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The effects of preservatives and temperatures on arachnid DNA

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Abstract. We tested the effects of different preservatives and temperatures on the yield of spider and scorpion DNA useable for PCR amplification. Our experiment was designed to simulate conditions in the field and laboratory over a six-week time period, testing the preservatives RNA*later*®, propylene glycol, and various ethanol concentrations. Three replicates of each preservation treatment were stored at five different temperature treatments; –80°C, –20°C, 2–4°C, 19–24°C, and 40°C. DNA was extracted and quality was assessed by electrophoresis on mini-gels, and by PCR amplification of high copy mitochondrial DNA fragments (cytochrome oxidase subunit I) and low copy nuclear DNA fragments (actin). Results show that RNA*later*® and propylene glycol are significantly better than the other preservatives for high quality DNA preservation and that tissue is best stored at –80°C or –20°C. Storage in 95% ethanol is appropriate if specimens are stored at –20°C or –80°C. We believe our results can help guide biologists in choosing preservatives and temperatures for DNA-based research on arachnids, other arthropods and invertebrates in general.

Additional keywords: DNA degradation, DNA preservation, PCR, scorpion, spider.

Introduction

A majority of studies investigating the effects of different preservation techniques on arthropod DNA extraction and quality have been on insects (e.g. Post *et al*. 1993; Riess *et al*. 1995; Dillon *et al*. 1996; Austin and Dillon 1997; Quicke *et al*. 1999; Rubink *et al*. 2003). It is possible to obtain DNA from dried insect specimens (Post *et al*. 1993; Dillon *et al*. 1996; Austin and Dillon 1997; Quicke *et al*. 1999) but this is not an option for arachnids, some insects and many other arthropods, which are typically stored in ethanol to facilitate identification using structures that shrivel when dried (e.g. the epigyne and the pedipalpal bulb of spiders). Ethanol can affect DNA preservation because of oxidative and hydrolytic effects, and the effects of ethanol and other preservatives have been tested to some extent on insects (Post *et al*. 1993; Riess *et al*. 1995; Dillon *et al*. 1996; Quicke *et al*. 1999; Rubink *et al*. 2003). There have been few studies on the effects of preservatives on non-insect arthropods (e.g. A'Hara *et al*. 1998; Gurdebeke and Maelfait 2002) and only limited comparisons of different ethanol concentrations at different temperatures. This study tests the effects of preservatives (including ethanol at two concentrations) at several temperatures on two arachnids that differ in size and body type.

Various claims have been made as to the minimum requirements necessary for the preservation of arachnid tissue that will yield useable DNA, but empirical studies on the effect of different DNA preservatives have been limited. A'Hara *et al*. (1998) found that spider DNA degraded substantially when stored in either ethylene glycol or 70% ethanol at room temperature. Gurdebeke and Maelfait (2002) tested the effects of three different pitfall trap preservatives (70% ethanol, 4% formaldehyde, and a modified Carnoy's solution) on the DNA of the spider *Coelotes terrestris* (Wider, 1834). They found that DNA could not be isolated from modified Carnoy's solution (acetic acid + TE buffer) and that it was only possible to generate a RAPD profile from specimens preserved in 70% ethanol but not from specimens preserved in 4% formaldehyde. Carnoy's solution was also found to be an unsuitable tissue preservative for insect DNA (Post *et al*. 1993; Riess *et al*. 1995).

Spiders and other arachnids to be used in DNA studies are typically stored cold (–20°C or –80°C) in 95–100% ethanol (e.g. Hedin 1997; Wheeler and Hayashi 1998; Vink *et al*. 2002). DNA degradation has been reported in spiders stored in 70–75% ethanol (Hormiga *et al*. 2003; Vink and Paterson 2003) and A'Hara *et al*. (1998) found substantial degradation of spider DNA that had been stored in 70% ethanol at room temperature for less than a month. Short segments (< 300 bp) of mitochondrial DNA (mtDNA) have been amplified from wolf spiders (Lycosidae) stored in 70% ethanol for up to two years (Colgan *et al*. 2002). However, longer segments of mtDNA (≥ 1000 bp) are now more commonly used in DNA based phylogenetic studies (e.g. Hedin and Maddison 2001; Bond 2004), requiring DNA that has not been degraded by poor storage methods. This is also the case for low copy-number nuclear genes, which are being used more

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frequently in molecular phylogenetic studies (e.g. Maddison and Hedin 2003*a*, 2003*b*; Arnedo *et al*. 2004).

