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TECHNICAL ADVANCES

Laser-assisted microdissection for the study of the ecology of parasites in their hosts

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Abstract

The population biology of internal parasites is difficult to study because the adult parasites are often inaccessible, deep within the host's body. Developing stages, such as eggs in the faeces or larvae in the skin are more easily obtained, but are difficult to handle because they are often very small and with a tough cuticle. This has limited their use in molecular ecology for estimating population biology parameters of the adults (their parents). We have used *Onchocerca ochengi* (a filarial nematode parasite of cattle) to describe a novel and generally applicable method of easily and conveniently isolating individual larvae (microfilariae) from the host using laser-assisted microdissection. Furthermore, we have been able to improve the isolation of DNA by using the laser to bisect the larva to release DNA from the tissues enclosed within the parasite cuticle, and in this way we have achieved amplification of fragments over 1400 bp, and routinely PCR-amplified single-copy sequences from 5% of the DNA from a single larva (the equivalent of approximately 15 nuclei), and regularly from 0.5%.

Keywords: DNA profiling, filariae, laser microdissection, microfilariae, *Onchocerca ochengi*, PCR

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Introduction

The population biology of parasites is, in general, difficult to study because significant parts of the life cycle can be spent within the host. These can present various sampling problems. Typical examples can be found among the filariae, which are helminthic, nematode worms which parasitize a wide range of terrestrial vertebrates, including amphibia, reptiles, birds and mammals. They can be transmitted by various biting arthropods including ticks, mosquitoes, blackflies, midges and horseflies, and they can be important in animal (and human) health (Muller 1979) and have a significant impact on the host population. The adult parasites are usually found in various deep tissues, and are therefore inaccessible without killing the host to sample them. However, the larvae (microfilariae) are much more easily accessible because they occur in superficial tissues (e.g. skin and capillary blood) to be available to the blood-sucking vector. Standard methods for parasitological study involve taking a small skin biopsy or blood smear from the living host and identifying and counting the microfilariae. These methods present many inadequacies. Microfilariae of closely related parasite species can be difficult to distinguish using light microscopy, for example, *Mansonella ozzardi* and *Onchocerca volvulus* in humans (Post *et al*. 2003), or *Onchocerca gutturosa* and *Onchocerca lienalis* in domestic cattle (Muller 1979). Sampling microfilariae can yield estimates of the density of microfilariae, but not the numbers of adult parasites, so that, for example, the lifespan and reproductive biology of the parasite will remain unknown. However, these are very important parameters in understanding the population ecology and transmission of the parasites (Basáñez & Boussinesq 1999), and could be assessed by the application of the molecular markers to individual microfilariae, for example DNA barcoding for species identification and microsatellite profiling to identify sibling groups (and hence, numbers and identity of adult parasites). However, microfilariae are difficult to work with. They are very small (see Fig. 1a),

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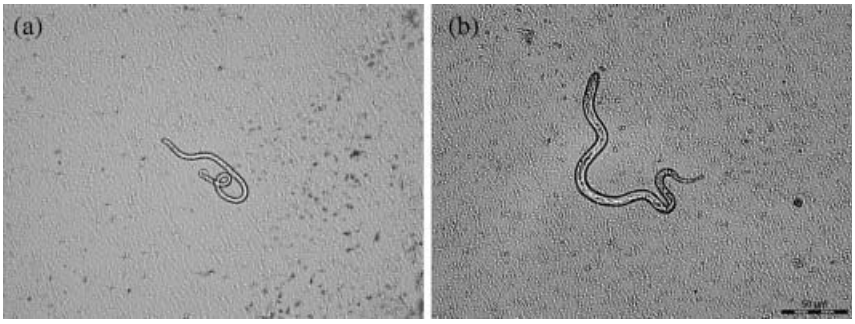

Fig. 1 Microfilariae of (a) *Onchocerca gutturosa* and (b) *Onchocerca ochengi* dried onto a membrane slide at the same magnification.

