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Rapid decline of PCR amplification from genomic extracts of DESS-preserved, slide-mounted nematodes

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Summary – Many studies use integrative methods to study both morphology and gene sequences of nematode species, yet there is little evidence to indicate the optimum criteria for merging taxonomic and molecular protocols. Preliminary evidence suggests that standard methods of desiccation and slide mounting nematode specimens in glycerin can sporadically result in degradation of genomic DNA. A time series experiment was constructed in order to assess whether this degradation of genomic DNA could be recorded and quantified. Two groups of nematode specimens were desiccated, mounted on slides, and stored at either 4°C or at room temperature for specified time intervals. Genomic DNA was extracted and PCR was conducted to amplify a section of the 18S rRNA gene at each time point. The resulting gel photographs were used to record the outcome of PCR and quantify the strength of amplification if successful. Results from slide-mounted specimens were compared to PCR products derived from unmounted nematodes extracted directly from the preservative solution at specified time intervals. The desiccation and slide mounting process appears to reduce overall band intensity after 1 day in slide mounts. Unmounted specimens consistently exhibit high success rates of PCR and a high overall DNA content per band at all time points, whereas slide-mounted nematodes show a decrease in the number of successful PCRs and weakening band intensity as time progresses. Results clearly indicate a steady degradation of genomic DNA in nematodes stored in slide mounts for more than 2 weeks, whereas unmounted specimens extracted from preservative solution showed no decline in PCR success or quality. We suggest a maximum storage period of 2 weeks on slides if mounted nematodes are to be used for molecular analyses.

Keywords – DESS, DNA, glycerin, integrated methods, molecular, morphology.

The use of molecular data in nematode studies is now ubiquitous, yet it is still routine for researchers to corroborate DNA sequences with morphology in order to assess the biological relevance of molecular patterns (Griffiths *et al*., 2006; Stock & Nadler, 2006; Bhadury *et al*., 2008). DNA barcoding studies promote visual identification of specimens as well as the retention of 'morphological voucher images' to record taxonomic features before nematodes are destroyed for DNA extraction (De Ley *et al*., 2005; Bhadury *et al*., 2006a). These barcoding studies have also provided empirical evidence concerning the resolution of different genetic loci used in species identification, with the nuclear 18S ribosomal subunit gene currently recommended as the ideal barcoding locus for nematodes (Bhadury *et al.*, 2006a).

Despite the frequent integration of morphological and molecular protocols in nematode studies, there is little published information to suggest how these two disparate approaches might best be optimised to obtain the most robust data. Taxonomic protocols are designed to maximise the clarity of specimens viewed under a microscope while preservatives and mounting methods are determined based on the resulting physical effects, ideally 'hardening' nematode anatomy and not introducing any preservation artefacts. The ideal protocol will provide unambiguous presentation of morphology and will cater for long-term storage of nematode specimens. In contrast, the success of molecular techniques hinges on the chemistry of reactions; PCR reactions are only successful if the concentrations of different ingredients are balanced at specific ratios. For reagents that are added to enhance reactions (such as formamide or $MgCl_2$), there is usually only a narrow concentration range within which any given substance can aid DNA amplification, with excess amounts of

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such substances becoming inhibitory (Saunders & Parkes, 1999). Taxonomic protocols are quite 'dirty' in comparison to most molecular protocols, which aim for purity and strict control of all reaction components. The ideal taxonomic protocol will provide unambiguous presentation of morphology and will cater for long-term storage of nematode specimens, regardless of the molecular impacts effected during this process.

For nematode studies, the currently favoured preservative is DESS, a dimethyl sulphoxide (DMSO)/EDTA solution saturated with sodium chloride. DESS offers the advantage of preserving both morphology and DNA with one solution, as opposed to previous sampling methodology which required collection of separate subsamples in ethanol and formalin for integrative studies (Yoder *et al.*, 2006). The latter situation could lead to potential discrepancies if species assemblages in subsamples differed, as well as preventing analysis of both morphology and DNA sequences from single nematodes. Although several studies have successfully amplified DNA from formalin-fixed samples (Thomas *et al*., 1997; Rubtsova *et al*., 2005), formalin is neither recommended nor widely used as a preservative when fresh material is being collected for molecular work. This study was aimed at optimising protocols for DESS-preserved material, and we will not discuss the separate problems encountered with amplifying DNA from formalin-fixed samples.

