Discrimination between six species of canine microfilariae by a single polymerase chain reaction

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Abstract

Canine dirofilariasis caused by *Dirofilaria immitis* is usually diagnosed by specific antigen testing and/or identification of microfilariae. However, *D. immitis* and at least six other filariae can produce canine microfilaremias with negative heartworm antigen tests. Discriminating these can be of clinical importance.

To resolve discordant diagnoses by two diagnostic laboratories in an antigen-negative, microfilaremic dog recently imported into the US from Europe we developed a simple molecular method of identifying different microfilariae, and subsequently validated our method against six different filariae known to infect dogs by amplifying ribosomal DNA spacer sequences by polymerase chain reaction using common and species-specific primers, and sequencing the products to confirm the genotype of the filariae. We identified the filaria in this dog as *D. repens*. This is the first case of *D. repens* infection in the United States.

Additionally, we examined microfilariae from five additional antigen-negative, microfilaremic dogs and successfully identified the infecting parasite in each case. Our diagnoses differed from the initial morphological diagnosis in three of these cases, demonstrating the inaccuracy of morphological diagnosis. In each case, microfilariae identified morphologically as *A. reconditum* were identified as *D. immitis* by molecular methods. Finally, we demonstrated that our PCR method should amplify DNA from at least two additional filariae (*Onchocerca* and *Mansonella*), suggesting that this method may be suitable for genotyping all members of the family *Onchocercidae*.

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1. Introduction

Canine heartworm disease is generally diagnosed by antigen testing for *Dirofilaria immitis*, and/or identification of microfilariae in the blood of infected dogs. However, other filariae, including *Acanthocheilonema* (*Dipetalonema*) *reconditum*, *Dirofilaria* (*Nochiella*) *repens*, *Acanthocheilonema* (*Dipetalonema*) *dracunculoides*, *Cercopithifilaria grassi*, *Brugia malayi*, *Brugia pahangi*, *Brugia ceylonensis*, and approximately 1% of *Dirofilaria immitis* infestations, can produce persistent microfilaremias with negative heartworm antigen tests (Courtney et al., 1993; Fischer et al., 2002; Genchi, 2003). In such cases, diagnosis relies on examination of the microfilariae, generally using a modified Knott’s test, or alkaline phosphatase staining (Chalifoux and Hunt, 1971; Knott, 1935). These methods are imperfect, and rely on specialist training to accurately differentiate the filariae. However, in regions where one of these filariae is not enzootic, parasitologists may exclude that filaria from consideration, or fail to recognize it, especially if a history of potential exposure is not provided (Cordonnier et al., 2002).

Accurate identification of filarial species in dogs can be clinically important, because of the zoonotic concerns and therapeutic implications. While *A. reconditum* and *A. dracunculoides* have few (if any) clinical consequences, *D. repens* infestations have been associated with subcutaneous granulomas and pruritus in dogs (Baneth et al., 2002; Bredal et al., 1998; Manuali et al., 2005; Tarello, 2003), and subcutaneous, conjunctival and pulmonary nodules, often confused with neoplastic tumors, in humans (Cordonnier et al., 2002; Pampiglione et al., 1996, 2001; Romano et al., 1976; Vakalis and Himonas, 1997). To reduce the risk of zoonotic transmission, treatment of *D. repens* infestations with filaricidal agents, or at least microfilaricidal agents, is recommended (adulticidal therapy can be postponed until clinical signs occur if risk of treatment is deemed to outweigh risk of disease). Adult *D. repens* have been successfully treated with melarsomine hydrochloride (Immiticide, Merial; two injections of 2.5 mg/kg IM 24 h apart) (Baneth et al., 2002). Microfilariae are susceptible to microfilaricidal doses of avermectins (ivermectin 50 μg/kg PO or SQ; doramectin 400 μg/kg SQ; effects of milbemycin have not been investigated) (Baneth et al., 2002; Tarello, 2003). On the other hand, treatment of *A. dracunculoides* or *A. reconditum* infestations is not usually required, so they should be positively identified to avoid inappropriate adulticide therapy.

