Eur. J. Entomol. **111**(2): 175–179, 2014
doi: 10.14411/eje.2014.024
ISSN 1210-5759 (print), 1802-8829 (online)

A goodbye letter to alcohol: An alternative method for field preservation of arthropod specimens and DNA suitable for mass collecting methods

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Key words. Insects, beetles, DNA preservation, flight intercept traps, Malaise traps, pitfall traps

Abstract. Despite its limitations, ethanol remains the most commonly used liquid for the preservation of arthropod specimens and their DNA in the field. Arthropod ecology and taxonomy have witnessed a substantial increase in the use of various trapping and molecular methods in the past two decades. However, the methods of collecting and the preservation liquids most widely used in arthropod traps do not properly preserve DNA. Trap-collected specimens are typically of limited utility for molecular studies due to the poor preservation of DNA. A stable and cheap substance that can be used to trap arthropods in and preserve their DNA is therefore needed. Here we test whether (i) 2% SDS, 100mM EDTA, (ii) 1% SDS, 50mM EDTA and (iii) 0.66% SDS, 33mM EDTA can preserve DNA of small and medium-sized beetles for one, four and eight weeks. Preservation of DNA was tested using PCR amplification of parts of the mitochondrial cytochrome c oxidase I (Cox1) and nuclear 28S rRNA genes. All the solutions tested preserved DNA for at least up to eight weeks and we recommend 2% SDS and 100mM EDTA as a cheap, stable and easily transportable alternative to ethanol for preserving specimens and their DNA collected in the field. This solution is also suitable for using as the collection and preservation liquid in arthropod traps.

INTRODUCTION

Bottles and drums of alcohol for preserving samples have been an indispensable part of entomological equipment since the early days of insect exploration (e.g. Wallace, 1853). The increasing use of various trapping (e.g. Malaise, flight intercept, pitfall traps) and rearing methods (e.g. Pokon et al., 2005; Hulcr et al., 2007; Vodka et al., 2009) over the past few decades has led to a corresponding increase in the demand for liquids suitable for collecting and preservation of specimens in the field. The trapping and rearing of insects often occurs over long periods of time and therefore hundreds to thousands of liters of preservation liquids are needed for extensive sampling programmes. The transportation and storage of these liquids may become one of the most expensive and logistically challenging parts of fieldwork, especially in remote areas. They also pose safety problems for air transport. Denatured ethanol, formaldehyde and ethylene glycol are the most often used collecting and preservation liquids. However, they tend to degrade the DNA in the specimens to various extents.

The use of molecular methods in arthropod ecology and systematics has greatly increased over the past years. Decreasing costs have made DNA analyses available for various applications, such as the identification of morphologically similar species, clarification of species concepts in taxonomically poorly known groups, matching adult and subimaginal stages of the same species and identifying trophic interactions (e.g. Sheppard & Harwood, 2005; Miller, 2007; Kent, 2009; Hrcek et al., 2011; Lehmann et al., 2012). Molecular methods require specimens in which the DNA is well-preserved. Deep freezing and rapid drying are the most effective methods for storing specimens (Post et al., 1993) but mostly unavailable in the field. Analytical-grade ethanol has become the most commonly used preservation liquid for the storage of arthropod samples to be used in DNA analyses (Reiss et al., 1995), although other liquids are also used (e.g. Bisanti et al., 2009; Stoeckle, 2010; Szinwelski et al., 2012). The use of ethanol, however, has several limitations, including the poor efficiency of low concentrations of ethanol in preserving DNA (Bisanti et al., 2009; Nagy, 2010). This limits its use in various insect trapping and rearing devices as their design mostly does not prevent the rapid evaporation of ethanol during the course of sampling. When ethanol is used as a preservative, samples need to be collected frequently, often daily, if they are intended for DNA analyses (Szinwelski et al., 2012). This may be a problem, particularly when attempting an extensive sampling programme using many traps, or traps that are difficult to access, such as those placed in the forest canopy. Apart from being volatile, analytical quality ethanol is also expensive. Last but not least, although the psychoactive effects of ethanol on humans may sometimes be useful during fieldwork and research in general (Jarosz

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et al., 2012) they make the logistics of its storage, transportation and use more complicated.

Today, there is no widely available liquid that can be used to preserve samples of arthropods in traps, which will preserve DNA for long periods of time, i.e. at least one to two weeks and thus allowing less frequent visits to traps. Consequently, the unstable quality of samples obtained from traps limits their use in subsequent molecular studies of arthropod ecology and systematics. Therefore, there is a demand for a stable, cheap and easily transportable liquid that will preserve arthropod specimens, including their DNA. Further requirements include it should be soluble in water and effective at low concentrations, thus allowing in situ preparation of the large volumes of liquid required for large-scale and long-term collecting of arthropods and in rearing programmes.