The adoption of DESS preservative facilitates and encourages the collection of morphological and molecular data from individual nematodes. However, this approach may be problematic; the most sensitive molecular procedures are situated furthest downstream in the overall process. Nematodes are exposed to many compounds during extraction and slide mounting, including colloidal silica (Ludox), ethanol, paraffin, glycerin and filtered tap water (non-distilled). Any of these compounds may subtly interact with nematode tissue and affect the chemistry of subsequent molecular reactions.

Previous work suggested that standard taxonomic slide preparations of nematodes mounted in glycerin could be adversely affecting DNA integrity, thus reducing subsequent PCR success (Meldal, 2004; Cook *et al*., 2005). We have also encountered frequent problems in obtaining reliable and consistent PCR results from DESS-preserved specimens stored in slide mounts and determined that a formal study was needed to assess the impact of standard methodology. To determine the true cause of the recurrent molecular problems, a time series experiment was designed to test the effect of the slide mounting process and account for any preservative effects.

Materials and methods

Fresh estuarine sediments were collected in February 2008, at the mouth of the River Torridge in Devon, southwest England (51°1′54″N, 4°12′12″W) and immediately fixed in DESS preservative. All samples were collected using an equal ratio of preservative to sediment. Each sample was thoroughly shaken after collection to distribute the preservative. The meiofauna fraction was extracted *via* decantation and floatation in Ludox using a 45 μm sieve according to the methods of Somerfield *et al.* (2005). All collected sediments and extracts were stored at 4°C when not needed. Nematodes were individually picked out of the extracted fraction under a dissecting microscope and transferred to a watch glass containing dehydrating solution (90% distilled water, 5% molecular grade glycerin and 5% molecular grade ethanol) before being placed into a desiccator for 48 h. Specimens were subsequently mounted on glass slides in anhydrous glycerin and sealed with a wax ring.

Two test groups were set up to examine whether the storage temperature of slide-mounted specimens would impact PCR success. Desiccation and slide storage were both done at 4°C for one set of nematodes and these processes were conducted at room temperature for a second set of nematodes. Picking and slide mounting of all specimens took place at room temperature as these procedures could be completed relatively quickly. At each predetermined interval in the time series (Table 1), ten slide-mounted nematodes from each temperature treatment were removed from slides and transferred into 1.5 ml microcentrifuge tubes. Genomic DNA was extracted *via* proteinase K digestion following the methodology of Holterman *et al*. (2006). Individual nematodes were picked out of slide mounts and transferred to 1.5 μl microcentrifuge tubes containing 25 μl sterile water. Lysis buffer (containing 0.2 M NaCl, 0.2 M Tris-HCl (pH 8.0), 1% β-mercaptoethanol and 800 μg ml^{-1} proteinase K) was added in an equivalent volume (25 μl). The final reaction volume was incubated for 2 h at 65°C and 750 rpm in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany), followed by a final 5 min at 100°C and 750 rpm to inactivate the proteinase K enzyme. Final lysates were stored at −20°C. To test for potential effects of the preservative solution over time, individual nematodes

Table 1. *Sampling intervals for slide mounting experiment.*

	Sampling time series							
	1 day	3 days	1 week	2 weeks	1 month	2 months	3 months	6 months
Room temperature	✓	✓	✓	✓	✓	✓	✓	✓
Cold treatment	✓	✓	✓	✓	✓	✓	✓	✓
Picked from DESS	×	×	✓	×	✓	×	✓	✓

All slides were prepared together at the start of experiment and a subset of nematodes was picked out at intervals as time progressed. Specimens picked from DESS were taken from the sample extracted on the dates indicated and genomic DNA was immediately extracted. Unlabelled bands on the gel photos (3-month and 6-month time points) were not part of this experiment.

were picked straight out of meiofauna extracts at 1 week, 1 month, 2 months and 6 months after collection. At each time point, genomic DNA was extracted from individual nematodes and PCR was conducted.