We aimed to test the effects of different preservatives and temperatures on the yield of spider and scorpion DNA useable for PCR amplification of high copy mitochondrial DNA and low copy nuclear DNA. The experiment was designed to simulate conditions in the field over a six-week period. An additional experiment is underway to test DNA preservation over a one-year period with preservatives and temperatures commonly found in invertebrate collections and will be reported in a later publication. Our results will be valuable for arachnologists and other invertebrate researchers wanting to successfully preserve tissues in the field for long-term storage of high quality DNA.

Materials and methods

Arachnids

Two arachnid species were used in this experiment: 150 specimens of *Pardosa falcifera* F.O. Pickard-Cambridge, 1902 (Araneae: Lycosidae) and 90 specimens of *Smeringurus mesaensis* (Stahnke, 1957) (Scorpiones: Vaejovidae). *Pardosa falcifera* specimens were collected live from Pine Valley Creek, San Diego County, California, USA (32°50′09″N 116°32′35″W) on Sept 27 2003 and Oct 19 2003. Specimens of *S. mesaensis* were collected live from near Snow Creek Road, Riverside County, California, USA (33°54′49″N 116°40′04″W) on Sept 26 2003. These two arachnids represent a diversity of body types and sizes: *Smeringurus mesaensis* is large (average body length 28.4 mm, $n = 27$) and heavily sclerotised; *Pardosa falcifera* is small (average body length 6.4 mm, $n = 23$) and soft bodied. Voucher specimens of both species have been deposited at the Smithsonian National Museum of Natural History, the American Museum of Natural History and the Department of Biology, San Diego State University.

Specimen preservation

For all treatments except the RNA*later* (Ambion, http://www.ambion.com/, verified June 2005) treatment, spiders were placed directly in 1.5 mL of preservative and scorpions were submerged in 40 mL of preservative after being euthanased by freezing (as per Prendini *et al*. 2002). For all treatments the ratio of tissue volume to preservative volume was ~1:8. Six preservation methods were tested:

1. Spiders and scorpions placed in 95% ethanol and left in 95% ethanol for six weeks. Storage of arachnids and other arthropods in 95–100% ethanol is standard for DNA based studies (e.g. Hedin 1997; Wheeler and Hayashi 1998; Vink *et al*. 2002).

2. Spiders and scorpions placed in 70% ethanol and left in 70% ethanol for six weeks. Museum specimens are usually stored in 70–75% ethanol in order to keep specimens flexible for morphological work (e.g. Martin 1978).

3. Spiders and scorpions placed in 95% ethanol and transferred to 70% ethanol after one day and left in 70% ethanol for six weeks. This method was used because the initial saturation with 95% ethanol may preserve the DNA while the storage in 70% ethanol may keep the specimens flexible for morphological work.

4. Spiders and scorpions placed in 70% ethanol and transferred to 95% ethanol after one week and left in 95% ethanol for five weeks. This method was to simulate the scenario of a researcher doing general collecting into 70% ethanol and then sorting and storing samples for DNA work within a one-week time frame.

5. Spiders and scorpions placed in 99.5+% propylene glycol (Sigma-Aldrich, http://www.sigmaaldrich.com/, verified June 2005) and left for six weeks, then transferred to 95% ethanol for one day at 4°C before extraction. Propylene glycol can be used as a capture medium in pitfall traps, is much less toxic than ethylene glycol, and has been shown to effectively preserve DNA in honey bees (*Apis mellifera* L., 1758) (Rubink *et al*. 2003). The transfer to 95% ethanol followed the protocol of Rubink *et al*. (2003), which allowed the specimen to be dried before DNA extraction.

6. Whole spiders, euthanased by freezing, were placed in 1 mL of RNA*later* (as per manufacturer's protocol, which recommends at least five times the volume of RNA*later* to one volume of tissue). The patella, tibia, basitarsus, telotarsus and pretarsus of leg 4 of the scorpions were placed in 1 mL of RNA*later*. Specimens were left in RNA*later* for six weeks.

In addition to the six treatments above, two further treatments were used to simulate collection of specimens into sub-optimal volumes of ethanol. Two spiders were each placed in 300 μL of 95% ethanol and left for six weeks. Thirty spiders were placed in 5 mL of 70% ethanol, stored for one week at room temperature, and then transferred to 1.5 mL of 95% ethanol for five weeks. Both treatments simulated a collector having limited amounts of ethanol in the field, and reflecting possible constraints in the amount of cargo or weight they can transport.