Over the last decade, several investigators have examined the molecular differentiation of filariae, using Southern blotting to identify DNA repeats from *D. repens* (Chandrasekharan et al., 1994) or polymerase chain reaction (PCR) to differentiate either *D. repens* or *A. reconditum* from *D. immitis* (Bredal et al., 1998; Favia et al., 1996, 1997; Mar et al., 2002; Vakalis et al., 1999). However, no existing studies examined the ability of PCR to differentiate between more than two filariae. While it would be possible to run multiple PCR reactions using published primer sequences to identify a specific filaria, a single differentiating reaction would provide an expeditious means of obtaining a diagnosis. Additionally, sequences are not available for all filariae of interest at this time, so species-specific reactions may fail in these cases.

This study was prompted by several clinical cases requiring accurate identification of microfilariae in dogs. These dogs had negative heartworm antigen tests and, in several instances, discrepant morphological identifications of the microfilariae. Using molecular techniques, we sought to accurately identify the filarial species in each case, and subsequently developed a PCR-based method that distinguishes between the six species tested to date (*D. immitis*, *A. reconditum*, *D. repens*, *A. dracunculoides*, *B. pahangi*, *B. malayi*) with a single primer pair. Our technique simplifies filarial identification, compared with previous studies.

2. Materials and methods

2.1. Case reports

2.1.1. Case 1

A 2-year-old intact male Labrador Retriever, born in the Czech Republic, but exported as a puppy to The Netherlands, was presented to the referring veterinarian (MF) for routine evaluation after importation from The Netherlands in January 2004 to Canada and subsequently New York State (United States) in April
2004. The dog was clinically normal and had never been tested for heartworm or administered heartworm preventative. The veterinarian identified a microfilaremia on routine heartworm screening, and performed a heartworm antigen test using a Dirocheck ELISA (Symbiotics Corporation, San Diego, CA), which was negative. Repeated antigen testing at several diagnostic laboratories failed to diagnose *D. immitis* infestation. Consequently, the veterinarian submitted blood for morphological classification of microfilariae to two diagnostic laboratories. Examination of the microfilariae using modified Knott’s tests resulted in discrepant diagnoses: one laboratory identified the microfilariae as *A. reconditum*, while the other identified them as *D. repens*.

2.1.2. Case 2
An 11-year-old male neutered mixed breed dog was presented to a referring veterinarian in San Diego, California, for routine evaluation. As with the first case, a microfilaremia was detected, but antigen testing for *D. immitis* was negative on multiple occasions. The microfilariae were identified as *A. reconditum* by a commercial diagnostic laboratory.

2.1.3. Case 3
An 8-year-old dog (signalment unknown) was presented to the referring veterinarian in Utica, New York, for evaluation of hematuria secondary to prostatitis. Microfilariae were detected on urinalysis, and subsequently on examination of blood. Antigen tests for *D. immitis* were negative. The microfilariae were morphologically identified as *D. immitis* with a Knott’s test.

2.1.4. Case 4
An 8-year-old female spayed miniature schnauzer was presented to the referring veterinarian after adoption from an animal shelter in Clemmons, North Carolina. Again, a microfilaremia was detected and identified morphologically as *A. reconditum*, but antigen testing for *D. immitis* was negative on multiple occasions.

2.1.5. Case 5
A 1-year-old male intact Pug was presented for heartworm testing in Modesto, California. Microfilaremia was detected and identified morphologically as *A. reconditum*, but antigen testing for *D. immitis* was negative.

2.1.6. Case 6
An 8-year-old male neutered Malamute was presented for heartworm testing in Vallejo, California. Microfilaremia was detected, and identified morphologically as *A. reconditum* but antigen testing for *D. immitis* was negative.

2.2. Sample preparation

In all six clinical cases, 1 ml of blood (anticoagulated with heparin or EDTA) was submitted.

