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Optimizing conflicting tasks in the analysis of parasitic worms: morphological imaging, DNA yield, specimen and DNA preservation

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Abstract

DNA barcoding is a promising identification tool in many organismal groups, especially where larval stages can hardly be identified to species level based on other approaches and expertise is restricted to few persons. The quality standards for building up a reference database, however, are strict and difficult to achieve in small animals such as parasitic helminths. Here we present two methodological approaches that improve DNA quality and quantity while preserving a voucher, which enables a repeatability of the primary morphological identification. In the first experiment, we attempted to preserve unscathed voucher specimens by obtaining DNA using an insect preparation needle. In the second experiment, we evaluated the impact of different staining and fixing solutions on DNA quality after three days, one month and two years. We found that it is possible to obtain sufficient DNA with an insect preparation needle. Furthermore, we found that borax carmine had a major impact on DNA quality after one month. No positive PCR could be obtained from samples that had been stored in glycerol for two years. We discuss implications for the treatment of helminths for DNA barcoding initiatives.

Key Words: DNA barcoding, DNA extraction, PCR, helminths, storage

Introduction

Since its introduction in 2003, DNA barcoding has enjoyed an ever-increasing popularity as an innovative taxonomic identification tool (DeSalle & Goldstein 2019), using DNA sequences as identifiers for species assignment (Hebert et al. 2003a, b; Stoeckle 2003). In most animal groups, sequences of the cytochrome c oxidase subunit I (*COI*) gene are used as such identifiers, i. e. standard DNA barcodes. Mitochondrial DNA (*mt*DNA) has certain advantageous characteristics: apart from the lack of introns and of recombination, *mt*-sequences are particularly suitable for DNA barcoding purposes due to their higher substitution rates compared to genes in the nuclear genome (Hebert et al. 2003a; Waugh 2007; Taylor & Harris 2012).

The primary intention of DNA barcoding is species identification based on DNA sequences. A sequence of an undetermined sample (Waugh 2007) is blasted against a reference database, yielding a taxonomic assignment. This database contains reference barcoding sequences derived from reliably identified specimens with existing voucher specimens. The respective voucher should be available in a scientific collection for repeatability of morphological determination and (optimally) also for additional molecular genetic analysis. Unfortunately, only a few DNA barcodes of helminths have

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been generated up to now, preventing a reliable genetic identification of helminths. This is even more frustrating because it would enable linking larval stages or tissues of parasitic helminths to sequences generated from reliably identified adults (Alcántar-Escalera et al. 2013). The first efforts to apply a molecular genetic approach on larval stages of helminths also revealed new information about geographic distribution, intermediate host specificity, and host range (Locke et al. 2011; Reier et al. 2019).

In 2007 the international database Barcode of Life Data System (BOLD) was established, and many projects worldwide have contributed to it. In Austria, the Austrian Barcode of Life (ABOL) initiative was founded in 2014, and the present study was part of the ABOL pilot study "parasitic worms". The BOLD criteria also apply to the ABOL database. Accordingly, apart from gaining enough DNA to generate a DNA barcode, a requirement is that the examined specimens are preserved as vouchers in a scientific collection under the best possible conditions (Ratnasingham & Hebert 2007). Additionally, high-quality photographs of reference specimens are desirable, ideally showing the relevant morphological traits of the species. Due to the often small size and the weakly defined morphological characters of parasitic helminths, it can be difficult to apply these standards on single individuals of such species. Nevertheless, using whole specimens for extraction should be avoided in a DNA barcoding project, and exceptions should only be made if no alternative procedure is appropriate. Thus, alternative DNA extraction approaches are needed to establish DNA barcodes of parasitic helminths.