which makes them easily lost in handling procedures, and it is difficult to ensure that only single specimens are transferred to polymerase chain reaction (PCR) tubes (for example). They can stick to the sides of plasticware (such as pipette tips) and glassware (Oskam *et al*. 1996). Access to their DNA also presents particular problems. They have a tough nematode cuticle which has proved difficult to break open reliably and routinely, and has required the development of special treatments such as chitinase-digestion (Sim *et al*. 1989) or treatment with DTT with freeze-thaw cycles (Unnasch & Meredith 1996). Furthermore, microfilariae contain only small quantities of DNA. For example *O. volvulus* has approximately 280 nuclei (Post 2005) (equivalent to 560 copies of the nuclear genome, assuming that all the nuclei are diploid), and *Onchocerca ochengi* is more or less the same size and expected to be similar. Analogous problems and opportunities are presented by other groups of parasites in their living hosts. For example, intestinal helminths are routinely studied by searching the host's faeces for (embryonated) eggs, and they have been shown to regulate wildlife populations (Tompkins & Begon 1999). The purpose of this study is to present a method for molecular microscopy which overcomes many of these technical problems using laser-assisted microdissection.

Laser microdissection microscopes are available off-the-shelf from the major manufacturers, and they have become routine tools in many areas of cell biology, particularly cancer biology. They allow the microscopic identification of morphologically distinct groups of cells (or even single cells) which can then be dissected away using the laser for subsequent genetic or proteomic characterization (Ellsworth *et al*. 2003), and the technique is being introduced slowly into parasitology (Jones *et al*. 2004).

Onchocerca parasites of cattle are frequently used as biological models in parasitological research (Muller 1979), and four species are known in northern Cameroon (Wahl *et al*. 1994). Adults are either found in nodules under the skin (*O. ochengi* and *O. dukei*) or in the walls of the aorta (*Onchocerca armillata*) or along the neck ligaments (*O. gutturosa*), but the microfilariae are all found in the skin. These species cause little noticeable disease, but *O. armillata* is occasionally associated with rupture of the aorta and the skin nodules reduce the value of the hides. These parasites are not known from wild animals. The closely related *O. volvulus* causes river blindness, which is a major tropical disease in humans. We have explored the potential of laser-assisted microdissection for the genetic analysis of individual microfilariae of *O. ochengi* from mixed infections in cattle in northern Cameroon.

Materials and methods

Collection of biological material

Samples of skin approximately 10 × 5 cm just anterior to the udder were taken from the hides of cattle postmortem at Ngaoundere abattoir in Cameroon on 29 January 2008. The skin was incubated in phosphate-buffered saline (PBS, pH 7.4) for several hours and microfilariae which emerged were pipetted and spread *en masse* onto Leica steel-frame 0.9 µm POL-membrane slides, which were allowed to dry for 1–2 h at ambient temperature. Membrane slides are metal frames with a plastic membrane stretched over a window in the middle of the slide, so that microfilariae can dry onto the membrane and can be clearly seen by transmission light microscopy. Slides were stored at room temperature over silica gel. They were further processed after approximately 5 months.

Laser-assisted microdissection

Dry preparations were placed inverted and without a coverslip on the stage of a Leica LMD6000 laser dissection microscope and viewed on a computer monitor. Individual microfilariae were identified according to Wahl *et al*. (1994) (Fig. 1) and *Onchocerca ochengi* chosen for isolation (Fig. 1b). Microfilariae were circumscribed with the mouse and subsequently cut around with an ultraviolet laser (Fig. 2c–e), which is controlled by the system software to follow the line defined by the mouse. The sample, still attached to the cut fragment of plastic membrane, falls by gravity into a PCR tube cap, where it can be observed through the microscope to confirm that it has been captured (Fig. 2f). Static electric charge was sometimes found to be a problem,