Treatment temperatures

Three replicates of each preservation treatment were subjected to five different temperature treatments over the six week time interval: 40°C; 19–24°C (room temperature); 2–4°C (standard refrigerator); –20°C (standard freezer); and –80°C. All specimens were kept in darkness for the duration of the experiment. Temperatures were chosen to represent conditions found in the field and in laboratories. A temperature treatment of 40°C was added as a possible extreme that may be encountered when specimens are stored in hot climates.

DNA extraction and comparison

DNA was extracted from two legs of each spider specimen (usually left legs 3 and 4) and from one patella (left leg 4) of each scorpion specimen using DNeasy tissue kits (QIAGEN, http://www.qiagen.com/, verified June 2005) as per manufacturer's protocol. Muscle tissue in arthropod legs is a good source of both nuclear DNA and mtDNA (Prendini *et al*. 2002). Extracted DNAs were dried down using a Savant Speed Vac SPD101B (Thermo, http://www.thermo.com, verified June 2005), then resuspended in 100 μL of AE elution buffer (QIAGEN) and stored in a –20°C freezer.

DNA yield and quality were compared by electrophoresis of extractions (10 μL of spider DNA or 2 μL of scorpion DNA) on 0.8% agarose mini-gels in 1X TRIS-Acetate-EDTA buffer. DNA was stained using ethidium bromide, and visualised and photographed on an Eagle Eye II system (Stratagene, www.stratagene.com, verified June 2005). Each mini-gel was run with a standardised 0.7 μL of molecular weight marker (Hyperladder I, Bioline, www.bioline.com, verified June 2005), which allowed comparison across different gels.

DNA quality was also assessed via PCR amplification of high copy mtDNA cytochrome oxidase subunit I (*COI*) fragments and low copy nuclear DNA (nuDNA) actin fragments. *Ex Taq* DNA polymerase (Takara, www.takara-bio.co.jp, verified June 2005) was used in all PCR amplifications as we have found that this enzyme formulation is very reliable in amplifications involving DNA of varying quality and concentration. Controls were used in all PCR experiments (i.e. water only, no extraction aliquot). The primers used to amplify a 1058 bp *COI* fragment were C1-J-1718-spider (5′-GGNGGATTTGGAAATTGRTTRGTTCC-3′) and C1-N-2776-spider (5′-GGATAATCAGAATANCGNCGAGG-3′), which were modified from those published in Simon *et al*. (1994) in order to better amplify arachnid DNA. The primer pair used to amplify fragments (between 800 and 3000 bp) of the three to four actin copies were actin-F (5′-ACNAACTGGGATGATATGGAGAA-3′) and actin-R (5′-CCNCCRATCCANACGGAR-

TACTT-3′). These primers were designed using spider cDNA library sequence data for various spider taxa (CJV, CYH and MCH unpublished). We used degenerate actin primers for testing low copy genes rather than species-specific primers because degenerate actin primers would provide a more conservative test of DNA quality.

In order to statistically compare results, actin PCR reactions were classified into three categories: no product, weak product, and strong product (–1, 0, 1 respectively) and were compared using a similarity matrix measuring the association between objects. This was calculated with SIMIL 3.01 in the R Package (Legendre and Vaudor 1991) using a Euclidian distance (D01 in Legendre and Legendre 1998). Mantel tests (Mantel 1967) were then performed with MANTEL 3.01 in the R Package, to test correlations between the similarity matrices and a model matrix (see Legendre and Legendre 1998) coding for (*1*) type of organism (scorpion or spider), (*2*) all pairs of temperatures and (*3*) all pairs of preservatives, using 9999 permutations.

Results

DNeasy tissue kits can recover DNA fragments up to 50 kb in size but most large fragments are ~30 kb (QIAGEN 2003). Although the mini-gel resolution was not sensitive enough to provide assessment of DNA quantity in nanograms, it was possible to grade DNA into four categories (see Table 1), reflecting differences in DNA quantity and maximum size.

Mitochondrial *COI* fragments were amplified from DNA extractions of all treatments, including specimens stored for six weeks in 70% ethanol at 40°C. Therefore, mtDNA amplification success provided no discrimination of the relative success of different treatments. Representative fragments were sequenced to confirm that no DNA other than the arachnid specimens was amplified. Sequences are available on GenBank (accession numbers AY843441–AY843446). There was no indication of multiple bands when visualising the *COI* PCR products, no sign of multiple peaks at any position in the sequencing results, and the sequence coded as expected. Thus, we are certain that our amplifications were of the target mitochondral *COI* gene and not non-target nuclear pseudogenes.

