Short Report: Intraspecific Genetic Variation and Phylogenetic Analysis of *Dirofilaria immitis* Samples from Western China Using Complete ND1 and 16S rDNA Gene Sequences

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Abstract. *Dirofilaria immitis* (heartworm) is the causative agent of an important zoonotic disease that is spread by mosquitoes. In this study, molecular and phylogenetic characterization of *D. immitis* were performed based on complete ND1 and 16S rDNA gene sequences, which provided the foundation for more advanced molecular diagnosis, prevention, and control of heartworm diseases. The mutation rate and evolutionary divergence in adult heartworm samples from seven dogs in western China were analyzed to obtain information on genetic diversity and variability. Phylogenetic relationships were inferred using both maximum parsimony (MP) and Bayes methods based on the complete gene sequences. The results suggest that *D. immitis* formed an independent monophyletic group in which the 16S rDNA gene has mutated more rapidly than has ND1.

INTRODUCTION

Dirofilaria immitis (heartworm) is a parasitic nematode that causes harmful diseases in mammals and is spread by mosquitoes. The usual host is dogs; however, foxes, coyotes, cats, red pandas, and humans are also infected.¹ Canine heartworm disease causes pulmonary hypertension because adult worms live mainly in the pulmonary arteries, where their presence incites the formation of reactive vascular lesions that result in pulmonary hypertension.^{2,3} Heartworm disease is globally distributed, and is especially widespread in tropical and subtropical areas.⁴ As a common amphixenosis, this disease is now receiving widespread attention, and increasing numbers of cases have been reported in recent years.^{5–9} In some areas, disease is present in up to 30% of human serum samples.¹⁰ Because of the wide epidemic range and multiple hosts, heartworms are now considered to be among the most harmful *Dirofilaria* nematodes infecting man and other mammals.

Recent breakthroughs have been reported for research on the *D. immitis* transcriptome,¹¹ secretome,¹² nuclear genome,¹³ and microRNAs.¹⁴ Mitochondrial DNA (mtDNA) sequencing has been used for identification of filariae and for investigating genetic variability and phylogenetic relationships; however, these studies were limited to mitochondrial DNA gene partial fragment analysis.^{15,16} In this study, we determined the complete nucleotide sequences of ND1 and 16S rDNA *D. immitis* isolates from seven different dogs, and reconstructed phylogenetic trees using maximum parsimony and Bayes methods. This work provides foundation data for diagnosis, prevention, and control of heartworm disease.

MATERIALS AND METHODS

Ethics statement. Animals from which specimens were collected were handled in accordance with the animal protection laws of the People's Republic of China (draft released on September 18, 2009). The owners of the dead dogs gave

permission for sample collection, and the welfare of the animals was ensured during the sampling process.

Specimen collection. Six live heartworms (DiC1–6) were collected during necroscopy of recently dead dogs from Cangxi, Sichuan Province, and one other sample (DiP1) was obtained from Pengshan, Sichuan. Isolates were washed three times in saline and stored at –20°C until needed for nucleic acid extraction.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing. The genomic DNA of each isolate was extracted using proteinase K digestion and phenol-chloroform extraction.¹⁷ Complete 16S and ND1 gene fragments were amplified from each sample by PCR using forward (16S-S, 5'-CAGTGTCTGAGATTTGTGGGCTA-3'; ND1-S, 5'-ATGGCCTAAAGGGGCGTAAG-3'), and reverse (16S-AS, 5'-ACTCCTCATGATCTGCAATCACACATA-3'; ND1-AS, 5'-TAGACTGAAGCCCCTGATGC-3') primers, which were designed based on published heartworm mitochondrial genomes in GenBank (accession no. NC_005305.1) using Primer premier software version 5.0 (Premier Biosoft International, Palo Alto, CA). The PCRs were carried out in a final volume of 25 µL, and contained 12.5 µL of 2 × Taq PCR Master Mix buffer (TIANGEN, Beijing, China), 1 µL of genomic DNA, 1 µL of each primer, and 9.5 µL of ddH₂O. The PCR program was as follows: 5 min at 95°C, followed by 35 cycles of 35 s at 94°C, 35 s at 61.3°C (16S) or 58.7°C (ND1), 35 s at 72°C, and a final extension of 5 min at 72°C. For each set of PCRs, positive and negative (no DNA template) controls were included. To assess quality, 5 µL of each PCR product was run on a 1.0% agarose gel. The PCR products were purified using a TIANGel Midi Purification Kit (TIANGEN), and submitted to Invitrogen Trading (Shanghai, China) for sequencing, which was performed three times. Reference sequences were obtained from GenBank using the basic local alignment search tool (BLAST), and the sequence of an isolate (DiStd) from Victoria, Australia was included (Table 1).