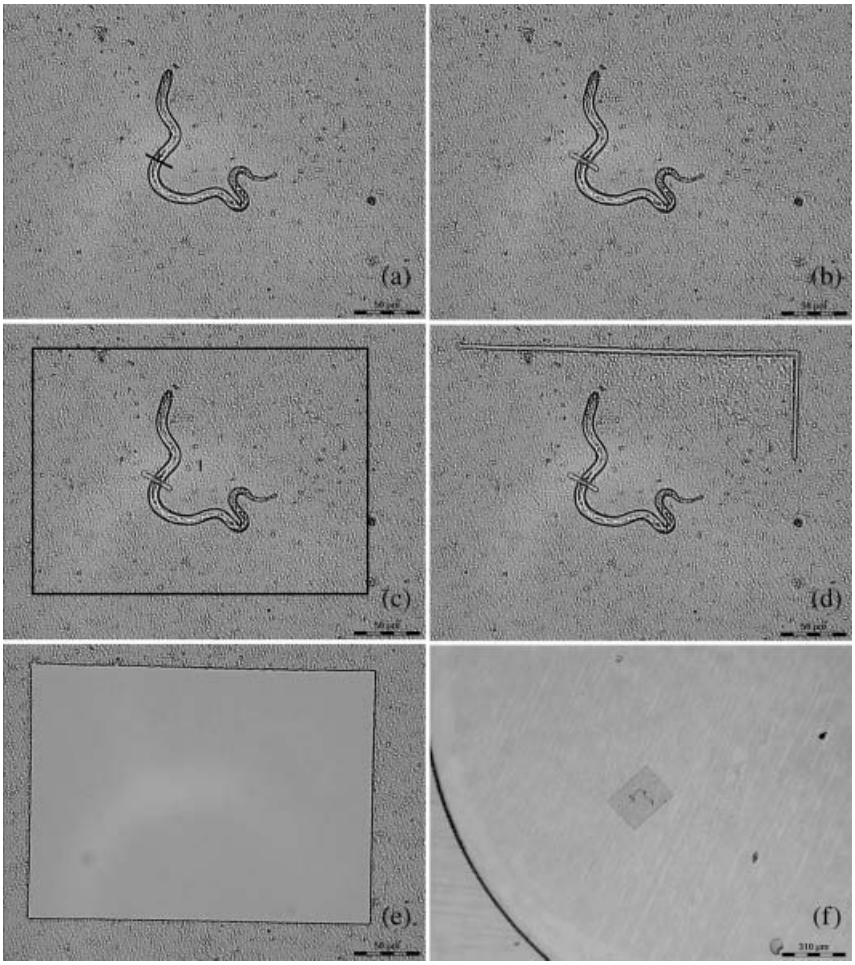

Fig. 2 Isolation of a single microfilaria of *Onchocerca ochengi* by laser-assisted microdissection. (a) Bissection line defined by the mouse, and (b) the laser cut. (c) microfilaria circumscribed by the mouse (d) laser in the process of cutting (e) laser cut complete, and (f) microfilaria having fallen into the PCR tube below.

preventing the cut membrane from falling directly into the PCR tube cap. To minimize this problem, the components of the system (microscope, slides and PCR tubes) were subjected to a flow of neutralizing ionized air (Charles Water bench top ionizer 9229) for 5 min before microdissection, and fragments of membrane to be cut out were made significantly larger than the microfilaria itself so as to increase their weight and hence chances of free fall. Microfilariae were collected individually or in batches of 10. In some cases, microfilariae were bisected using the laser before capture (Fig. 2a, b). Microfilariae were taken immediately on to DNA extraction.

DNA extraction

DNA to be used as template for PCR was prepared from laser-captured microfilariae using the QIAamp DNA Micro kit (QIAGEN) following the 'isolation of genomic DNA from laser-microdissected tissues' protocol, with QIAamp MinElute columns and DNA recovered in 30 μL or 50 μL of sterile distilled water. Alternatively, microdissections were incubated in 30 μL or 50 μL of proteinase K extraction buffer (400 μg proteinase K/mL, in 100 mM Tris-HCl pH 8.0) at 56 °C for at least 3 h according to Roehrl *et al*. (1997), and used without further purification.

DNA detection

In total, 11 sets of PCR primers were used to amplify eight genes (listed in Tables 1 and 2) from *O. ochengi* DNA extracts. For all genes, the presence of amplifiable *O. ochengi* DNA was scored by the presence of a PCR amplicon of expected size visualized on 0.8% or 2% agarose gels (depending upon fragment size) stained with ethidium bromide and observed using a Gene Genius Bioimaging System (Syngene), with confirmation of samples by direct Sanger-sequencing (except O-150, see below), using both the forward and reverse primers that had been used to amplify them, in an ABI 96-head capillary 3730xl DNA Analyser. PCR clean-up, quantification and sequencing was all carried out at the Natural History Museum's sequencing facility.