To test preservation of DNA, the metazoan specific primers G18S4 (5′-GCTTGTCTCAAAGATTAAGCC-3′) and 22R (5′-GCCTGCTGCCTTCCTTGGA-3′) (Blaxter *et al.*, 1998) were used to amplify a *ca* 400 bp product of the nuclear 18S rRNA gene under the following profile: 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 45 s, extension at 72°C for 2 min, with a final extension of 72°C for 10 min. The PCR reaction was done using a DyNAzyme EXT PCR kit (New England Biolabs, Ipswich, MA, USA), with a final reaction volume of 25.75 μl. Each reaction contained 2 μl of genomic DNA, 18.25 μl sterile water, 0.4 μM of each primer (Integrated DNA technologies, Coralville, IA, USA) 2.5 μl 10× DyNAzyme EXT Buffer containing $MgCl_2$ (final reaction volume 1.5 mM $MgCl_2$), 0.5 μl dNTP mix containing 10 μM each nucleotide, and 0.5 μl Dynazyme EXT DNA polymerase (0.5 enzyme units in final reaction volume). The varying sizes of individual nematodes presumably resulted in different amounts of starting template in individual PCR reactions. In order to save costs and emulate standard protocols, no attempt was made to measure the DNA content of nematode genomic templates. Positive and negative controls were amplified alongside all reactions containing experimental samples. Samples were subsequently visualised by running 5 μl PCR product on a 1.5% agarose gel containing ethidium bromide. The same volume of a quantitative ladder, Hyperladder V (Bioline, London, UK), was run alongside all samples in order to estimate the amount of amplified DNA based on the resulting gel photographs.

Results

Results clearly demonstrate reduced PCR success over time for preserved nematodes mounted in glycerin, with eventual failure of PCR amplification. Figure 1 shows the size markers used in this study. Figure 2 displays results obtained from unmounted nematodes amplified directly from DESS preservative. Successful amplification can be observed at all time points with no obvious decrease in band intensity. Visual inspection of gel photographs reveals that band intensity is noticeably weaker for nematodes mounted on slides after 1 day, compared to PCR products from individuals picked straight out of DESS. Similar band intensities are seen for PCR products of specimens mounted for 3 days (Fig. 3), 1 week and 2 weeks (Fig. 4). After 1 month and 2 months in slide mounts, PCR amplification of 18S almost fails completely, apart from a few weak bands still visible on the gel photograph (Figs 5, 6). A sudden, universal, low-level amplification is seen in all slide-mounted specimens extracted at a time point of 3 months (Fig. 7). This anomalous result was initially suspected to be laboratory contamination, yet the negative control shows no sign of any contaminant and the positive control confirms suc-

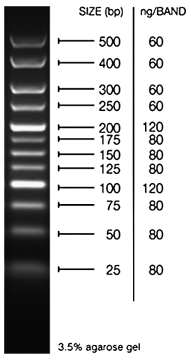

Fig. 1. *Size markers used in this study.*

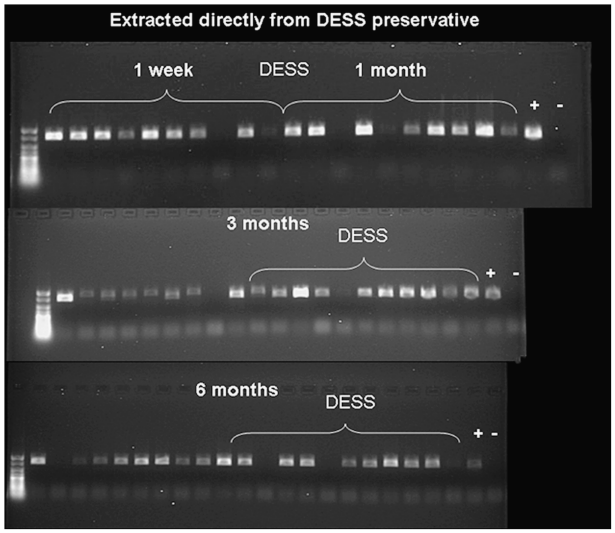

Fig. 2. *PCR amplification for unmounted nematodes picked out of DESS preservative and directly digested for molecular work.*

