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Comparison of different protocols for DNA preparation and PCR amplification of mitochondrial genes of tardigrades

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ABSTRACT

Phylogenetic relationships and molecular taxonomy within the Tardigrada have been given a lot of attention in recent years. Here I present the first comparison of different protocols for DNA preparation by investigating six commercial available DNA extraction kits and the CTAB method. Successful extraction of DNA from tardigrades depends strongly on the life-stage (embryo, adult), and on the condition of the specimens, respectively on the preservation (anhydrobiotic, ethanol). Although the extraction kits showed differences in the amount of extracted DNA, in all cases fresh tissue of live animals or embryos resulted in the best quality and quantity of DNA. A lesser amount of DNA was extractable from anhydrobiotic animals and embryos and the results of specimens fixed in ethanol were unsatisfactory. All used commercially available DNA extraction kits and PCR cocktails have been focused on vertebrate tissues, blood, cultured cells, bacteria and yeast. However, I used successfully the kits according to the manufacturer's instruction without changes in the protocols for DNA extraction of tardigrades. Commercial kits provide a simple and convenient way to isolate pure genomic DNA of high-quality from tardigrades. Furthermore I tested eight different Taq polymerase enzymes for PCR amplification of mitochondrial genes of tardigrades. Each of the enzymes resulted in a PCR product, and even if the amount of the PCR products was quite different, it was possible to use it successful for direct sequencing. Summarizing, the successful PCR of the target DNA depends on the purity and quality of the DNA template and for this the species preservation is more critical than the extraction method or the PCR cocktail which can be optimized.

Key words: DNA isolation, cox1, molecular methods, Tardigrada, PCR

1. INTRODUCTION

Molecular methods have opened up a wide range of new approaches to invertebrate research, particularly with regard to molecular phylogenetic and taxonomic studies. Several molecular fingerprint systems have been proposed and tested for various numbers of invertebrates and protozoa, including length polymorphism in polymerase chain reaction-amplified gene segments, restriction fragment length polymorphisms (RFLP) (Szalanski *et al*. 1999; Torres *et al*. 2000; Wang & Porter 2004), randomly amplified polymorphic DNA (RAPD) (Gawel & Bartlett 1993; Bidochka *et al*. 1994; Winder *et al*. 2005; Djadid *et al*. 2006), amplified fragment length polymorphisms (AFLP) (Van Der Wurff *et al*. 2003; Hayashi *et al*. 2004; Wang & Porter 2004; Winder *et al*. 2005; Slotman *et al*. 2006) and riboprinting (Clark *et al*. 1989, 1995; Clark & Diamond 1991, 1997; Schlegel *et al*. 1991; Steinbrück *et al*. 1991; Clark & Pung 1994; De Jonckheere 1994; Schill & Steinbrück 2007).

Most studies focused on 18S rDNA to improve the understanding of the phylogenetic relationship between tardigrades and arthropods (Garey *et al*. 1996, 1999; Moon & Kim 1996; Aguinaldo *et al*. 1997; Giribet & Ribera 2000; Giribet *et al*. 2000; Shultz & Regier 2000; Giribet & Wheeler 2001). Scientific work on the phylogeny of the phylum Tardigrada is rather in the early stages and few studies on molecular signature sequences like the small subunit ribosomal RNA (SSU; 18S) (Garey *et al*. 1999; Blaxter *et al*. 2003; Jørgensen & Kristensen 2004; Guidetti *et al*. 2005) or the mitochondrial cytochrome c oxidase (COI) gene (Guidetti *et al*. 2005) are published. In addition, the molecular mechanisms involved in the capability to undergo an anhydrobiotic stage are the focus of research (Schill *et al*. 2004).

However, the study of tardigrade species is severely hampered by a general problem which is frequently encountered in the molecular work with small invertebrates. The extraction and purification of intact DNA is often extremely difficult due to two major problems: the minute size of the individuals and the low amount of total DNA in invertebrates (Redi & Garagna 1987; Bertolani *et al*. 1994; Garagna *et al*. 1996). Additional problems are encountered with the step of preservation, the handling of specimens in the field or laboratory, the choice of the extraction protocol or commercial kit, or the polymerase enzymes and the sequencing reactions. In this publication, I focused the attention on how tardigrades in different life-stages should be preserved best and which kind of extraction kits as well as enzymes work successfully without major optimization of existing protocols.

