European Journal of Soil Biology 57 (2013) 13–18

Contents lists available at SciVerse ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Storage and shipping of tissue samples for DNA analyses: A case study on earthworms☆

Daniela Straube*, Anita Juen

Institute of Ecology, University of Innsbruck, Technikerstrasse 25, 6020 Innsbruck, Austria

ARTICLE INFO

Article history:
Received 28 November 2012
Received in revised form
5 April 2013
Accepted 11 April 2013
Available online 25 April 2013
Handling editor: Stefan Schrader

Keywords:
Lyophilization
Preservation method
DNA extraction
Field samples
DNA quality

ABSTRACT

Nowadays, molecular analyses play an important role in studies of soil dwelling animals, for example in taxonomy, phylogeography or food web analyses. The quality of the DNA, used for later molecular analyses, is an important factor and depends on collection and preservation of samples prior to DNA extraction. Ideally, DNA samples are frozen immediately upon collection, but if samples are collected in the field, suitable preservation methods might be limited due to unavailability of resources or remote field sites. Moreover, shipping samples over long distances can cause loss of DNA quality e.g. by thawing or leaking of preservation liquid. In this study we use earthworms, a key organism in soil research, to compare three different DNA preservation methods – freezing at −20 °C, storing in 75% ethanol, and freeze drying. Samples were shipped from the United States of America to Austria. The DNA of the samples was extracted using two different extraction methods, peqGOLD™ and Chelex® 100. The DNA amplification success was determined by amplifying four DNA fragments of different length. The PCR amplification success is significantly influenced by preservation method and extraction method and differed significantly depending on the length of the DNA fragment. Freeze drying samples was the best preservation method when samples were extracted using the silica based extraction method peqGOLD™. For samples that were extracted with Chelex® 100, storage in ethanol was the best preservation method. However, the overall amplification success was significantly lower for the extraction procedure based on Chelex® 100. The detection of the small DNA fragments was higher and independent from the extraction method, while the amplification success was significantly reduced for the longer DNA fragments.

We recommend freeze drying of DNA samples, especially when they have to be shipped for longer distances. No special packaging or declaration is needed for freeze dried samples, and the risk of thawing is excluded. Storage of freeze dried samples also reduces costs because samples can be kept at room temperature in a desiccator. It should be noted, that the extraction methods showed significant differences in DNA amplification success. Thus, the extraction method should be taken into account when choosing the preservation method.

1. Introduction

DNA analyses have become an indispensable tool in soil zoology and soil ecology, especially in studies on trophic interactions [1], soil monitoring [2], and relationships within and among populations [3]. A critical factor for DNA analyses is the collection and the treatment of the samples prior to these analyses. Ideally, samples are taken in a laboratory under optimal conditions, where they can be processed immediately, but for most investigations on soil organisms samples have to be collected directly in the field. To avoid loss in DNA quality, it is therefore essential to optimize the preservation of samples and to find efficient and reliable transport methods. Earthworms play a major role in the composition of soil fauna, as they breakdown and recycle organic material, support plant growth due to their nutrient rich casts, improve soil quality [4], and help aerate the soil. Because of their diversity and importance in soils, earthworms have been studied for decades and molecular tools are increasingly applied to solve taxonomic and phylogenetic questions [5,6], track predation on earthworms [7], reveal the phylogeography and mechanisms of distribution [8], or improve our understanding on litter decomposition [9]. Therefore, samples of earthworm tissue were chosen for the following experiments on preserving and shipping samples for subsequent DNA analyses. There is a variety of methods to preserve

* Corresponding author. Tel.: +43 (0) 512 507 51678; fax: +43 (0) 512 507 51799.
E-mail addresses: Daniela.Straube@uibk.ac.at, Daniela.Straube@gmx.net (D. Straube), Anita.Juen@uibk.ac.at (A. Juen).

1164-5563/$ – see front matter © 2013 The Authors. Published by Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejsobi.2013.04.001

tissue and DNA samples that are well tested and frequently used including freezing, drying, and storage in ethanol or buffer [10–12]. Among the most often used preservation method of samples collected for DNA analyses is freezing. Freezing at −80 °C or in liquid nitrogen (−196 °C) [11,13] is most often used for long term storage; for short term storage −20 to −28 °C is preferred [10,14]. When samples are collected in the field, it is often difficult to freeze them immediately, and there is a risk of thawing during transport, especially with long distances. Alternatively, samples can be stored in absolute or in 75% ethanol. Ethanol was used successfully in various studies to preserve samples for PCR, sequence analyses or microsatellite amplification [11,15–19], however, several studies observed considerable DNA degradation in tissue and environmental samples stored in 75% ethanol [10,13]. Similarly, contradictory results are reported from drying samples [15,20,21]. Differences in preservation success may be explained partially by different methods of drying – air-drying, oven drying, chemical drying or freeze-drying [17,18,22]. Unfortunately, there are no systematic studies on stability and damage of DNA in dried samples [23] that would allow a general conclusion. In any case, both methods, preservation in ethanol or by drying, are cost efficient because storage is possible at room temperature.