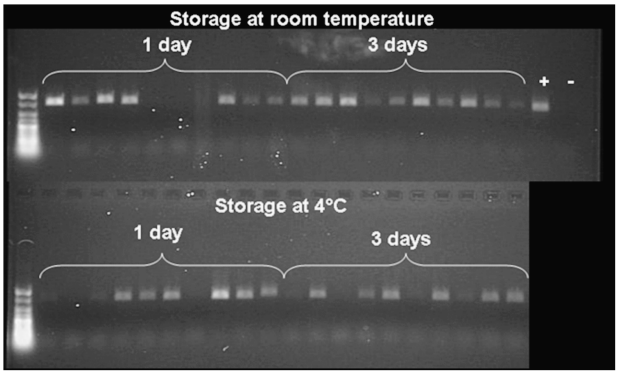

Fig. 3. *PCR amplification of slide-mounted specimens at 1-day and 3-day time points.*

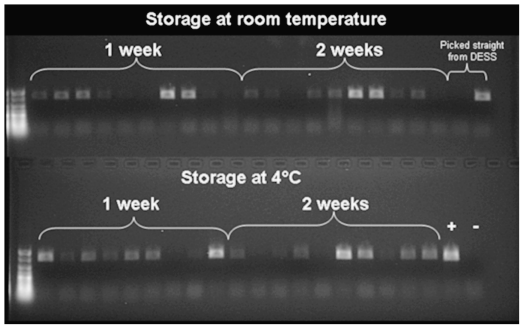

Fig. 4. *PCR amplification of slide-mounted specimens at 1-week and 2-week time points.*

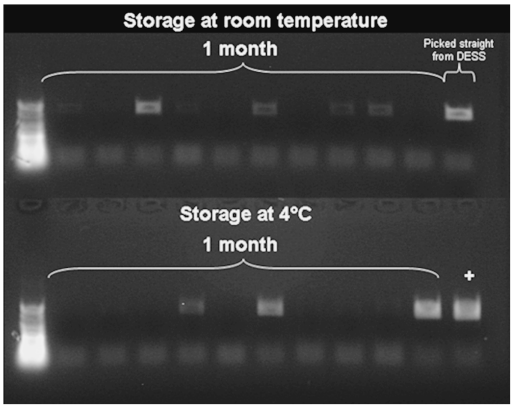

Fig. 5. *PCR amplification of slide-mounted specimens at the 1-month time point.*

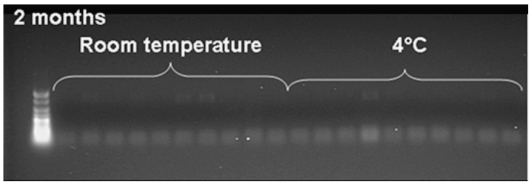

Fig. 6. *PCR amplification of slide-mounted specimens at the 2-month time point.*

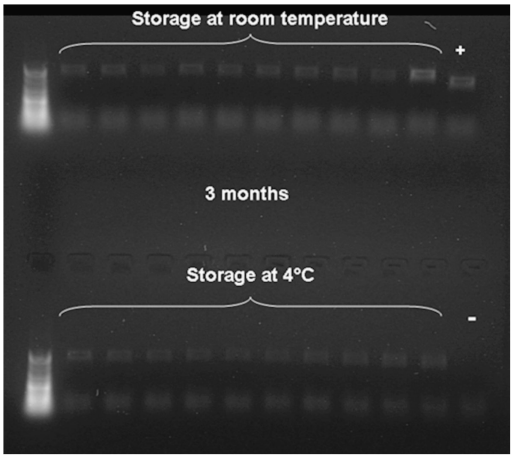

Fig. 7. *PCR amplification of slide-mounted specimens at the 3-month time point.*

cess of the PCR reaction. At 6 months (Fig. 8), only one very faint band is visible amongst 20 specimens amplified.