We used blood from a microfilaremic dog with *D. immitis* infestation and from a dog with *A. reconditum* infestation as positive controls. In both controls antigen testing and Knott’s testing provided the filarial diagnosis. The sample of *A. reconditum*-infested blood was obtained by one of the authors and morphologically identified as *A. reconditum*. Fresh adult *B. malayi* and *B. pahangi* worms were generously provided by T. Klei of Louisiana State University. Blood from a dog infested with *A. dracunculoides* was generously provided by Francisco Alonso de Vega of Murcia University in Spain after morphologic diagnosis with alkaline phosphatase staining.

Finally, we used DNA from a non-infected dog as a negative control in all PCR reactions.

2.3. DNA extraction

In all clinical cases, the *D. immitis* positive control specimen and the *Brugia* specimens, 500 µl anticoagulated blood (or five adult *Brugia* filariae) were suspended in 500 µl lysis buffer (50 mM Tris, pH 8.0; 100 mM EDTA, 100 mM NaCl, 1% SDS) and digested with 10 µl proteinase K (10 mg/ml) for 16 h at 55 °C, followed by a standard phenol/chloroform extraction method, as described previously (Sambrook and Russell, 2001).

The *A. reconditum* and *A. dracunculoides* positive control specimens were submitted on filter paper as dried blood spots. DNA was extracted using a QIAmp DNA Blood Mini Kit DNA extraction kit (Qiagen Inc., Valencia, CA) according to manufacturer’s instructions.
2.4. Polymerase chain reaction filarial genotyping

We designed primer pairs that spanned the internal transcribed spacer region 2 (ITS2) of the ribosomal DNA of either *D. immitis*, or *A. reconditum*, and a primer pair spanning the 5S ribosomal intergenic region of *D. repens* based on previously published sequences (Table 1) (Favia et al., 2000; Mar et al., 2002). These primers were used to specifically genotype each filarial species. Additionally, we designed a primer pair that would amplify fragments of different fragment length from both *D. immitis* and *A. reconditum* (DIDR-F1, DIDR-R1) (referred to as the pan-filarial primers throughout the remainder of the manuscript).

For additional independent genotyping validation, we designed primers from published sequences of the cytochrome oxidase subunit 1 (COI) gene for each of these species (Table 1).

Each PCR reaction consisted of 1.5 mM MgCl$_2$, 250 μM dNTPs, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 U of Taq polymerase and 1 μl of DNA solution in a total volume of 20 μl. The PCR procedure consisted of a denaturing step at 94 °C for 2 min and 32 cycles of denaturing (30 s at 94 °C), annealing (30 s at 60 °C for ITS2-based primers and 30 s at 58 °C for COI-based primers) and extension (30 s at 72 °C); a final extension (7 min at 72 °C) and a soak at 4 °C in an Eppendorf Mastercycler thermal cycler (Brinkmann Instruments Inc., Westbury, NY). The PCR product (10 μl) was examined on a 1.5% agarose gel, and fragments of predicted size were extracted from both reactions and purified using a QiaQuick PCR purification kit (Qiagen Inc., Valencia, CA). The PCR products were ligated into pGEM-T Easy Vector System 1 TA cloning vectors (Promega US, Madison, WI) and cloned (Sambrook and Russell, 2001). Candidate clones were screened by restriction digests, and clones with expected digestion fragments were sequenced at the institutional sequencing facility on an Applied Biosystems Automated 3730 DNA sequencer.

### Table 1

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* a D.imm-F1, D.imm-R1, A.rec-F1 and A.rec-R1 are identical to ITS2F-Di, ITS2R-Di, ITS2F-Dr and ITSR-Dr primers published by Mar et al. (2002).

b Predicted product size based on Genbank sequence.
Analyzer (Applied Biosystems, Foster City, CA) using universal M13 forward and reverse primers. Sequence results were compared with those of other species within the Genbank data bank at the National Center for Biomedical Information.

Additionally, we examined DNA quality by performing PCR for canine actin on each sample, to ensure sufficient template for the microfilarial PCR.