2. METHODS

2.1. Tardigrade species and preservation

Individuals of the common Eutardigrada species, *Milnesium tardigradum* Doyère, 1840 were used to investigate the best method of preservation in a DNA-friendly fashion for successful amplification reactions. The species was cultured as described by Schill *et al*. (2004) in 6 cm plastic culture dishes on a 4 mm layer of 3% agar and a layer of Volvic™ water. For food, rotifers of the species *Philodina citrina* Ehrenberg, 1832 were provided twice a week, which were cultured separately in Volvic™ water and fed with the green algae *Chlorogonium* sp.

In order to investigate the best method of preservation, single specimens at the age of 25 days and blastula stages were used. For each of the seven tested extraction methods five specimens each were used alive or preserved in 75% ethanol and stored for 14 days, or dried and subsequently stored for 14 days at room-temperature (RT). For drying, the animals and embryos were transferred with a Pasteur pipette to a 0.44 mm smooth Whatman® filter paper (Schleicher & Schuell, Dassel, Germany) 0.25 cm^2 in size. After any excess water had been removed, the moist but not wet filter paper was placed in a 0.5 ml plastic tube. Then the open tubes were placed immediately in a closed box containing a saturated KNO_3 salt solution, providing an equilibrium relative humidity of 94.5% at RT.

2.2. DNA isolation

For the DNA isolation and purification of the alive, frozen, dried or ethanol-fixed specimens I used Wizard® Genomic DNA Purification Kit and Wizard® SV Genomic DNA Purification System (both from Promega, Madison, U.S.A.), DNeasy® Tissue Kit (Qiagen, Hilden, Germany), E.Z.N.A.® Tissue DNA II Kits (PeqLab, Erlangen, Germany), GenElute™ Mamalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, U.S.A.), NucleoSpin® Tissue (Macherey-Nagel, Düren, Germany) and the CTAB extraction method (Jones & Walker 1963). DNA was extracted from single tardigrades and embryos according to the manufacturer's instruction. To release the DNA, the tardigrade's cuticle was ground by hand with a plastic pestle in a 1.5 ml Eppendorf tube (Eppendorf, Hamburg, Germany). The Wizard® Genomic DNA purification and Cetyltrimethylammonium bromide (CTAB) extraction were conducted without columns and are based on precipitations. CTAB is a cationic detergent which has the useful property of precipitating nucleic acids from homogenates at low ionic strength. With all other kits the DNA was bound to a membrane in a column. All sorts of contamination were removed by subsequent steps of washing. Finally, the DNA was eluted twice from the membrane with pure water or an elution buffer. After each extraction the concentration and purity of nucleic acids were judged by measuring the absorbance using a ND-1000 Spectrophotometer (PeqLab, Erlangen, Germany).

2.3. DNA amplification

Successful PCR amplification of the genes of interest is dependent upon various factors, particularly the purity, the quality and the quantity of the DNA template and therefore upon the original material, the preservation and DNA isolation. Furthermore, there are differences in the Taq DNA polymerases, which were originally isolated from *Thermus aquaticus* Brock and Freeze, 1969 (see Saiki *et al*. 1988), and in the complex PCR cocktails containing magnesium ions, primers and enhancing reagents from different commercial suppliers. I evaluated the effectiveness of eight polymerases using DNA, isolated from pooled alive *M. tardigradum* from a clonal strain with the NucleoSpin® Tissue Kit to ensure a constant amount and quality of template DNA for the comparison of different Taq DNA polymerases. I tested amplification of the DNA using GoTaq® DNA Polymerase (Promega, Madison, U.S.A.), EXL™ DNA Polymerase (Stratagen, La Jolla, U.S.A.), TaKaRa Ex Taq™ (TaKaRa Bio, Shiga, Japan), Taq DNA Pol (Qbiogene, Morgan Irvine, U.S.A), BIO-X-ACTShort DNA Polymerase (Bioline, London, U.K.), Genaxxon DNA Polymerase (Genaxxon BioScience, Biberach, Germany) and Taq DNA Polymerase (Eppendorf, Hamburg, Germany), Taq polymerase (PeqLab, Erlangen, Germany). Each enzyme and PCR cocktail was used according to the manufacturer's instructions in a final volume of 50 µl and was not optimised for tardigrades. For the mitochondrial cytochrome c oxidase (*cox1*) gene, I used the primers to amplify a 658 bp fragment of the COI gene (Folmer *et al*. 1994). The PCR thermal regime consisted of one cycle of 1 min at 94 °C; five cycles of 1 min at 94 °C, 1.5 min at 45 °C and 1.5 min at 72 °C; 35 cycles of 1 min at 94 °C, 1.5 min at 50 °C and 1 min at 72 °C and a final cycle of 5 min at 72 °C.