The overall success of PCR reactions is summarised in Figure 9. The amount of DNA in successful PCR reactions was subsequently quantified by comparing band intensities of nematode samples with the appropriately

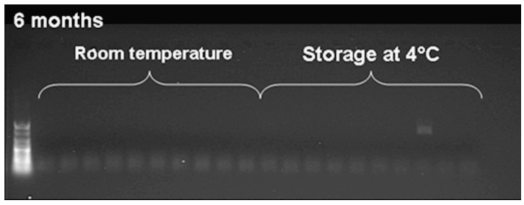

Fig. 8. *PCR amplification of slide-mounted specimens at the 6-month time point.*

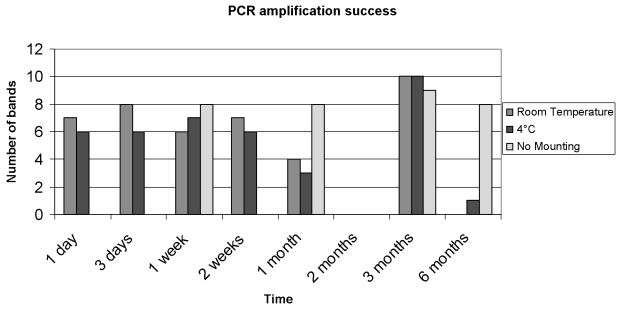

Fig. 9. *PCR amplification success over time for slide-mounted and unmounted nematodes. Bars represent the number of nematodes from which the 18S gene was successfully amplified. Note that unmounted nematodes were only amplified at the 1-week, 1-month, 3-month and 6-month time points.*

sized band of the quantitative ladder. The 400 bp band of Hyperladder V (representing 60 ng of DNA) was used as a marker for evaluating the strength of the nematode PCR products on all gels run. Gel bands that were equally intense or brighter than the ladder band were classed as containing equal or greater amounts of DNA ($\geqslant$60 ng), whilst weaker bands were classed as containing less DNA (<60 ng) than the ladder. All category assignments were done by eye after visual inspection of each gel photo.

For slide-mounted specimens, both temperature treatments show similar success of overall PCR amplification for each time point (Fig. 9). However, quantification of these PCR products reveals that the storage of slide-mounted nematodes at 4°C resulted in more robust PCR amplifications. Room temperature specimens often contained a substantial proportion of weak bands (<60 ng) with these low intensity bands becoming the dominant category at later time points (Fig. 10). The cold treatment group exhibited a greater proportion of strong band intensities ($\geqslant$60 ng) at each time point (Fig. 11) compared with specimens kept at room temperature. For unmounted nematodes that were amplified straight from DESS preservative, band intensity is overwhelmingly strong ($\geqslant$60 ng) at

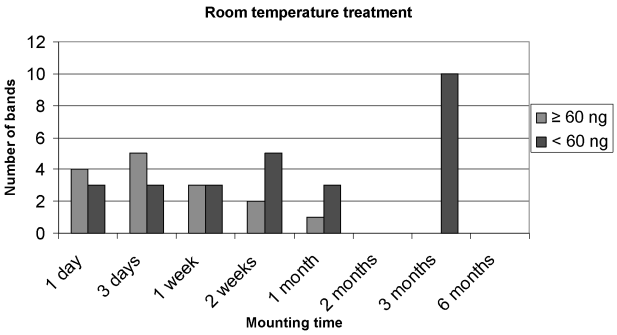

Fig. 10. *Quantification of DNA content in bands appearing on gel photographs for slide-mounted nematodes stored at room temperature. DNA content estimated per 5 µl PCR product.*

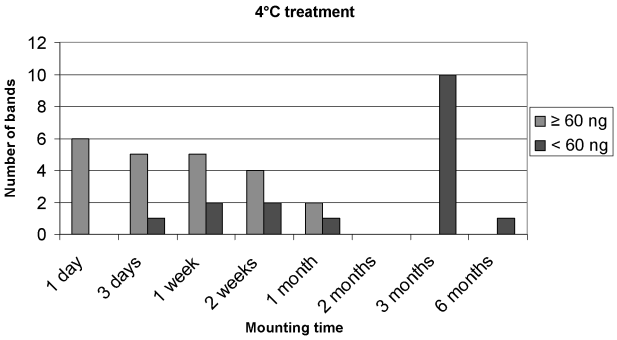

Fig. 11. *Quantification of DNA content in bands appearing on gel photographs for slide-mounted nematodes stored at 4°C. DNA content estimated per 5 µl PCR product.*