Obtaining sufficient DNA from specimens, which should be vouchered, is not the whole problem in DNA barcoding parasitic helminths. Proper species identification requires sample preparation, which itself often conflicts with DNA yield and DNA quality. Chemicals such as glycerol and borax carmine are important in the preparation procedure to investigate relevant morphological traits under the light microscope and to capture microphotographs showing all specific helminth characters (Berland 1984). In biological collections, formalin is sometimes added to the fixative and to mounting media to prevent mould and bacterial contamination, and to ensure a long-term preservation (comp. Gatenby & Beams 1950; Adam & Czihak 1964; Piechoki 1975). The impact of such chemicals on DNA yield and DNA quality of parasitic helminths is still poorly investigated. The formaldehyde contained in formalin leads to a cross-linking between proteins and DNA as well as protein-protein linking (Schander & Kenneth 2003). Moreover, formaldehyde presumably leads to fragmentation of DNA (Zimmermann et al. 2008; Hykin et al. 2015). These properties may cause problems in PCR amplification and DNA extraction (Srinivasan et al. 2002). Some studies indicated that such cross-linkage might be broken up using specific chemical agents and a prolonged digestion with Proteinase-K in DNA extraction (minimum 6 h) (Schander & Kenneth 2003). Nevertheless, the impact of formalin used in low concentrations and over the short term are unknown. Some studies indicated that PCR amplification is not always inhibited in samples containing traces of formalin (Schiller et al. 2014; Jaksch et al. 2016). Glycerol is used as a preservative for micro-preparation as well as a medium for clearing specimens (Berland 1984). Storage of DNA standards for quantification in real-time PCR in 50% glycerol-double-distilled water is used in medicine (Schaudien et al. 2007; Röder et al. 2010), but to our knowledge no data are available on the impact on DNA preservation regarding storage of specimens in glycerol for morphological investigations. The same applies for borax carmine, which

is often used to stain helminths. We report here on our experiments conducted during a DNA barcoding study on the phylum Acanthocephala (parasitic helminths). We aimed to improve the methods in treating these small and poorly investigated animals. This may help to establish an optimized procedure enabling (1) proper morphological imaging, (2) DNA extraction from a minimal amount of tissue, (3) sufficiently good conservation of the voucher specimens, and (4) preservation of DNA for molecular genetic analyses. To overcome the dilemma between the need for sufficient DNA quality versus preservation of such small voucher specimens, we conducted experiments on DNA yield. The aim was to gain sufficient DNA for DNA barcoding while using the smallest possible tissue sample and to get a better insight into the effects of the chemicals used in preparing (conservation, staining and embedding) helminth samples.

Material and methods

Altogether 51 specimens of the species *Pomphorhynchus bosniacus* Kiskároly & Čanković, 1967 (Acanthocephala) from the intestine of a common barbel, *Barbus barbus* (L.) were analysed (24 in the MSQ experiment and 27 in the staining experiment). The specimens are deposited in the wet collection of the Natural History Museum Vienna (NHMW ZooEV InvNr. 21.274).

DNA extraction of minimal sample quantities (MSQ experiment)

To test an alternative DNA extraction approach on parasitic helminths, we conducted an experiment involving DNA extraction from minute tissue quantities gathered by a needle. The first trial was implemented with a blood lancet on three specimens of *P. bosniacus* (Acanthocephala) as test run (for details see below). The next trial was performed on 20 specimens of *Pomphorhynchus bosniacus* obtained from a single fish specimen (*Barbus barbus*, Danube River, Vienna) with insect preparation needles (0.45 × 38 mm). The tip of the needle was stabbed through the tissue of the parasitic worms, twisted carefully in the pseudocoel and transferred immediately into the ATL lysis buffer of the *QIAmp DNeasy Blood and Tissue Kit* (QIAGEN, Hilden, Germany) without removing any visible tissue. By applying this method, only cells directly attached to the needle are used for further DNA extraction steps, which were conducted following the protocol of the manufacturer. For a higher DNA yield, *QIAmp MinElute* columns of the *QIAamp DNA Micro Kit* (QIAGEN, Hilden, Germany) were used and DNA was eluted in 20 µl AE buffer. As a positive control sample, a piece of tissue of an ethanol-preserved specimen was used for DNA extraction. DNA quality of selected samples was quantified by inspecting the DNA aliquot on a 1 % agarose gel and by measuring selected samples with *Invitrogen Qubit® 3.0 Fluorometer* (Thermo Fisher Scientific Inc., Waltham, USA).

Tests for differently stained and fixed samples

We conducted a test series to determine the effect of different chemicals used for morphological identification on the DNA quality of helminths. For the experiment, we used 27 individuals (including one control) of *P. bosniacus* obtained from a single fish specimen (*Barbus barbus*). Figure 1 shows the experimental procedure: Each animal was incubated in an embryo dish in a pre-mixed solution with the following compositions:

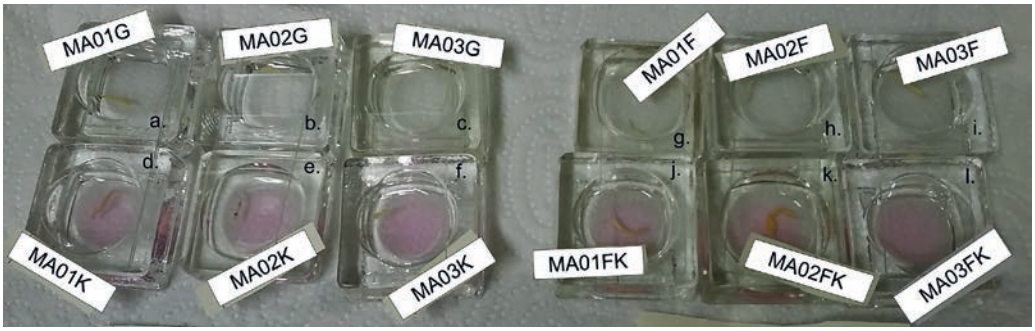

Fig.1: Experimental assembly of the one-month approach (same assembly as for three days). In each dish one specimen was added using four different solutions: 1000 µl glycerol + ethanol (50:50) (letters a.–c.), 1000 µl glycerol + ethanol (50:50) + 1 µl borax carmine (d.–f.), 1000 µl glycerol + ethanol (50:50) + 10 µl formalin (g.–i.) and 1000 µl glycerol + ethanol (50:50) + 10 µl formalin + 10 µl borax carmine (j.–l.). The sample lab-IDs for the one-month approach are indicated at each dish.

1. Glycerol + ethanol: 1000 µl solution (50:50) (a., b. and c. in Figure 1)
2. Glycerol + ethanol + borax carmine: 1000 µl glycerol-ethanol solution (50:50) + 10 µl borax carmine (d., e. and f. in Figure 1)
3. Glycerol + ethanol + formalin (4 vol% formaldehyde in distilled water): 1000 µl glycerol-ethanol solution (50:50) + 10 µl formalin (numbers g., h. and i. in Figure 1). The addition of 1% formalin to glycerol is meant to ensure long-term preservation without damage due to bacterial or fungal contamination.
4. Glycerol + ethanol + formalin + borax carmine: 1000 µl glycerol-ethanol solution (50:50) + 10 µl formalin + 10 µl borax carmine (j., k. and l. in Figure 1)

We conducted the same approach twice under different conditions. In the first trial, the animals remained for one month in the solution, in the second one for three days. One animal preserved in 80% ethanol was used as a positive control specimen. In addition, two specimens were kept in glycerol (as in 1. but after the evaporation of the ethanol) for two years on a microscope slide at room temperature for a long-term assessment.

All specimens were washed twice in ethanol for five minutes before molecular genetic analysis. DNA was extracted by cutting off a piece of tissue (from the body centre), and further steps were the same as described for the MSQ experiment.

PCR amplification and sequencing

We followed the protocol of Reier et al. (2019) for DNA amplification, using the forward primer *H14AcanCOIFw1* (TTCTACAAATCATAARGATATYGG) and the reverse primer *H14AcanCOIRv2* (AAAATATAMACTTCAGGATGACCAAA) to yield a 711 bp PCR product of the *COI* region of *mt* DNA (length of DNA barcode 661 bp). We used 3–5 µl of DNA template for samples extracted by needle and 1 µl of DNA template for all other extractions. PCR amplification was performed under the following conditions: 94°C for 7 min, 40 cycles of (94°C for 30 s, 48°C for 1 min and 75°C for 1 min) and 75°C for 10 min. Samples of the MSQ experiment that showed negative results in the first trial

were repeated up to three times. All positive PCR products of the PCR tests with differently stained and fixed material were sent for sequencing including the positive control. Of the MSQ tests, four PCR products were sequenced. PCR products were sequenced (both directions) by Microsynth (Balgach, Switzerland) using the PCR primers.

The sequences and the sample information were uploaded to the BOLD database with the accession numbers ACANT008-18 – ACANT023-18.

Results & discussion

DNA extraction of minimal sample quantities (MSQ experiment)

A test run was performed on extractions from samples gathered by the blood lancet. PCR from these three DNA solutions revealed positive results, i. e., a clear PCR product of the expected size. Furthermore, the positive control extracted with a standard protocol from a piece of tissue (DNA concentration: 11.9 ng/µl) yielded a positive PCR result. Of the 20 extractions performed with a needle, 16 had a positive result, indicating that in principle it is possible to obtain enough tissue by using a needle. Nonetheless, only five samples showed strong DNA bands on the agarose gel, whereas eleven showed only faint bands. Sequencing of three of the PCR products generated in this experiment confirmed the *COI* barcode of *Pomphorhynchus bosniacus*, but one of them showed several double peaks in the 3' section of the chromatogram. That chromatogram, which was obtained from a weak band amplified from a DNA template solution of 1.47 ng/µl, does not meet the quality criteria for complete and reliable DNA barcodes. The other two *COI* barcodes obtained from a strong DNA band (DNA concentration of template: 5.87 ng/µl) and from a faint DNA band (DNA concentration of template: 3.57 ng/µl) were of sufficient quality.