An aqueous mixture of the chelator sodium dodecyl sulfate (SDS) with the detergent ethylenediaminetetraacetic acid (EDTA) may serve as such an alternative. It is known that this mixture preserves DNA and is often used at concentrations of 2% SDS + 100 mM EDTA (Asahida et al., 1996; Votýpka et al., 2010). Using a dry mixture of SDS/EDTA, the aqueous solution can be easily prepared in large quantities in the field, thus avoiding the need to transport large volumes of liquid. The solution would be suitable for the open containers used for collecting arthropods in various traps since only water would evaporate, increasing the concentration of the active ingredients. An aqueous solution of SDS/EDTA might be suitable as a collection or preservation liquid in various arthropod collecting traps, as long as it effectively preserved DNA in low concentrations and under ambient temperatures. The aim of this study is to test this possibility.

We examined the effectiveness of solutions of SDS/EDTA in preserving arthropod DNA over prolonged periods of time. Small and medium-sized beetles were stored in three concentrations of the SDS/EDTA aqueous solution at tropical temperatures either for one, four or eight weeks.

METHODS

Small and medium-sized beetles were stored in three different concentrations of the solution for three different periods of time. In particular, nine individuals of the ~2 mm long pollen beetle, *Meligethes aeneus* (Fabricius, 1775) (Coleoptera: Nitidulidae), and nine individuals of the ~14 mm long jewel beetle, *Lamprodilla rutilans* (Fabricius, 1777) (Coleoptera: Buprestidae), were stored in three different solutions: (i) 2% SDS and 100mM EDTA; (ii) 1% SDS and 50mM EDTA; (iii) 0.66% SDS and 33mM EDTA. All solutions were diluted with distilled water. In all cases beetles were either stored for one, four or eight weeks at temperatures ranging from 20 to 35°C, which mimic tropical conditions. After this treatment they were transferred to a –18°C freezer for long term storage. Due to a problem with the amplification of *L. rutilans* DNA, three additional DNA samples extracted from fresh individuals were used to test for primer efficiency. Since DNA is unlikely to be preserved in wet samples without preservative, we did not test such a treatment.

Prior to DNA extraction, all beetles were also well preserved for morphological analysis and taxonomic identification as well as for long-term storage in museum collections.

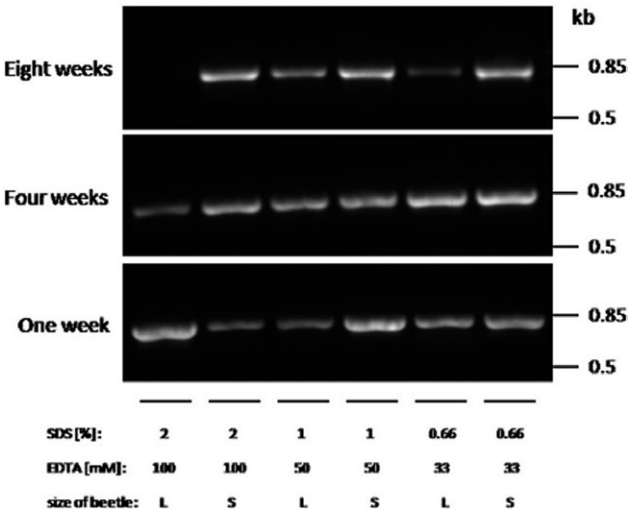

Fig. 1. Photographs of agarose gels in 1 x TAE buffer. The PCR products of the mitochondrial cytochrome *c* oxidase I gene obtained using primers C1-J-2183 and TL2-N-3014 and genomic DNA of beetles kept for either one, four or eight weeks at 20–35°C. Beetles were previously stored in one of three different solutions, either 2% SDS, 100mM EDTA, or 1% SDS, 50mM EDTA (diluted 1 : 1 in distilled water) or 0.66% SDS, 33mM EDTA (diluted 2 : 1 in distilled water). L – *Lamprodilla rutilans* (medium-sized beetle); S – *Meligethes aeneus* (small-sized beetle).