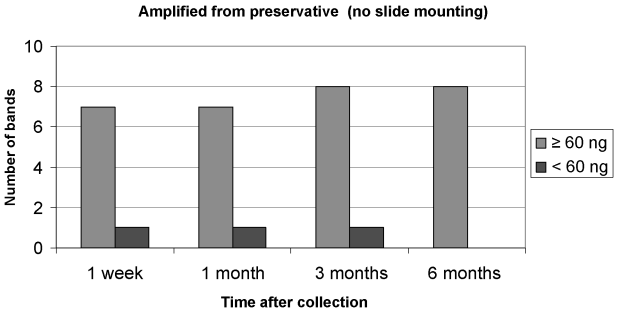

Fig. 12. *Quantification of DNA content in bands appearing on gel photographs for unmounted nematodes amplified directly from DESS preservative. DNA content estimated per 5 µl PCR product.*

all time points with very few weak bands amongst all PCR products obtained (Fig. 12). Amplification of unmounted nematodes remains strong at time points where the PCR success of slide-mounted specimens is reduced (2 months) or has virtually failed (6 months).

Discussion

Slide mounting is likely to remain an important step for integrative studies, although the exact methodology currently varies between studies. Techniques currently used include temporary mounts of live nematodes (De Ley *et al.*, 2005), semi-permanent (Bhadury *et al.*, 2006a, 2008), or permanent (Eyualem & Blaxter, 2003) mounts of specimens in glycerin. Although the nematodes picked straight out of DESS display high success rates of PCR at all time points, the morphology of these non-desiccated, preserved nematodes is less than ideal for identification and video capture. However, the clarity of morphology in DESS-preserved specimens can equal or exceed that of formalin-fixed specimens if nematodes are subject to desiccation and subsequently mounted in glycerin (Yoder *et al.*, 2006).

Results from the time series experiments demonstrate that the success rate of PCR amplification of nematode DNA falls sharply after 2 weeks of storage in slide mounts, with eventual failure of PCR amplification. The low level amplification seen at the 3-month interval is unlikely to represent nematode DNA, given the reduced PCR success for most slide-mounted nematodes at 1 month and overall failure of amplification at 2 and 6 months. Considering that the negative control is blank, any contaminant would need to be present in the nematode genomic template and not present in the master mix of PCR reagents. While it cannot be determined for certain what these bands represent, environmental fungal contamination is suspected. The bands obtained from experimental samples at the 3-month time point are slightly larger in size then expected (compared with the nematode positive control); at previous time intervals for slide-mounted and unmounted samples, the positive control is equivalent in size to the experimental bands. The authors have noted similar weak banding patterns amplified from degraded marine nematodes removed from slide mounts. Further attempts at sequencing such bands usually resulted in failed sequencing reactions or intermittent short sequence reads. Sequence similarity searches on GenBank indicated that the resulting contaminant sequences matched to marine fungi (Bhadury *et al.*, unpubl.). Certain regions of the 18S rRNA gene are highly conserved across eukaryotes and supposedly nematode-specific primers have been known to co-amplify fungal 18S genes from environmental samples (Bhadury *et al.*, 2006b). Fungi are a well known source of food for terrestrial nematodes (Munn & Munn, 2002) and the ubiquity of fungi in marine and estuarine ecosystems (Hyde *et al.*, 1998) makes them a likely source of food for nematodes in those environments as well. Thus, genomic extraction protocols which involve the digestion of whole specimens may also inadvertently extract the DNA of any fungi that may be present within the digestive tract.

There is a clear need for specific taxonomic protocols that will facilitate the success of molecular work on mounted specimens, especially considering the continued expansion of molecular work on free-living nematodes. Previous studies have indicated that nematode degradation in glycerin is unpredictable and DNA can be affected at times ranging from days to months (Cook *et al.*, 2005). We have also found similar variation in success: DNA has degraded after only a few weeks on slides for some specimens, whilst for other nematodes PCR amplifications and sequencing have been successful even though specimens were kept stored in slide mounts for several months. Meldal (2004) kept specimens mounted in glycerin for a maximum of 2 weeks before removing them for molecular work. Results from the present study further support this timeframe as an acceptable window for storing nematodes in slide mounts.