There was no significant difference between DNA preservation method for spiders and scorpions; therefore, temperature and preservative results were compared using Mantel tests as outlined above without distinction between spiders and scorpions. The successful PCR amplification of actin fragments correlated with results from the DNA mini-gels (correlation coefficient $R = 0.726$, $P < 0.00001$) and was the best indicator of relative DNA quality (Table 1). Statistical pairwise comparisons of actin PCR results for preservatives and temperature are shown in Tables 2 and 3 respectively. RNA*later* and propylene glycol were significantly better than all other preservatives and there was no significant difference between these two preservatives; however, only RNA*later* was successful at preserving scorpion DNA at 19–24°C. There was no significant difference between the preservation of DNA in 95% ethanol and 70% ethanol. Initial saturation of specimens in 95% ethanol followed by storage in 70% ethanol was not significantly worse than other ethanol treatments. The least effective preservation treatment was preservation in 70% ethanol with subsequent transfer to 95% ethanol. We are uncertain as to why this treatment should perform worse than specimens preserved in 70% ethanol without subsequent transfer. There were no significant differences between the two sub-optimal treatments and their regular counterpart treatments (95% ethanol and 70% ethanol followed by 95% ethanol).

Except for one scorpion sample, there was no successful amplification of actin from any samples stored at 40°C and every temperature was significantly better than 40°C (Table 3). There was no significant difference between specimens stored at 4°C and 19–24°C. Specimens stored at –20°C and –80°C were significantly better than specimens at all other temperatures but there was no significant difference between –20°C and –80°C over the six week trial.

Discussion

It was possible to amplify ~1000 bp fragments of high copy mtDNA (*COI*) from arachnids stored for six weeks in all common preservatives over a wide range of temperatures. However, to maximize the probability of successful amplification of low copy nuDNA, we suggest that specimens be preserved in RNA*later* (US$240/500 mL) or propylene glycol (≤ US$30/500 mL). It should be noted that RNA*later* is designed to preserve RNA and we did not test whether propylene glycol effectively preserves RNA. It was encouraging to see that propylene glycol effectively preserved high quality DNA at room temperature, as this preservative can be used in pitfall trapping (Rubink *et al*. 2003). Our results were based on 99.5+% laboratory grade propylene glycol, but Rubink *et al*. (2003) successfully used propylene glycol antifreeze (5% water and 3% proprietary additives) to preserve DNA in the laboratory and the field.

It is also preferable to store specimens at –20°C or –80°C. Other researchers have also found that –80°C is the best temperature for insect specimen storage (Post *et al*. 1993; Riess *et al*. 1995; Dillon *et al*. 1996). We found that storage in high percentage ethanol is best if specimens are stored at –20°C or lower. Post *et al*. (1993) found that Diptera were best preserved at 4°C in 100% ethanol, but they did not test specimens stored at –20°C in 100% ethanol, which may have produced better results. We recommend that if 95% ethanol is to be used, specimens should be refrigerated or, preferably, frozen as soon as possible. Dillon *et al*. (1996) found that 100% ethanol preserved Hymenoptera effectively for up to 16 months. However, they did not test for low copy nuclear DNA, which were significantly degraded after just six weeks in the same conditions. Although DNA degradation occurs in tissue stored at room temperature over six weeks in 95% ethanol, we have found that little degradation occurs over 24 h but DNA does start to degrade after five days (personal observation). When collecting in the field where a freezer is not available, we recommend collecting specimens into RNA*later* or propylene glycol in order to best preserve DNA.

Table 1. Results for DNA mini-gels and actin PCR amplification

Preservative treatment	40°C				19–24°C				2–4°C				–20°C				–80°C			
	Mini-gel			Actin	Mini-gel			Actin	Mini-gel			Actin	Mini-gel			Actin	Mini-gel			Actin
Scorpion																				
RNA*later*	1	1	1		3	3	3		2	2	2		3	3	3		3	3	3	
Propylene glycol	0	0	0		0	0	0		2	3	3		2	3	3		3	2	2	
95% alcohol	0	0	0		1	1	2		1	1	2		3	3	3		3	3	3	
70% alcohol	0	0	0		0	1	1		0	1	0		3	3	3		3	3	3	
95% then 70% alcohol	0	1	1		1	0	1		0	0	0		1	2	2		2	1	2	
70% then 95% alcohol	0	0	0		1	0	1		0	0	0		0	0	0		0	0	0	
Spider																				
RNA*later*	0	0	0		2	2	2		2	2	2		2	2	1		3	3	3	
Propylene glycol	0	0	0		3	2	2		2	3	3		3	2	2		0	1	1	
95% alcohol	0	0	0		1	1	1		2	2	2		2	1	0		2	2	1	
70% alcohol	0	0	0		1	1	1		2	3	2		3	3	3		2	2	2	
95% then 70% alcohol	0	0	0		1	1	0		1	1	1		0	0	0		1	1	1	
70% then 95% alcohol	0	0	0		0	0	0		0	0	0		0	0	0		0	0	0	
Sub-optimal 95% alcohol	0	0	0		0	0	0		2	1	2		3	2	2		3	3	2	
Sub-optimal 70% then 95% alcohol	0	0	0		1	0	0		2	1	1		0	0	0		0	0	0	