Five sets of primers were used to amplify multicopy DNA from *O. ochengi*. Mitochondrial DNA deriving from

Table 1 Number of PCRs producing a visible amplicon of multiple-copy DNA. The percentage is the amount of the DNA preparation used as template for the PCR, followed by the proportion of reactions which yielded a visible amplicon

DNA: numbers of microfilariae: laser	QIAGEN method single mf bisected	Roehrl method single mf bisected	QIAGEN method batch 10 mfs bisected	Qiagen method batch 10 mfs unbisected
Mt 12 s rDNA	16.7%: 15/15	16.7%: 6/6	16.7%: 1/1	16.7%: 1/1
(expected size: 518 bp)	1.7%: 15/15	1.7%: 6/6		
Mt 16 s rDNA	16.7%: 15/15	16.7%: 6/6	16.7%: 1/1	16.7%: 1/1
(expected size: 486 bp)	1.7%: 15/15	1.7%: 6/6		
Mt NADH ND5	16.7%: 15/15	16.7%: 6/6	16.7%: 1/1	16.7%: 1/1
(expected size: 471 bp)	1.7%: 15/15	1.7%: 6/6		
Nuclear 5.8 s rDNA	16.7%: 15/15	16.7%: 6/6	16.7%: 1/1	16.7%: 1/1
(expected size: 565 bp)	1.7%: 15/15	1.7%: 6/6		
O-150 satellite	16.7%: 15/15	—	16.7%: 1/1	16.7%: 1/1
(expected size: 150 bp)	1.7%: 15/15			

Table 2 Number of PCRs producing a visible amplicon of single-copy DNA. The percentage is the amount of the DNA preparation used as template for the PCR, followed by the proportion of reactions which yielded a visible amplicon

DNA: numbers of microfilariae: laser	QIAGEN method single mf bisected	QIAGEN method single mf unbisected	Roehrl method single mf bisected	Roehrl method single mf unbisected
Glutathione reductase (GD)	5%: 9/12	5%: 5/12		
GD7F + GD7R (exon only)	1.7%: 4/11			
(expected size: 135 bp)	1%: 5/12	1%: 1/12		
	0.5%: 2/8		0.5%: 0/8	0.5%: 0/8
Glutathione reductase (GD)	5%: 0/12	5%: 0/12		
GD5F + GD7R (exon + intron)	1.7%: 1/11			
(expected size: 556 bp)	1%: 0/12	1%: 0/12		
	0.5%: 3/8		0.5%: 4/8	0.5%: 0/8
Manganese superoxide dismutase	5%: 0/12	5%: 0/12		
(MnSOD)	1.7%: 0/11			
MnSOD4F + MnSOD4R (exon only)	1%: 0/12	1%: 0/12		
(expected size: 138 bp)	0.5%: 0/8		0.5%: 3/8	0.5%: 0/8
Manganese superoxide dismutase	5%: 0/12	5%: 0/12		
(MnSOD)	1.7%: 1/11			
MnSOD2F + MnSOD4R	1%: 0/12	1%: 0/12		
(exon + intron)	0.5%: 0/8		0.5%: 1/8	0.5%: 0/8
(expected size: 1454 bp)				
Glutathione S-transferase (GST2)	5%: 12/12	5%: 11/12		
GST26F + GST26R (exon only)	1.7%: 2/11			
(expected size: 120 bp)	1%: 9/20	1%: 1/12		
	0.5%: 8/8		0.5%: 5/8	0.5%: 3/8
Glutathione S-transferase (GST2)	5%: 9/12	5%: 6/12		
GST24F + GST26R (exon + intron)	1.7%: 1/11			
(expected size: 343 bp)	1%: 0/12	1%:0/12		
	0.5%: 3/8		0.5%: 0/8	0.5%: 0/8

the NADH dehydrogenase 5 subunit, and from both the 12S and 16S ribosomal gene sequences was amplified using PCR primers and reaction conditions described by Morales-Hojas *et al*. (2006). The chromosomal 5.8S ribosomal DNA gene was amplified using the PCR primers and protocol described by Morales-Hojas *et al*. (2007). Highly repetitive chromosomal DNA deriving from the O-150 repeat was amplified using the primers and reaction conditions described by Fischer *et al*. (1996). Because of known primer specificity, amplification of appropriate-sized O-150 repeats was deemed as sufficient evidence of *O. ochengi* DNA when using the Fischer *et al*. (1996) primers and protocol.