Aliquots of all PCR products were run with 1 µl SYBR Gold® I 1:1000 (Molecular Probes, Eugene, USA) on a 1% agarose gel for quality control. The signal intensity of the bands in the gel was quantified by a densitometric image analysis (Herolab E.A.S.Y., Germany). The signal value of the strongest band was artificially set to 100%. Weaker signals were related to this reference.

2.4. PCR product purification and sequencing

For standard sequencing I cleaned the *cox1* gene PCR products with the Rapid PCR Purification System (Marligen Bioscience, Ijamsville, U.S.A) which is designed for the rapid removal of PCR reaction components (primers, nucleotides, enzymes, salts and other impurities) from amplified DNA. The purified double stranded DNA was sequenced with an ABI 3730 Ana-

Tab. 1. The extractable DNA concentrations per specimen of *Milnesium tardigradum* with different methods. * = range of 0-10 ng DNA/specimen; ** =range of 10-20 ng DNA/specimen;*** = range of 20-30 ng DNA/specimen.

	Adult tardigrade			Tardigrade embryo	
	Active	Anhydrobiotic	Ethanol-fixed	Active	Ethanol-fixed
GenElute™ Mamalian Genomic DNA Miniprep Kit	***	***	**	***	**
NucleoSpin® Tissue	***	***	**	***	**
E.Z.N.A.® Tissue DNA II Kits	**	**	*	**	*
DNeasy® Tissue Kit	**	**	*	**	*
Wizard® Genomic DNA Purification Kit	*	*	*	*	*
Wizard® SV Genomic DNA Purification System	*	*	*	*	*
CTAB	*	*	*	*	*

lyzer at the custom DNA sequencing service AGOWA (Berlin, Germany) and identified using BLAST® (Basic Local Alignment Search Tool).

3. RESULTS

3.1. DNA isolation

Overall, the amount of purified and intact DNA was strongly dependent on the original material of the animals. Of course, fresh tissue of active animals or embryos will result in the best quality and quantity of DNA (Tab. 1). A lesser amount of DNA was extractable from anhydrobiotic animals. Even less was obtained from specimens fixed in ethanol. In general, due to the small amount of DNA in the adult tardigrades and in the embryos, it was not possible to determine the exact amount of extracted DNA nor the impurities with proteins and metabolites with the spectrophotometer. However, with the GenElute™ Mammalian Genomic DNA Miniprep Kit and the NucleoSpin® Tissue DNA extracting kit, I obtained the best results with live animals and embryos, as well as with the anhydrobiotic stages. The E.Z.N.A.® Tissue DNA II Kit and DNeasy® Tissue Kit worked marginally worse with fixed and dried specimens. The Wizard® Genomic DNA Purification Kit, Wizard® SV Genomic DNA Purification System and the CTAB extraction method produced less success for all samples and too small amounts of DNA.

3.2. DNA amplification

I evaluated the effectiveness of eight different polymerases using equal amounts of total DNA from *M. tardigradum* and tested the enzymes for their ability to amplify the *cox1* gene (658 bp). With all enzymes I received clear bands of amplification products, but differences in the strength of the signals (Fig. 1). I calculated the intensity of all bands in relation to the marker bands and the strongest signal of the *cox1* gene amplification was obtained with the PCR cocktail using the BIO-X-ACTShort DNA Polymerase (Fig. 2). PCR amplifications with the GoTaq® DNA Polymerase, TaKaRa Ex Taq™ and Taq DNA Pol worked, compared to the other enzymes, also excellent. The amount of PCR product with GoTaq® DNA Polymerase reached 82%, with the Taq DNA Pol 71%, and TaKaRa Ex Taq™ 62%. EXL™ DNA Polymerase and the Taq polymerase from PeqLab was satisfying, even though the signal was a little bit weaker (62%). With the Genaxxon DNA Polymerase and the Eppendorf Taq polymerase the signals of the corresponding bands were weakest and came up to 36% compared to the amplifications with the BIO-X-ACTShort DNA Polymerase.