2.5. Microfilarial phenotyping

In addition to the PCR genotyping, we performed a modified Knott’s test and wet smear on the blood submitted from Cases 1–3 and the positive control samples. The morphological diagnosis for the six clinical cases is the preliminary morphological diagnosis provided with initial sample submission and not the diagnosis obtained by one of the authors (MFF).

3. Results

All blood-derived DNA samples were positive for canine actin (not shown), indicating adequate DNA extraction.

Primers specific for *D. immitis* (D.imm-F1 and D.imm-R1), *A. reconditum* (A.rec-F1 and A.rec-R1) and *D. repens* (D.rep-F1 and D.rep-R1) amplified the expected products only from *D. immitis* (302 bp), *A. reconditum* (348 bp) and *D. repens* (247 bp, 153 bp) DNA, respectively (Fig. 1). The doublet amplicon obtained with primers specific for *D. repens* has been previously reported (Favia et al., 2000). There was marginal amplification of a product from *A. reconditum* DNA (247 bp) with *D. repens*-specific primers. No filariae-specific primer pairs amplified any products from DNA of the normal dog (negative control), or from DNA extracted from *B. pahangi*, *B. malayi* or *A. dracunculoides* (data not shown). These results confirmed the genotyping of *D. immitis*, *A. reconditum* and *D. repens* for subsequent analysis and the specificity of the primer sets.

The pan-filarial primer pair (DIDR-F1 and DIDR-R1) amplified the expected product from *D. immitis* DNA (542 bp), and *A. reconditum* DNA (578 bp) (Fig. 2). Unexpectedly, this primer pair also amplified a 484 base-pair product from the DNA extracted from Case 1 (*D. repens*-infested dog). The different sizes of the amplicons for all three filarial species allowed us to use this pan-filarial primer set to genotype the filarial species in Cases 2–6.

Filaria-specific primers for COI demonstrated specificity for each of the filarial species (Fig. 3), further confirming the genotyping of microfilariae from Case 1 as *D. repens* and validating the pan-filarial primer set results.

Results of PCR with pan-filarial primers from Cases 2–4 and 6 identified the microfilariae as *D. immitis* (Fig. 4) (confirmed with species-specific COI PCR); PCR from Case 5 identified the microfilariae as *A. reconditum* (also confirmed with species-specific COI PCR).

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**Fig. 1.** Gel electrophoresis of filarial PCR products in a 1.5% agarose gel using filarial-specific ITS2-region primers. Lanes 1, 6, 11, 16: MW marker (KB Plus); lanes 2, 7, 12: *D. immitis* DNA; lanes 3, 8, 13: *A. reconditum* DNA; lanes 4, 9, 14: *D. repens* (Case 1) DNA; lanes 5, 10, 15: *C. familiaris* DNA (negative control). Lanes 2–5: D.immF1/D.immR1 primer pair; lanes 7–10: A.recF1/A.recR1 primer pair; lanes 12–15: D.repF1/D.repR1 primer pair.

**Fig. 2.** Gel electrophoresis of filarial PCR products in a 1.5% agarose gel using pan-filarial ITS2-region primers. Lanes 1, 4: MW marker (KB Plus); lane 2: *A. reconditum* DNA; lane 3: *D. immitis* DNA; lane 3: *D. repens* (Case 1) DNA.
Sequencing of the two amplicons obtained with the D. repens-specific ITS2-region primers (D.rep-F1, D.rep-R1) unequivocally confirmed the presumptive diagnosis of D. repens microfilaremia in Case 1.

Sequencing of the 484 bp amplicon obtained with the pan-filarial primers identified a novel sequence that had high homology with the 5.8S and 28S ribosomal RNA sequences from D. immitis (present at the 5’ and 3’ termini of the amplicon), but only moderate homology with the central D. immitis ITS2 region (Fig. 5), and no appreciable homology with A. reconditum ITS2 region. This sequence was consequently labeled as the ITS2 region of D. repens, submitted to the Genbank data bank at the National Center for Biomedical Information and assigned the accession number AY693808.