Genomic DNA was isolated from whole pollen beetles and parts of the jewel beetles using High Pure Template Preparation Kits [Roche]. Approximately 100 ng of genomic DNA was used for PCR amplification of the mitochondrial cytochrome *c* oxidase I gene (Cox1) and 28S rRNA gene (28S). The Cox1 was amplified using the forward and reverse primers C1-J-2183 (alias Jerry) (5' CAA CAT TTA TTT TGA TTT TTT GG) and TL2-N-3014 (alias Pat) (5' TTC AAT GCA CTT ATT CTG CCA TAT TA) (Simon et al., 1994). PCR cycling parameters included a denaturation step at 95°C for 5 min, followed by 40 cycles of 92°C for 30 s, 50°C for 30 s, 72°C for 1 min 30 s, with a final elongation step at 72°C for 10 min. 20 µl of PCR product was loaded on a 0.9% agarose gel stained with ethidium bromide and visualized using a UV light. The 28S gene was amplified using the forward and reverse primers S3660 (5' GAG AGT TMA ASA GTA CGT GAA AC) and A335 (5' TCG GAR GGA ACC AGC TAC TA) (Sequeira et al., 2000). PCR cycling parameters included a denaturation step at 94°C for 5 min, followed by 3 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, with a final elongation step at 72°C for 10 min. The annealing temperature decreased by 2°C at the end of every 3rd cycle down to 44°C (total of 24 cycles with annealing temperatures 58–44°C), followed by 21 cycles with annealing temperature 42°C. 5 µl of PCR product stained with SYBR Green was loaded on a 1% agarose gel and visualized using a UV light.

RESULTS AND DISCUSSION

The size of the Cox1 fragments amplified was about 820 bp. Fragments of the Cox1 gene were amplified in most samples, with one important exception, namely the medium-sized beetle stored for eight weeks in 100mM EDTA and 2% SDS (Fig. 1). Fragments of gene 28S, ca 800 bp-long, were amplified in all DNA samples of the small-sized beetle (Fig. 2). The primers, however, apparently also an-

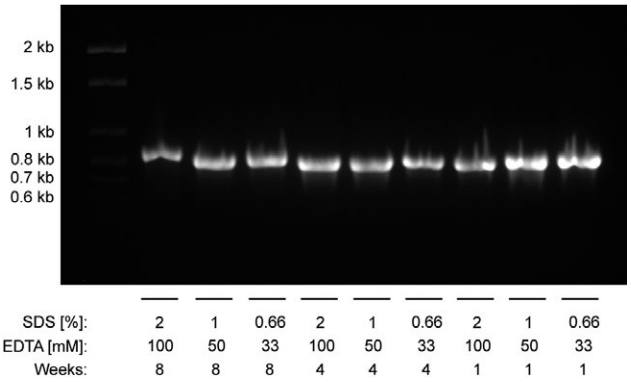

Fig. 2. Photograph of agarose gel in 1 x TAE buffer. The PCR products of 28S gene obtained using primers S3660 and A335 and genomic DNA of small-sized beetles (*Meligethes aeneus),* which were kept for either one, four or eight weeks at 20–35°C. Beetles were previously stored in one of the three different solutions, either 2% SDS, 100mM EDTA, or 1% SDS, 50mM EDTA (diluted 1 : 1 in distilled water) or 0.66% SDS, 33mM EDTA (diluted 2 : 1 in distilled water).

nealed to different DNA regions of the medium-sized beetle. The PCR thus resulted in several unspecified products, as the agarose gel showed > 1 visible band for each sample. This was also the case for three fresh DNA samples. It is likely therefore that the problem over amplifying 28S DNA occurred regardless of the treatment of the sample prior to DNA extraction and was caused by the primers used. We observed a smeared pattern of undigested DNA isolated from most small or mid-sized beetles, indicative of partial degradation of the DNA (data not shown). Nanodrop readings suggested relatively low DNA quality, perhaps due to contamination (Table 1). Nevertheless, the PCR amplification of medium-sized fragments of nuclear and mitochondrial genes was successful, except in the above mentioned cases. Moreover, a large amount of the amplicon was obtained, which was more than sufficient for direct sequencing.

Our results show that mitochondrial and nuclear DNA of beetles stored in any of the three different concentrations of detergent tested at rather high ambient temperatures (i.e. 20 to 35°C) remain sufficiently well preserved for use in traditional targeted PCR and sequencing. We did not explore the reasons behind the failure of the PCR to amplify Cox1 from DNA extracted from a large beetle stored for 8 weeks in the highest concentration of SDS + EDTA. However, given the fact that the protocols worked well for the same beetle species at lower concentrations and under the same conditions for DNA extracted from a small species of beetle, we assume that this failure was due to technical problems. Similarly, we did not explore the reasons behind the difficulties with the amplification of nuclear genes from the medium-sized beetle as protocol optimization including the search for more suitable primers was not an aim of this study. One of the aims was to establish whether even low concentrations of both chemicals are sufficient for DNA preservation, as this would significantly reduce the cost of preparing large quantities of the fixative solution in the field. The higher concentration of both chemicals is routinely used for preserving DNA samples (Votýpka et al., 2010, 2012) and we wondered whether lower concentrations would be as effective, as this would make the preparation of fixative in the field more cost-effective. Our results show that even 0.66% SDS/30 mM EDTA was sufficient for the preservation of amplifiable nuclear and mitochondrial DNA. The cost of SDS + EDTA is significantly lower than the prohibitively high cost of aqueous fixatives such as ethanol. Our observations were further confirmed by good PCR amplifications of Cox1 and 28S genes from beetles preserved in the above-mentioned solution for several weeks at the ambient temperatures (up to 35°C) of humid tropical climates.