DNA quality on mini-gel: 0 = very little or no DNA visible and no visible fragments larger than 1000 bp; 1 = weak streak of DNA and no fragments larger than 6000 bp; 2 = strong streak with fragments up to 10 kb; 3 = very strong streak with a large amount of DNA over 10 kb. Actin PCR product: white = none; grey = weak band; black = strong band.

Table 2. Pairwise comparison of preservative treatments
Statistical values were obtained by Mantel tests between similarity matrices of pairwise temperature comparisons and a model matrix (Legendre and Legendre 1998). The amount of variance expressed (R^2) was greater between highly contrasted treatments

	RNA*later*	Propylene glycol	95% ethanol	Sub-optimal 95% ethanol	70% ethanol	95% ethanol then 70% ethanol	70% ethanol then 95% ethanol
RNA*later*	–	–	–	–	–	–	–
Propylene glycol	n.s.	–	–	–	–	–	–
95% ethanol	$P < 0.0002$ $R^2 = 8.88$	$P < 0.0111$ $R^2 = 3.80$	–	–	–	–	–
Sub-optimal 95% ethanol	$P < 0.0205$ $R^2 = 3.26$	n.s.	n.s.	–	–	–	–
70% ethanol	$P < 0.0009$ $R^2 = 6.57$	n.s.	n.s.	n.s.	–	–	–
95% ethanol then 70% ethanol	$P < 0.0001$ $R^2 = 11.50$	$P < 0.0024$ $R^2 = 5.97$	n.s.	n.s.	n.s.	–	–
70% ethanol then 95% ethanol	$P < 0.0001$ $R^2 = 19.39$	$P < 0.0002$ $R^2 = 11.72$	n.s.	$P < 0.0056$ $R^2 = 4.20$	$P < 0.0186$ $R^2 = 3.51$	$P < 0.0190$ $R^2 = 3.35$	–
Sub-optimal 70% ethanol then 95% ethanol	$P < 0.0001$ $R^2 = 16.47$	$P < 0.0011$ $R^2 = 6.134$	n.s.	$P < 0.0023$ $R^2 = 6.44.$	n.s.	n.s.	n.s.

n.s. = Not significant.

Specimens should then be refrigerated or frozen as soon as possible and stored at –20°C on return to the laboratory.

We do not suggest that RNA*later* and propylene glycol be used to store specimens for morphological analyses. It appears that propylene glycol may cause soft tissue shrinkage in specimens (M. J. Ramirez, personal communication) and RNA*later* crystallises in 95% ethanol (personal observation). We recommend that legs be preserved for DNA work in RNA*later* or propylene glycol and the rest of the specimen stored in 70% ethanol for morphological work.

Although our results are based on arachnid species, we believe that these results are a significant contribution to our general knowledge of the preservation of arthropod tissue for DNA-based research and are potentially applicable to other arthropods and invertebrates. We hope that this will guide biologists in the best choice of preservatives and temperatures for preserving invertebrate tissue for current and future DNA-based research.

Table 3. Pairwise comparison of temperature treatments
Statistical values were obtained by Mantel tests between similarity matrices of pairwise preservative comparisons and a model matrix (Legendre and Legendre 1998). The amount of variance expressed (R^2) was greater between highly contrasted treatments

	40°C	19–24°C	2–4°C	–20°C
40°C	–	–	–	–
19–24°C	$P < 0.0002$ $R^2 = 8.76$	–	–	–
2–4°C	$P < 0.0001$ $R^2 = 12.45$	n.s.	–	–
–20°C	$P < 0.0001$ $R^2 = 26.62$	$P < 0.0002$ $R^2 = 9.50$	$P < 0.00001$ $R^2 = 7.23$	–
–80°C	$P < 0.00001$ $R^2 = 34.13$	$P < 0.0001$ $R^2 = 12.53$	$P < 0.00001$ $R^2 = 9.91$	n.s.

n.s. = Not significant.

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