Morphologic examination of the microfilariae from Case 1 using a modified Knott’s technique, showed a lack of a cephalic hook, a slightly tapering anterior end, and no consistent tail bend (Fig. 6). The concentration of microfilariae (abundant) and the motility of live microfilariae (not propulsive) on a wet smear, were most consistent with a diagnosis of D. repens. Examination of the microfilariae from Cases 2 and 3 by one of the authors (MFF) supported our molecular diagnosis of D. immitis.

Results of PCR from B. malayi, B. pahangi and A. dracunculoides DNA, using the pan-filarial primers, produced bands at 615 bp (B. malayi), 664 bp (B. pahangi) and 584 bp (A. dracunculoides) (data not shown). Sequencing of these amplicons revealed unique sequences, which were subsequently submitted to Genbank and assigned accession numbers AY988599 (B. malayi), AY988600 (B. pahangi) and DQ018785 (A. dracunculoides).

While PCR with pan-filarial primers failed to discriminate between A. reconditum and A. dracunculoides, restriction fragment length polymorphism PCR (RFLP-PCR) using MseII resulted in two fragments (251 bp and 333 bp) only in A. dracunculoides (data not shown) allowing discrimination between these two Acanthocheilonema species.

Sequence comparison of the ITS2 region amplified by our pan-filarial primers (DIDR-F1 and DIDR-R1) and previously published filarial sequences of this region demonstrated 77–86% similarity between species of the same genus (e.g. D. immitis and D. repens showed 77% similarity, all three Brugia species showed 86% similarity), but only 30–60% similarity between species of different genera (e.g. B. pahangi and A. reconditum showed only 30% similarity). Phylogenetic tree analysis also grouped sequences most closely by genus, i.e., species of the same genus grouped together.
Fig. 5. Nucleotide sequence alignment of 5.8S-ITS2-28S ribosomal DNA region of seven filariae. The 5.8S and 28S ribosomal DNA are identified above the 5' and 3' ends of the sequences; shaded sequences within the majority sequence are primer binding sequences for the pan-filarial primers.
Fig. 5. (Continued).
4. Discussion

We initiated this study to resolve a discordant diagnosis of filariasis in a dog, and successfully identified a *D. repens* infestation in a dog in the United States by PCR genotyping of microfilarial DNA extracted from the patient’s blood. We also resolved discordant diagnoses provided by standard morphological examination of the microfilariae (Knott’s test). Unexpectedly, the pan-filarial primers we designed (DIDR-F1 and DIDR-R1), based on sequence homology of *D. immitis* and *A. reconditum*, amplified not only the expected 542 and 578 bp products from these filariae, but also a 484 bp product from *D. repens*, that was present in the Case 1 of this study, a 584 bp product from *A. dracunculoides*, a 615 bp product from *B. malayi*, and a 664 bp product from *B. pahangi*. This permitted a single PCR reaction to differentiate between *D. immitis* and five other filariae found in dogs, removing the need to perform multiple species-specific genotyping reactions. We subsequently identified microfilariae from an additional four dogs as *D. immitis* and as *A. reconditum* in one dog with this pan-filarial primer set. Our molecular identification was concordant with independent morphological phenotyping of the microfilariae by an experienced parasitologist (MFF), but discordant with the initial morphological diagnosis in 3/5 cases, illustrating the inaccuracy of morphological diagnosis by minimally trained technicians. Finally, we were able to provide additional genotyping information for several filariae that usually require specialized diagnostic interpretation.

Previous investigators have utilized PCR technology to diagnose filarial infestations in dogs and humans (Baneth et al., 2002; Favia et al., 1996, 1997; Mar et al., 2002); however, in each of these studies, only two filarial species were compared. We designed our pan-filarial primers from previously submitted sequences, and optimized them for efficient amplification.

