclear and host-associated bacterial markers that we tested (Table 1 and Table S1). However, as mentioned above, there are limitations to the use of ethanol in the field. Travelling by airplane with ethanol at concentrations > 70% is currently illegal in the United States (International Air Transport Association 2012), and shipping high-concentration ethanol can be problematic, with regulations changing depending on the countries and carriers involved. This restriction does not apply to the less-volatile propylene glycol. In addition, it can be a challenge while abroad to gain access to high-concentration ethanol that has

not been denatured, a process that renders the ethanol unsuitable for specimen preservation (Waller and Strang 1996; King and Porter 2004). Another potential problem with using ethanol in the field is the tendency for ethanol to evaporate when left unattended for long periods of time. Other researchers have used 95% ethanol in pitfall traps for the purposes of preserving spiders for DNA analysis, and evaporation proved to be a problem as the ethanol became more dilute and lost its preservation capacity unless used in a trap with a funnel (Gurdebeke and Maelfait 2002). According to available material safety data sheets, the evaporation rate of ethanol and propylene glycol are 3.3 and 0.005 (BuAc = 1), respectively, suggesting that evaporation would not be an issue with propylene glycol in pitfall traps.

DMSO and RNAlater were both relatively poor solutions for DNA preservation of our specimens when the specimens were stored at room temperature. The lowest PCR success we observed was for samples collected originally in DMSO and then extracted 10 months later, with a little more than half of those samples yielding successful amplification of the nuclear LR gene. Transferring the ants from DMSO to ethanol seemed to have little effect on PCR success or DNA yield, at least in the time frame we examined. Of note, DMSO has been shown to alter the wing morphology in insects, and RNAlater can be difficult to rinse off of samples (personal observation), both of which can lead to problems in species identification. Finally, the transfer of specimens from their original collection buffer into fresh ethanol after 6 months did not produce any significant differences in DNA yield or PCR success.

Although we did not specifically test the effects of temperature or rupturing of specimens to ensure that the preservatives penetrated the insect tissues, these certainly can affect the success of preservation medium. Specimen storage temperature is important and several studies have demonstrated the effectiveness of ultracold storage for specimen preservation (Catzeflis 1991; Post *et al*. 1993; Reiss *et al*. 1995; Dillon *et al*. 1996; Caterino *et al*. 2000; Frampton *et al*. 2008). Our studies were purposely conducted at room temperature to mimic the storage conditions found in many institutions and museum collections around the world, but undoubtedly cold storage would improve the effectiveness of DNA preservation for many, if not all, of the preservation medium we tested. In addition, rupturing or breaking open the specimens could improve the effectiveness of DNA preservation by allowing the preservatives to more rapidly penetrate tissues.

In conclusion, our data suggest that while 95% ethanol is the ideal preservative for ant specimens collected in the field – as well as their associated microbes – for molecular analysis, propylene glycol is also a valuable and chemically stable alternative. This stability makes propylene glycol easier to ship or travel with, and also makes it less prone to evaporation when left unattended for long periods of time in passive collecting traps. While this study addresses ants specifically, the results should apply across arthropod taxa and their host-associated microbes.

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