Besides considering the quality of sample preservation, it may be necessary to calculate all costs and risks that are involved with transport, especially if transport requires several days. For this study we focused on three preservation methods and the effort and risks that arise if samples have to be shipped over long distances for several days. Samples were sent frozen, in 75% ethanol, and freeze-dried. Frozen samples can be shipped on ice, in liquid nitrogen or in dry ice, using special containers; however the needed equipment and the shipment service can become quite expensive, depending on the number of samples. The most cost efficient method is to send samples in maximal 2 kg dry ice. With extra insulation dry ice keeps the samples frozen for two to three days. The problem is that there is a considerable risk of thawing if the shipment is delayed due to customs.

The shipping duration is less important for samples preserved in ethanol. However, ethanol is a flammable liquid and therefore large amounts are classified as “dangerous goods” and require special packaging and transport. For samples that require less than a total amount of 500 ml ethanol at concentrations lower than 80%, a cheaper transport service is available. Shipping of dried samples causes the fewest problems; transport is not restricted by sample size or volume, and extended shipping duration is not a risk for the samples.

In the following experiment we tested three different preservation methods using pieces of earthworm tissue. Earthworms were easily available in high numbers from a breeder. In many zoological and ecological studies the initial sample quality can be reduced due to partial digestion, e.g. in fecal pellets. We simulated this situation by treating the tissue samples with sodium hypochlorite for different periods of time. After preserving the samples by freezing, in 75% ethanol or by freeze drying they were sent from North America to Europe using one of the widely available parcel services. As it is well known that the DNA extraction method is critical for the quality of a DNA sample, we tested two different extraction procedures and finally evaluated the three preservation methods based on the success in PCR amplifications targeting four DNA fragments that differed in length. The aim of this study was to find a preservation method that is reliable and allows cost efficient long time transport of the samples.

2. Material and methods

2.1. Earthworm samples

As the source for tissue samples, we used earthworms sold as live bait and later identified morphologically as *Eisenia zebra* (Michaelsen 1903). Seventy-five earthworms were rinsed under water and freeze-killed. A small piece, about one-sixth of the whole worm, cut from the middle of the earthworm body, was used for further treatments.

To simulate different sample quality with partially degraded DNA, earthworm pieces were treated with bleach (1% sodium hypochlorite). Five different bleaching durations were tested: 50, 30, 15, 5, and 0 (control) minutes. Each treatment was applied to 15 earthworm pieces. Finally, the earthworm pieces were individually put into 1.5 ml reaction vials and subjected to one of three preservation methods: freezing (F), ethanol (E), or freeze dried (D), ensuring that each of the 5 different bleaching treatments was represented by 5 samples in each of the preservation methods.

2.2. Storing and shipping methods

Ethanol samples were stored in 75% ethanol at room temperature. Frozen samples were kept in the freezer at −20 °C until shipped on dry ice. Freeze dried samples were stored in the freezer at −20 °C, and put frozen into the freeze drier, where they were vacuum dried using a Virtis 12ES (SPS SCIENTIFIC, Gardiner, NY, USA), at −50 °C and 30 mTorr pressure for two days. All samples were sent at the same day through FEDEX from Athens (Georgia, USA) to Innsbruck (Austria), and arrived without damage after 3 days. In the package of the frozen samples, no dry ice was left and the samples were thawed but still cold. Upon arrival they were immediately transferred to a −80 °C freezer, while the samples preserved in ethanol were kept at 4 °C in a refrigerator, and the dried samples were stored at room temperature.

2.3. DNA extraction

For the following analyses each of the 75 earthworm pieces was homogenized with glass beads in 300 μl PBS buffer (150 mM sodium phosphate, 150 mM NaCl, pH 7,2) and 5 μl Proteinase K (20 mg/ml) for 1 min at 5.000 rpm using a Precellys® Tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). From each homogenate 100 μl were used for a Chelex® 100 extraction and an additional 100 μl for a peqGOLD™ extraction. The Chelex® 100 extraction method was chosen because it is fast, cheap, and can be adapted to extract a large number of samples simultaneously [24]. After adding 200 μl of 10% Chelex® 100 to each sample, the samples were incubated overnight at 58 °C followed by 15 min deactivation of proteinase K at 94 °C. For the second extraction method a silica based extraction kit (peqGOLD™ Tissue DNA Mini Kit, PeqLab, Erlangen, Germany) was used, following the extraction protocol of the manufactures guidelines; with an incubation time of 1 h at 50 °C. This method was selected because it delivers high quality DNA, and the equipment needed is usually available in most laboratories. Extraction methods based on chloroform or CTAB are cheaper but were not considered as they require more handling and time. All extractions were done in a pre-PCR laboratory using a UV-equipped laminar flow hood. Extraction negative controls were included to check for cross-sample contamination, and all samples were finally stored at −28 °C.

The nucleic acid concentration of all extracts was measured using NanoDrop (ND-1000, NanoDrop Technologies, Inc., Wilmington, USA), following the manufacturer’s guidelines.

2.4. PCR and visualization of PCR products

To test the success of DNA preservation, all extracts were tested in PCRs. As it is known from earlier studies that smaller DNA fragments can be detected even in highly digested DNA samples, four DNA fragments of different length were amplified by using