Six sets of primers were designed from three different single-copy genes from *Onchocerca volvulus* (glutathione reductase, GD; manganese superoxide dismutase, MnSOD; and Glutathione S-transferase, GST2). All three genes were

previously reported as single copy on the basis of Southern blot hybridizations (Henkle-Dührsen *et al*. 1995; Liebau *et al*. 1996; Müller *et al*. 1997), and they were selected because their intron/exon structure was known and so the size of PCR fragments could be predicted. For reactions that were designed to amplify short fragments spanning only one exon, the PCR conditions used were: 2 min at 94 °C followed by 40 cycles of 94 °C for 45 s, 53 °C for 45 s and 72 °C 1 min 30 s, followed by a final 10 min round of synthesis at 72 °C. For reactions amplifying longer fragments, spanning introns, the PCR conditions were 2 min at 94 °C followed by 45 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C 3 min, followed by a final 15-min round of synthesis at 72 °C. In all the single copy gene amplification reactions, the reaction mixes were carried out in 50-μL volume composed of 0.25 μL of a forward and reverse primer (from a stock concentration of 100 pmol/μL); 5 μL of Bioline 10× NH_4 PCR buffer; 2 μL of 50 mM $MgCl_2$; 0.5 μL of 25 mM DNTPs, and 2 U of Bioline *Taq* polymerase.

Single-copy gene primers for PCR were designed with the help of MWG software, with one primer near the end of an exon and the second primer either at the other end of the same exon (to amplify a short fragment) or in another (nearby) exon or conserved upstream sequence (to amplify a longer fragment). Amplification from the 7th exon of the glutathione reductase gene was achieved with primers GD7F (5′-GTGGGAGGTGGTTACATAGC-3′) and GD7R (5′-CGTTGGCCCTCTATCAATGG-3′), and a larger fragment was amplified with the primers GD5F (from exon 5) (5′-CGACTTGATTCGAGGAAAAGC-3′) and GD7R. Amplification from the 4th exon of the manganese superoxide dismutase gene was achieved with primers MnSOD4F (5′-ACGGCTATAAAGAAGGATTTCG-3′) and MnSOD4R (5′-ACAACAAGCAAGTTGCAAAC-3′), and a larger fragment using primers MnSODF (from 5′UTR) (5′-AGGCCATCCATATCAGCTATC-3′) and MnSOD4R. Amplification from the 6th exon of the Glutathione S-transferase (GST2) gene was achieved with primers GST26F (5′-TTTCATATGCAGATTACGCCC-3′) and GST26R (5′-TTGGTCTATCCTTCATTCGTTG-3′), and a larger fragment with primers GST25F (from exon 5) (5′-CCAGGAGAATTGGCGAAATTTG-3′) and GST26R.

DNA detection sensitivity

DNA extracts from both bisected and uncut microfilariae and microfilariae prepared by both methods (see above) were diluted in sterile distilled water and then PCR tested for amplifiable *O. ochengi* DNA. The multiple-copy DNA primer sets were tested on QIAGEN DNA extracts from 15 individual laser-bisected microfilariae and two batches of 10 microfilariae (one of laser-bisected microfilariae and one with uncut microfilariae). Tests were performed with 5 μL (16.7%) of the DNA extraction (prepared by both methods) and with 10-fold diluted samples (1.67% of the microfilaria DNA extraction) in a single PCR. Parallel experiments were performed with six individual laser-bisected microfilariae with their DNA extracted by the Roehrl *et al*. (1997) method. In those cases where these amplifications yielded no visible amplicon, 5 μL of putative amplification product was used as template in a second round of amplification with the same PCR conditions, except that the number of rounds of amplification was reduced to 25. This second round of amplification was successful in 10–20% of cases which had not shown a visible amplicon after the first round.

Diluted extracts, representing 5%, 1.67%, 1% and 0.5% of the total microfilaria DNA, using the treatment methods detailed above, were also used in single-copy gene amplification experiments. In these experiments, 39 individual microfilariae were laser-bisected and 20 were left uncut. They were subjected to either the Roehrl or QIAGEN DNA extraction procedure and diluted according to Table 2.