The desiccation and slide mounting process appears to reduce overall band intensity immediately: after only 1 day in slide mounts there were fewer strong bands ($\geqslant$60 ng) in both temperature treatments compared to the first time point of the unmounted treatment. Unmounted specimens consistently exhibit high success rates of PCR and a high overall DNA content per band at all time points, whereas slide-mounted nematodes show a decrease in the number of successful PCRs and weakening band intensity as time progresses. Storing slide mounts at 4°C seems to increase the strength of PCR amplifications in the short term. The cold treated slides showed a higher proportion of strong bands ($\geqslant$60 ng) at each time point compared to the room temperature treatment, even though the total number of bands present per time point was almost always higher for the room temperature treatment. Strong bands are preferred if the PCR product is to be purified and sequenced; higher DNA content increases the chance of successfully sequencing the amplified gene region, although weaker bands can often return good quality sequences if the amount of starting template in the sequencing reaction is increased. The likelihood of a failed sequencing reaction increases as the quantity of DNA within a PCR reaction (and thus band intensity) decreases.

We have not found a suitable explanation that conclusively accounts for DNA degradation in slide-mounted nematodes. Glycerin and DMSO (a component of DESS preservative) can be used as additives to aid PCR success, but neither substance has been shown to inhibit reactions at high concentrations (Bickley & Hopkins, 1999). Sodium chloride, another component of DESS preservative, is known to inhibit PCR reactions at concentrations >25 mM (Bickley & Hopkins, 1999), but if this compound was solely responsible we would expect failure of all PCR reactions from preserved nematodes. One plausible explanation relates to the decreased thermal stability of DNA in the presence of glycerin and sodium ions (Sorokin *et al.*, 1997). Individual DNA strands are quite stable at high temperatures and it is standard procedure to keep DNA at 95°C for several minutes in PCR reactions. At this temperature, hydrogen bonds between complementary DNA strands are broken and double stranded DNA separates into single strands although the integrity of individual strands is not diminished (Turner *et al.*, 2000). Sorokin *et al.* (1997) note that glycerin normally enhances the thermal stability of DNA molecules, but when glycerin and sodium ions are present concurrently this stabilising effect is greatly reduced. Reduced thermal stability could increase the likelihood of individual DNA strands to denature when heated; this could prevent PCR amplification if the template DNA is structurally damaged. In DESS preservative, DMSO increases the porosity of cell membranes and allows NaCl to physically enter cells and inactivate nuclease enzymes (Yoder *et al.*, 2006). This preserves DNA integrity by preventing shearing, but also results in high cellular concentrations of sodium chloride. After desiccation has been carried out, glycerin has also been introduced to the nematode tissues. Slide mounting protocols require that slides are briefly placed on a hotplate at 65°C in order to melt the wax ring and secure the cover slip. This seemingly benign step may, in fact, have significant implications for the longevity of cellular DNA. The heating of desiccated specimens containing high cellular concentrations of glycerin and Na^+ ions may lead to physical alterations of DNA structure and/or chemical interactions resulting in decreasing integrity of DNA over time for mounted nematodes. Future studies will need to investigate whether DNA storage can be improved by avoiding heat during slide mounting.

The slide mounting process remains an undeniably essential component for morphological identification of nematode specimens. We currently utilise a timeframe of 3 days maximum from initial desiccation of nematodes until extraction of genomic DNA and have reliably amplified and sequenced gene fragments of 18S from hundreds of specimens processed in this way, with only occasional failures. After the extraction of genomic DNA has been completed, samples can be safely stored at −20°C until needed for molecular work, with no further need for concern regarding the integrity of nematode DNA.

For integrative studies, it is crucial to obtain correct taxonomic identifications and capture a record of digital voucher images if the specimen is going to be destroyed for molecular work. The desiccation and slide mounting process is especially necessary for inexperienced taxonomists who may not be able to decipher morphological features from specimens extracted straight out of preservative solution. Knowledge of nematode diversity remains sparse and reliable identifications derived from DNA sequences are unlikely to be accurate given the current depauperate state of nematode sequence databases. Collection of morphological data will continue to be utilised in nematode studies for years to come.

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