Overall, DNA extraction using needles is a very promising approach because the voucher specimens were only slightly damaged at one minuscule point of the midbody, which exhibits no taxonomically essential characters. We therefore recommend it for particularly rare (type and voucher) specimens. We suggest further testing and improving this method for DNA extraction of small animals (e. g. parasites, small molluscs) for DNA barcoding: Proteinase K treatment (e. g. overnight) during the DNA extraction process, as suggested by Schander & Kenneth (2003), could increase the DNA yield. Furthermore, other types of needles should be tested (e. g. hollow needles). Re-amplification employing nested primers could increase both the yield of PCR products and the quality of the subsequent sequencing.

PCR tests of differently stained and fixed samples

We obtained positive PCR results for samples treated with glycerol both for three days and one month (Table 1). In contrast, the results were negative after two years of preservation in pure glycerol. We therefore suggest transferring the specimens to 80 % ethanol immediately after the morphological analysis. Importantly, glycerol may prevent the ATL lysis buffer from permeating the tissue. This calls for at least two washing steps in ethanol before transferring the samples into the ATL lysis buffer for DNA extraction. The same results as for glycerol (three days, one month) were obtained for the samples

Table 1. Overview of the results of extracting DNA from differently stained and fixed tissues. Used solution, sample lab-IDs of individuals of the three-day approach (Lab-ID three days) and the one-month approach (Lab-ID one month). BOLD accession numbers, if available, are given. Legend: + positive results, -+ positive results exhibiting a poor PCR product, - negative results, asterisk: sequenced samples.

Solution	Lab-ID three days	BOLD accession number	Result PCR	Lab-ID one month	BOLD accession number	Result PCR
Glycerol + ethanol	DA01G*	ACANT008-18	+	MA01G*	ACANT017-18	+
	DA02G*	ACANT009-18	+	MA02G*	ACANT018-18	+
	DA03G*	ACANT010-18	+	MA03G*	ACANT019-18	+
Glycerol + ethanol + borax carmine	DA04K*	ACANT011-18	+	MA04K*	ACANT020-18	+
	DA05K*	ACANT012-18	+	MA05K		-
	DA06K*	ACANT013-18	+	MA06K		-
Glycerol + ethanol + formalin	DA07F*	ACANT014-18	+	MA07F*	ACANT021-18	+
	DA08F		+	MA08F*	ACANT022-18	+
	DA09F*	ACANT015-18	+	MA09F*	ACANT023-18	+
Glycerol + ethanol + formalin + borax carmine	DA10FK		-	MA10FK		-
	DA11FK		-	MA11FK		-+
	DA12FK*	ACANT016-18	+	MA12FK		-

where 1 % formalin was added (Table 1). The combination of formalin and borax carmine resulted in an immediate negative impact on DNA yield and quality. Only one sample of the three-day approach showed a positive result, and only one sample of the one-month approach was positive (Table 1) but the sequence was unreadable due to double peaks and a very low sequencing signal strength, possibly reflecting DNA degradation due to borax carmine. When mixed with glycerol, borax carmine showed positive results after three days, but negative results after one month. We conclude that borax carmine affects DNA quality and should not be used for morphological analysis if DNA extraction from the same sample is planned.

The positive control (ethanol-fixed but untreated sample) yielded a positive result in the PCR (BOLD accession ACANT024-18). Several studies indicate that the best option for preserving DNA quality is to freeze the specimen and / or tissue or to preserve it in ethanol (Schander & Kenneth 2003; Haring 2016). A long-term preservation (several decades) of good-quality DNA of samples stored in ethanol has been demonstrated (e. g. Zimmermann et al. 2008; Schiller et al. 2014; Haring 2016; Jaksch et al. 2016). Our results revealed that ethanol, glycerol and formalin used in the preparation of parasitic helminths do not impact DNA quality at least during a period of up to one month (cf. Table 1). All positive and subsequently sequenced samples matched the requirements for DNA barcodes and were genetically assigned to the target helminth species *P. bosniacus*. Overall, for an integrative taxonomic approach, we recommend that treatment of specimens with these staining and fixation solutions should be as short as possible to ensure sufficient DNA quality. Borax carmine should be avoided when sampling for DNA analysis. Thus, initial DNA sampling preceding further treatment is highly recommended.

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