Data analysis. Similar searches for ND1 and 16S rDNA single sample sequences were performed using the NCBI BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Analyses of multiple sequence alignments were performed using the ClustalW program.¹⁸ Amino acid sequences were inferred from the nucleotide sequences by consulting the

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TABLE 1
Species information and GenBank accession numbers

Parasite samples	Region	Host	GenBank accession no.	
			16S	ND1
Onchocercidae				
<i>Dirofilaria</i>				
<i>Dirofilaria immitis</i> /DiC1	China	Dog	KC846057*	KC846050*
<i>Dirofilaria immitis</i> /DiC2	China	Dog	KC846058*	KC846051*
<i>Dirofilaria immitis</i> /DiC3	China	Dog	KC846059*	KC846052*
<i>Dirofilaria immitis</i> /DiC4	China	Dog	KC846060*	KC846053*
<i>Dirofilaria immitis</i> /DiC5	China	Dog	KC846061*	KC846054*
<i>Dirofilaria immitis</i> /DiC6	China	Dog	KC846062*	KC846055*
<i>Dirofilaria immitis</i> /DiP1	China	Dog	KC846063*	KC846056*
<i>Dirofilaria immitis</i> /DiStd	Australia	Dog	NC_005305.1	
<i>Onchocerca</i>				
<i>Onchocerca flexuosa</i>	Europe	<i>Cervus elaphus</i>	NC_016172.1	
<i>Onchocerca volvulus</i>	Unknown	<i>Homo sapiens</i>	NC_001861.1	
<i>Loa</i>				
<i>Loa loa</i>	Unknown	<i>Homo sapiens</i>	NC_016199.1	
<i>Brugia</i>				
<i>Brugia malayi</i>	Unknown	<i>Homo sapiens</i>	NC_004298.1	
<i>Wuchereria</i>				
<i>Wuchereria bancrofti</i>	Unknown	<i>Homo sapiens</i>	JN367461.1	
<i>Wuchereria bancrofti</i> /TWG2008	Unknown	<i>Homo sapiens</i>	JF775522.1	
Ascarididae				
<i>Baylisascaris</i>				
<i>Baylisascaris schroederi</i>	China	<i>Ailuropoda melanoleuca</i>	NC_015927.1	

*New sequences determined in this study.

invertebrate mitochondrial genetic code, and estimates of evolutionary divergence between complete sequences were made using the Kimura 2-parameter model¹⁹ using the MEGA software package version 5.10.²⁰

The MP trees based on ND1 and 16S rDNA genes were constructed by MEGA 5.10 using the maximum parsimony method, Kimura's two-parameter model and 1,000 bootstrap replications. Bayes trees were constructed with MrBayes3.2.1 software. The evolutionary model was set as GTR + I + Γ , the analysis was run for 1,000,000 generations, burn-in time was for 250,000 generations, and sampling frequency was 1 in 100 generations.²¹

RESULTS

The ND1 and 16S rDNA complete gene sequences of the eight *D. immitis* isolates (seven from China, one from Australia) were aligned, and the nucleotide mutation sites were identified (Figure 1). Two sites in ND1 were non-synonymous substitutions. The nucleotide sequences of the 16S gene displayed a frequency of substitution of 1.13% (11 of 968 sites), and the corresponding frequency for ND1 was 0.56% (5 of 898).

Estimates of the evolutionary divergence among all eight *D. immitis* isolates were calculated based on the complete 16S and ND1 sequences, and were 0.00100–0.0052 (average = 0.0030) and 0.0000–0.0034 (average = 0.0014), respectively (Figure 2).

The phylogenetic analysis using 16S sequences showed that isolates from two different places (DiP1, DiC4) clustered together to form one small branch, and the other six isolates formed another branch (Figure 3A). Another species (*Baylisascaris schroederi*) was present in both constructed trees, and clustered with *D. immitis* in one tree, and with *Wuchereria* spp. + *Brugia malayi*; and *Onchocerca* spp. + *Loa loa* in the other.

The topology of the trees constructed with the ND1 sequences (Figure 3B) showed that all eight *D. immitis* isolates clustered together in a single group. All other species besides the *Baylisascaris schroederi* outgroup were present in both trees, with *D. immitis*, or with *Wuchereria* spp. + *B. malayi* + *L. loa* + *Onchocerca* spp.