3.3. Sequencing

Direct sequencing of all *cox1* gene PCR products with different polymerase enzymes resulted in sequences of good quality. I obtained the longest sequences of around 650 bp from the PCR products amplified with BIO-X-ACTShort DNA Polymerase, GoTaq® DNA Polymerase, Taq (PeqLab), EXL™ DNA Polymerase. Due to the differences in the amount of PCR product obtained with the various enzymes, the amounts were sufficient for sequencing and quality comparable. Using the TaKaRa Ex Taq™, Taq DNA Pol, BIOGenaxxon DNA Polymerase and Taq DNA Polymerase from Eppendorf I did not see the first 80 bp compared to the sequencing reactions with the other enzymes. However, with all used enzymes I have got consistent sequences with a minimum of 570 bp in length by direct sequencing.

4. DISCUSSION

The aim of this study was to compare different procedures for nucleic acid isolation in order to test their efficiency and practicability in obtaining PCR-compatible material from limno-terrestrial tardigrades. This is the first comparative approach for studying tardigrade DNA extraction methods and the associated PCR and sequencing reactions.

To obtain good quality DNA from tardigrades the preservation is one of the critical factors. Whenever possible fresh animals or eggs, respectively embryos should be used for DNA extraction, which is in general recommended by several molecular standard protocols (e.g. Sambrook & Rusell 2001). Unfortunately, working with fresh material is sometimes not possible in the field. However, tardigrades are well known for their ability to undergo a cryptobiotic stage. Thus, this capability can be used to preserve the living cells. The animals can become slowly dehydrated and subsequently stored for a long time. Usually many small invertebrates

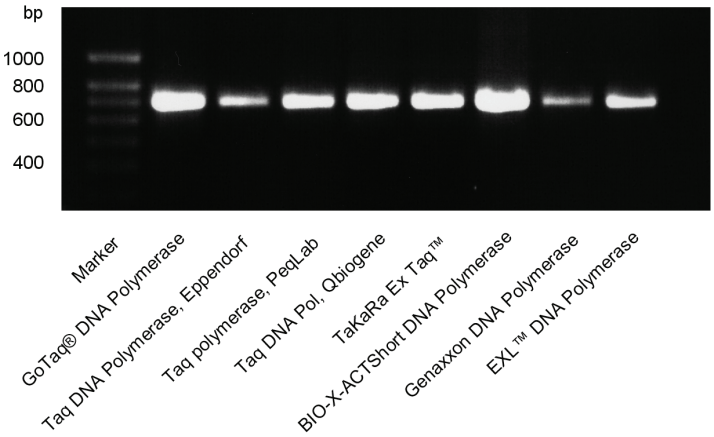

Fig. 1. Amplification products of the *cox1* gene from active adults of *Milnesium tardigradum* with eight different PCR enzymes. DNA were extracted with the NucleoSpin® Tissue Kit.

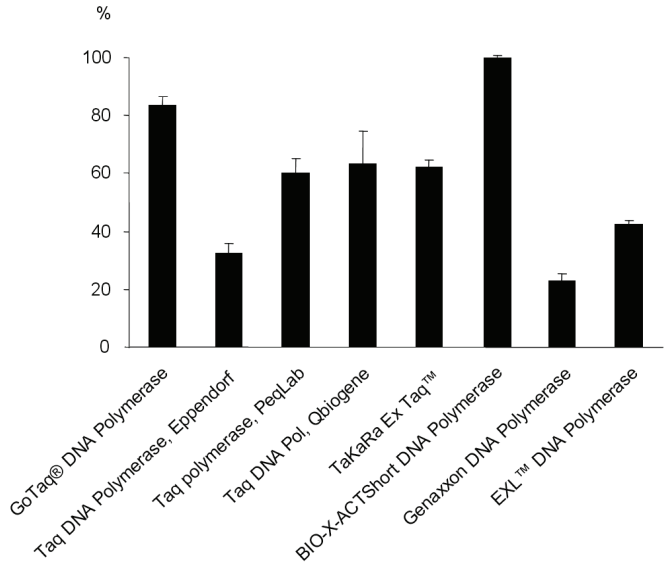

Fig. 2. Densitometric analysis of the *cox1* gene amplification products from active adults of *Milnesium tardigradum* with eight different PCR enzymes. The signal value of the strongest band was artificially set to 100%. Weaker signals were related to this reference. All data are means ± SD.

are fixed in ethanol for the later species identification and the mounting on slides.