The need to correctly identify the filarial species is becoming relevant throughout the world, because of the increased frequency of transportation of dogs between continents and countries (Genchi, 2003; Zahler et al., 1997). While *D. repens* has been considered an exotic parasite in some parts of the world, such as the United States and Australia, and consequently not considered in the differential diagnosis of canine filariasis in these continents, this case illustrates the potential diagnostic complications that can arise, and provides a simple molecular strategy for correctly identifying the filaroid. The potential mosquito vectors of *D. repens*—*Aedes aegypti* (Cancrini et al., 2003), *Ae. albopictus* (Anyanwu et al., 2000) and *Ae. vexans*—are enzootic in the United States and *Ae. vexans* is enzootic in the region of New York State where this dog currently lives (Nassau County Department of Health, 2003). Similarly, *Ae. aegypti* is enzootic in Australia; *Ae. albopictus* is not found in Australia. Thus, the potential exists for spread of *D. repens* in these regions. Whether the strains in the United States or Australia can incubate larvae and transmit the infestations is unknown. Interestingly, *Ae. aegypti* is
relatively resistant to infestation with *D. immitis*, and is considered an inefficient intermediate host for transmission of heartworm disease (Apperson et al., 1989).

The geographic region in which Case 1 acquired the infection is unknown. *Dirofilaria repens* has not been reported in The Netherlands, but has been identified in the Czech Republic (where this dog was born), Hungary, Italy, Greece and France (Cordonnier et al., 2002; Elek et al., 2000; Pampiglione et al., 2001; Vakalis and Himonas, 1997; Vasilkova et al., 1992).

The microfilariae from Case 2 were initially incorrectly identified as *A. reconditum*, partly based on a negative antigen test, while the microfilariae in Case 3 had been identified correctly as *D. immitis*. In Cases 2 and 3, examination by an experienced parasitologist (MFF) correctly identified the microfilariae as *D. immitis*. Causes for antigen-negative *D. immitis* microfilaremia include prior adulticide therapy without subsequent microfilaricidal therapy or natural death of adult heartworm with persistence of microfilariae (which have a lifespan of approximately 2 years) (Otto, 1977). Additionally, at least one product insert (Dirocheck Canine Heartworm Antigen Test Kit Product Insert, Synbiotics, San Diego, CA) also lists subthreshold antigenemia, immune clearance of antigen–antibody complexes, false positive microfilarial results (due to contamination of reagents used for sample preparation), transfusions from microfilaremic dogs, or destruction of antigen due to improper storage as potential causes of discordant results. While a *Dirofilaria* microfilaria does not necessarily endanger the health of the infested patient, the patient can act as a reservoir for further infestation of other dogs if the intermediate mosquito host is present. Microfilaricidal therapy can resolve this situation.

The microfilaremia in Case 3 was initially identified during a routine urinalysis for hematuria (microfilaruria). We believe this is the first reported case of microfilaruria in a dog. A prior report exists of microfilaruria in a cat (Beaufils et al., 1991).

We did not test the discriminatory ability of our pan-filarial primer set against all filariae that are known to infest dogs or cats (Genchi, 2003). At least one additional “Dipetalonema-like” filaria exists in Europe and Africa—*Cercopithifilaria* (*Acanthocheilonema*) *grassi*—but has not been reported in the United States. It is possible that our pan-filarial primer set will fail to discriminate this filaria from *D. immitis*, however, *D. immitis*-specific primer sets should correctly genotype the filarial species in question if this becomes necessary. Further studies are required to validate the pan-filarial primers against *C. grassi*. Additionally, reports exist of novel filariae in dogs in Ireland and the United States (Jackson, 1975; Pacheco and Tulloch, 1970). Techniques such as those described in this report might be useful in genotyping such filariae.