Hence, we conclude that the mixture of 100mM EDTA and 2% SDS is suitable as a fixative for beetles and possibly also other small to medium-sized arthropods. Even the SDS/EDTA solution with the lowest tested concentration effectively preserved DNA. Samples in traps should thus withstand even substantial dilution of the collecting liquid (due to rain, for example), if solutions containing 100mM EDTA and 2% SDS are used.

All of the three concentrations of SDS/EDTA that we tested preserved beetle DNA at temperatures recorded in tropical environments (20 to 35°C) for one to eight weeks, which is sufficient for most arthropod traps, as these are

Table 1. Nanodrop readings of the concentration and quality of genomic DNA extracted from medium-sized jewel beetles (*Lamprodilla rutilans*, Buprestidae) and small-sized pollen beetles (*Meligethes aeneus*, Nitidulidae) previously stored in three different concentrations of the SDS/EDTA solution under ambient temperatures typical of a humid tropical climate for three different periods of time.

Storage time	8 weeks			4 weeks			1 week		
	DNA[1]	260/280	260/230	DNA[1]	260/280	260/230	DNA[1]	260/280	260/230
Medium-sized beetle									
2% /100mM*	79.1	1.45	0.78	63.6	1.41	0.77	124.2	1.47	0.96
1% /50mM*	53.8	1.37	0.75	62.1	1.44	0.77	49.2	1.42	0.77
0.66% /33mM*	64.8	1.41	0.81	12.5	1.27	0.84	29.3	1.35	0.77
Small-sized beetle									
2% /100mM*	33.1	1.34	0.78	95.3	1.41	0.83	59.1	1.4	0.79
1% /50mM*	33.1	1.39	0.77	45.2	1.41	0.78	57.1	1.42	0.75
0.66% /33mM*	71.6	1.37	0.78	45.1	1.35	0.77	55.7	1.43	0.75

* SDS/EDTA concentration in preservation liquid; [1] genomic DNA concentration [ng/µl].

often inspected weekly or fortnightly. The preservation of DNA by this method may, however, differ among taxa. The SDS/EDTA solution is considered unsuitable for insect preservation, because it slowly penetrates through the cuticles of intact specimens (Quicke et al., 1999) and is thus rarely used for arthropods (e.g. Moreau et al., 2013). It is important to note that we tested the effectiveness of SDS/EDTA solution only on beetles. However, these insects are amongst those with the thickest cuticles of any arthropod. SDS/EDTA solution is therefore also likely to preserve the DNA of most other arthropods, although this assumption remains to be tested.

On the other hand, EDTA is a surface-active agent and SDS an anionic surfactant used to open proteins to access DNA. Hence, it is likely that biological samples stored in this solution will eventually deteriorate. Samples that are likely to be stored for a long time prior to DNA extraction should therefore be kept dry or frozen, while specimens intended for museum collections should be transferred to ethanol. Although non-poisonous to humans at low concentrations, higher concentrations of SDS/ETDA may cause skin irritation and can be harmful to aquatic ecosystems. When working with the dry mixture of SDS/EDTA it is recommended that protective gloves and glasses are worn, contact with mucous membranes avoided and general hygienic rules should be followed (SCBT, 2013a, b). The dry mixture of SDS/EDTA is easy to transport, store and prepare in the field by adding water. For example, only 20 g of SDS and 37.2 g of EDTA are needed for 1 l of 2% SDS, 100mM EDTA solution. This mixture is an effective DNA preservative even at low concentrations and the active substances do not evaporate.

ACKNOWLEDGEMENTS. This paper is dedicated to the memory of Pavel Pokluda (1985–2012), an outstanding biologist, who tragically died during fieldwork in Papua New Guinea. We thank S.T. Segar (University of South Bohemia, České Budějovice) for language correction, R. Březová (Institute of Organic Chemistry and Biochemistry, Prague) for consultations on SDS/EDTA toxicity and C. Hawks and S.E. Miller (Smithsonian NMNH, Washington DC) for comments on the manuscript. L.C. was supported by the Czech Grant Agency (P504/12/1952) and Technology Agency of the Czech Republic (TA02021501). J.L. was supported by the Czech Grant Agency (204/09/1667) and a Praemium Academiae award, and is a Fellow of the Canadian Institute for Advanced Research. V.N. was supported by the Czech Grant Agency (206/09/0115).

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Received April 26, 2013; revised and accepted December 31, 2013
Prepublished online March 6, 2014