This study indicated that fresh material provided the highest amount of DNA, independently of the extraction kit. The extraction of dehydrated specimens seems to be more difficult, may be due to the fact that cellular components are protected and stabilized during the anhydrobiotic stage. Up to now the mechanisms for this are poorly understood. This problem can be bypassed through rehydration before the animals are processed with an extraction method. Nevertheless, during storage for a long time the DNA will become degraded due to hydrolysis and oxidation (Lindahl 1993) as well as exposure to ultraviolet light (Eglinton & Logan 1991). Thus, the specimens pass through a chemical and physical ageing process which also causes a reduced survival rate in tardigrades after rehydration (Bertolani *et al*. 2004). For degraded template DNAs other pairs of primers for small amplicons or other methods for typing of degraded DNA should be used, which are common in the field of forensic research (Timken *et al*. 2005; Meissner *et al*. 2007).

The DNA extraction from ethanol-preserved tardigrades was possible; however, the yield was relatively low. In general, ethanol-fixed specimens are poorer targets for analysis. Other preservation methods, like formaldehyde, should be avoided (Prendini *et al*. 2002; Schander & Halanych 2003). However, even for this material are special protocols available, but they were not subject of this study.

Besides different commercially available extraction kits, mentioned above, which are designed for the simple, rapid isolation of a reproducible PCR quality genomic DNA in as little as a few minutes up to a few hours, and the CT method, Floyd *et al*. (2002) suggested another method for DNA extraction from small invertebrates. He picked up nematodes directly into 20 µl of 0.25 M NaOH in 0.2 ml tubes and kept them at room temperature for 3-16 h Subsequently, this lysate was heated for 3 min at 95 °C. To neutralize the base, 4 µl of HCl and 10 µl of 0.5 M Tris-HCl buffered at pH 8.0 were added. Five µl of 2% Triton X-100 was also added, and the lysate was heated for a further 3 min at 95 °C and stored at -20 °C. Blaxter *et al*. (2003) used this method with tardigrades to generate a so called molecular operational taxonomic unit (MOTU), which is a 500 bp long sequence of the 18S rDNA. In other experiments I have had difficulties with this method (unpublished data).

In agreement with the results of Hajibabaei *et al*. (2005), I found that the NucleoSpin® Tissue kit is one of the best for tardigrade DNA isolation. They reported that the NucleoSpin® kit was the most effective for pieces of birds and fish, and for archived moths, too. Smith *et al*. (2005) used the NucleoSpin® kit in 95% ethanol preserved ants from Madagascar for assessing this hyper diverse arthropod group with DNA barcoding. In the study of Hajibabaei *et al*. (2005), the DNeasy kit was less effective than the NucleoSpin kit despite their similar technology. However, Nichols (2004) used the DNeasy kit successfully for studies on monophyly on the order level and interrelationships within the class Demospongiae. Monaghan *et al*. (2005) used the Wizard® SV Genomic DNA Purification System for DNA-based species delineation in tropical beetles and have got good results in contrast to these results, independently of the preservation method.

All the kits used for extraction of DNA from tardigrades are not developed for invertebrates and of course, it is possible to adapt single parts of the protocols for special applications. The condition of the DNA is one of the determining factors for a successful PCR and depending upon two factors: the quality and the quantity of the template DNA. If less DNA is available for an amplification, the whole genome amplification from small samples using Multiple Displacement Amplification (MDA) (Dean *et al*. 2002) is a powerful tool to provide highly uniform amplification across the entire genome, with negligible sequence bias (Hosono *et al*. 2003).

There are many Taq polymerase enzymes available from different suppliers and the used enzymes are just a random choice. As we can see, all the enzymes resulted in a PCR product. However, the amounts of PCR products obtained in this study were different but sufficient for direct sequencing. Hajibabaei *et al*. (2005) figured out that *cox1* gene sequences sometimes recovered from sequences where no PCR product was evident on the agarose gel.

5. CONCLUSIONS

Molecular methods for species identification by using short DNA sequences, known as barcode, signature sequences or molecular operational taxonomic unit, have been proposed and initiated to facilitate biodiversity studies and identify different life-stages. Regardless of the sequence of interest, this investigation showed that the preservation of the tardigrade samples (active or dehydrated animals, as well embryos) and the right extraction protocol is the key factor for good results. I used all protocols and kits according the manufacturer's instruction and non of them were optimized for tardigrades or rather generally for invertebrates. In general, for best results modifications of protocols are of course necessary depending on the species and life-stage.

With four of seven different extraction methods, DNA has been successfully extracted with suitable quality. However, according the present study I recommend to keep sampled tardigrades for DNA extraction alive or let them undergo into an anhydrobiotic state. The same is valid for embryos, but depending on the stage of development, because a further developed embryo has more cells and subsequently more genomic DNA. The fixation with ethanol should be avoided.

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