The *B. malayi* sequence we obtained from adult filariae showed only 86% similarity with a published *B. malayi* sequence (Genbank # AF499130), comparable to the degree of similarity between our *B. pahangi* sequence and the published *B. malayi* sequence (AF499130), or between our *B. pahangi* and *B. malayi* sequences. This differed substantially from the similarity between the published sequences for *B. malayi* (AF499130) and *B. timori* (AF499132), which the authors claimed were 99.5% similar, with only 4 base-pair substitutions along the 600 base-pair region (Fischer et al., 2002). Several reasons exist for these discrepant results. Fischer et al claimed that this degree of similarity was unique to these two species, however, they did not examine DNA from adult *Brugia* species, but only from microfilaria, and by supposition based on epidemiological data. Thus, their initial phenotypic classification, which they used as a gold standard for that study, could be erroneous. We could find no study by these authors that confirmed their results using adult *B. malayi* and *B. timori*. Given the much lower degree of similarity between species of a single genus detailed in this report (e.g. *D. immitis* and *D. repens*, *B. malayi* and *B. pahangi*, or *A. reconditum* and *A. dracunculoides* all showed intra-genus, interspecies variation of approximately 20%, similar to the difference we observed between *B. malayi* and the previously published *B. timori*), we suspect that the sequence for *B. malayi* (AF499130) published by Fischer et al. (2002) is incorrect and is in fact the sequence for *B timori*. Examination of the data presented by these authors indicates a broad genetic variation of the Hha1 tandem repeat in these species, which also suggests a misclassification of microfilariae.

A less plausible alternative is that inter-species variation of the ITS2 region between *B. malayi* and *B.
timori (<0.5%) is less than the intra-species variation of *B. malayi* from different regions (>20%). Several studies of ITS2 regions in various helminths suggest that while intra-species variation can occur, it is less than inter-species variation, and that in most cases intra-species variation is limited to single nucleotide polymorphisms (Conole et al., 1999; Gasser et al., 1999a,b; Huby-Chilton et al., 2001; Newton et al., 1998; Woods et al., 2000) and previous studies of other filariae show similar results (Gasser et al., 1996).

These findings agree with our observations of ITS2 variability in filariae and support our genotypic identification of *B. malayi*. Finally, it is possible that the sample of adult *B. malayi* provided for our study was incorrectly identified; however, phenotypic classification of filariae is generally more robust using adult filariae than microfilariae. The reason for the discrepancy between our data and that of Fischer et al. (2002) remains to be elucidated.

Finally, we examined the ability of our pan-filarial primer sequences to amplify the 5.8S-ITS2-28S region from two additional filariae in *silico*. Analysis of published sequences for *Onchocerca volvulus* (Genbank # AF228575), and *Mansonella ozzardi* (Genbank # AF228554) demonstrated that our pan-filarial primers should amplify 470 and 430 bp amplicons, respectively. This suggests that our primer set might be uniquely capable of amplifying DNA that can be used to genotype all filariae of the family Onchocercidae.

We recommend using the pan-filarial ITS2-region DIDR-F1 and DIDR-R1 primers with DNA from *D. immitis* (as a positive control and amplicon size standard) against which to compare amplicons from clinical cases of microfilaremia that test negative for *D. immitis* antigen. Amplicons approximately 40 base-pairs larger than the *D. immitis* amplicon (542 base-pairs) are diagnosed as *A. reconditum* (578 base-pairs) or *A. dracunculoides* (584 base-pairs), while those approximately 60 base-pairs smaller than *D. immitis* are diagnosed as *D. repens* (484 base-pairs). Discrimination of *Brugia* species by comparing amplicons is limited because *B. malayi* (615 base-pairs) is similar to *B. timori* (625 base-pairs), but these species should be easily differentiated from *B. pahangi* (664 base-pairs). However, PCR-RFLP will discriminate *B. malayi* and *B. timori*. If further confirmation is required, either species-specific PCR can be performed, using the published primer pairs from this and other studies, or the amplicons can be sequenced, however, we feel that simple gel electrophoresis should be sufficient for diagnosis in most cases. Additionally, we feel that clinicians and parasitologists should be alerted to the possibility that *D. repens* infestation might be an emerging condition with zoonotic potential in the United States.

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**References**