All *O. ochengi* DNA PCR assays were carried out alongside negative controls, in which template DNA was substituted with sterile distilled water. Where possible, positive controls were included which consisted of 5 μL of previously successful amplicons that had been diluted up to 1000-fold (according to band intensity).

Results

The conditions under which PCR amplicons were obtained are summarized in Tables 1 and 2. It is notable that all multicopy DNA was visibly amplified from all specimens (and batches of specimens) under all conditions tested, including when less than 2% of the DNA extract from a single microfilaria was used as template (probably equivalent to only six nuclei).

The results from the single copy genes were more complicated. It is not clear that either method of DNA preparation was more successful. For example, 16/48 (33%) successful PCRs were obtained from 0.5% of QIAGEN method DNA from single-bisected microfilariae compared with 13/48 (27%) from the Roehrl *et al*. method. However, bisecting the microfilaria seemed to be more successful because using both QIAGEN (5% and 1%) and Roehrl (0.5%) DNA preparations, 57 out of 200 (29%) specimens which had been laser bisected yielded a PCR product, compared with 27 out of 192 (14%) microfilariae which had not been bisected. The results clearly varied between genes. The MnSOD gene only yielded five amplicons out 166 trials (3%), whereas GD yielded 34/166 (21%) and GST2 yielded 70/174 (40%). The results were more successful for the smaller amplicons for each gene [exon-only amplifications = 80/257 (31%); exon + intron amplifications = 29/249 (12%)]. The results for GST2 are particularly striking, because 13/16 bisected microfilariae yielded an amplicon,

even when the amount of DNA template (0.5% of total) was probably equivalent to only three genomes.

Direct Sanger sequencing of PCR products (seven to 12 sequences for each multicopy DNA, and one to four for each single-copy DNA), followed by database searches, confirmed that all 11 primers had successfully amplified the targeted DNA sequence. For all the multicopy genes, the best database match was with an *Onchocerca ochengi* DNA sequence. For all six different single-copy gene amplicons, there were no previous sequences from *O. ochengi* in the databases for comparison, and the best database match was with the orthologous *Onchocerca volvulus* DNA region from which the primers had been designed. Identity between the new *O. ochengi* sequences and the available *O. volvulus* sequences ranged from 98.4% to 99.9%. Nucleotide substitutions (potential single nucleotide polymorphisms, SNPs) were detected among the sequences of all the multicopy DNAs. For example, among the 12 12 s mt rDNA sequences, nine nucleotide positions were found to vary with 98–100% identity between sequences.

One sequence from each of the targeted genes has been deposited in the GenBank and European Molecular Biology Laboratory (EMBL) databases with accession numbers FM206484 (12 s rDNA), FM206485 (16 s rDNA), FM206483 (ND5), FM206482 (ITS +5.8 s rDNA), FM206480 (MnSOD), FM206481 (GD) and FM206479 (GST2).

Discussion

The novel features of the method are the use of laser-assisted microdissection to isolate individual microfilariae for molecular analysis, and the laser bisection of the specimens to enhance the release of DNA from the tissues enclosed within the cuticle. It is difficult to compare other methods, because the great difficulties involved have discouraged researchers from even attempting to work with single microfilariae. The only relevant study showed a failure to amplify the O-150 repetitive DNA sequence from 22% of individual microfilariae (Oskam *et al*. 1996), compared with 0% in our study. We have detected nucleotide substitutions which are likely to be SNPs (present in amplicons sequenced in both directions and in more than one microfilaria), and potentially useful for population studies, and we should easily be able to detect molecular markers, such as barcoding loci, for species identification. Nuclear repetitive DNA (ITS-5.8 s rDNA) yielded uninterpretable sequences in five out of 12 specimens, which is likely to be the result of direct sequencing when there is intragenomic variation.

The success rate for single-copy genes varied between the three genes tested, but routine amplification could be achieved using 5% of the total DNA extract (thus allowing for up to 20 separate reactions) for two genes, and it has proved possible to amplify up to 1500 bp. The parasites had been stored on dry membrane slides for 5 months before microdissection and DNA analysis, and it is possible that the success rate might be improved with a shorter storage time. The success rate is also likely to be improved through the careful choice of genes, primers and PCR optimization, and should be sufficient for microsatellite profiling of individual microfilariae to study the population biology of their parents.

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