DISCUSSION

By comparing the complete sequences of all ND1 and 16S rDNA genes from eight *D. immitis* isolates, we determined an average mutation rate of 0.86% (16 substitution sites of 1,866 sites) across both genes. Both complete genes contained replacements, and the mutation rate of the 16S gene (1.13%) was higher than that of the ND1 gene (0.56%). Estimates of evolutionary divergence performed using the Kimura two-parameter model were in the range of 0.0000–0.0052,

	ND1	16S
	1 3 6 7 8 9 7 8 4 6 5 8 9 6 3	2 3 4 4 5 5 5 6 7 3 3 4 6 9 5 6 9 6 4 9 1 6 5 5 7 0 0 7 9 0
DiC1	TTGTT	ATGATCAGCTT
DiC2		. . A . . T C
DiC3	. C . . .	. CA . . T
DiC4	. . . CC	. . AG . T
DiC5		. . A . . TG . T . .
DiC6	C	G . A . . T
DiP1		. . AG . T
DiStd	. . A . .	. . A . AT . T . A .

FIGURE 1. Nucleotide mutations of mitochondrial ND1 and 16S genes in eight *Dirofilaria immitis* samples. The substitution sites are numbered from the initiation codon of each gene. Numbers are shown vertically. Dots indicate nucleotides identical to those of DiC1. Non-synonymous substitution sites are shown with a gray background.

A		DiC1	DiC2	DiC3	DiC4	DiC5	DiC6	DiP1	DiStd
16S	DiC1		0.0018	0.0018	0.0018	0.0021	0.0015	0.0022	0.0023
	DiC2	0.0031		0.0015	0.0014	0.0018	0.0010	0.0019	0.0021
	DiC3	0.0031	0.0021		0.0014	0.0018	0.0010	0.0019	0.0021
	DiC4	0.0031	0.0021	0.0021		0.0017	0.0010	0.0011	0.0021
	DiC5	0.0041	0.0031	0.0031	0.0031		0.0015	0.0021	0.0022
	DiC6	0.0021	0.0010	0.0010	0.0010	0.0021		0.0015	0.0018
	DiP1	0.0041	0.0031	0.0031	0.0010	0.0041	0.0021		0.0023
	DiStd	0.0052	0.0041	0.0041	0.0041	0.0052	0.0031	0.0052	
B	ND1	DiC1		0.0000	0.0016	0.0000	0.0010	0.0000	0.0011
		DiC2	0.0000		0.0016	0.0000	0.0010	0.0000	0.0011
		DiC3	0.0011	0.0011		0.0019	0.0016	0.0011	0.0015
		DiC4	0.0022	0.0022	0.0034		0.0019	0.0016	0.0019
		DiC5	0.0000	0.0000	0.0011	0.0022		0.0010	0.0011
		DiC6	0.0011	0.0011	0.0022	0.0034	0.0011		0.0015
		DiP1	0.0000	0.0000	0.0011	0.0022	0.0000	0.0011	
		DiStd	0.0011	0.0011	0.0022	0.0034	0.0011	0.0022	0.0011

FIGURE 2. Estimates of evolutionary divergence between complete sequences of mitochondrial (A) 16S and (B) ND1 rDNA genes. Analyses were conducted using the Kimura two-parameter model. Standard error estimate(s) are shown above the diagonal. All positions containing gaps and missing data were eliminated. Evolutionary analyses were performed using MEGA5.

and this was higher for the 16S gene (0.0030) than for ND1 (0.0014). These results suggest that the 16S rDNA gene is more likely to undergo mutation in the mitochondrion of *D. immitis*, leading to higher diversity.

The gene sequences investigated in this work were from closely related species representing the main lineages of the *Onchocercidae* family, along with the *Baylisascaris schroederi* outgroup. The phylogenetic trees constructed using two different methods gave the same results; groupings of species other than the outgroup (*Baylisascaris schroederi*) were present in both *D. immitis* and *Wuchereria* spp. + *B. malayi* + *L. loa* + *Onchocerca* spp. trees. This is in accordance with other reports on the phylogeny of the *Filarioidea* superfamily, based on molecular approaches.^{15,16,22–24} The ND1 and 16S rDNA gene complete sequences are therefore suitable markers for phylogenetic analysis of these organisms. The phylogenetic

tree constructed with the 16S sequences showed more detail than did the tree based on ND1, in particular regarding the topology of the two samples DiP1 and DiC4. Additionally, the branches of the 16S trees were more likely to diverge.

Examination of ND1 and 16S rDNA complete gene sequences suggests these are both suitable markers for phylogenetic analysis of nematodes. The reconstructed phylogenetic trees based on these complete sequences also suggest that *D. immitis* is an independent monophyletic group separate from the other filariae. The 16S rDNA gene has a higher mutation rate, and the higher genetic divergence indicates that this gene is more sensitive for molecular phylogenetic analysis of nematodes. Because of the limited sample size and the possibility that regional differences may have an influence on the outcome, we intend to perform a more extensive study in the future.

FIGURE 3. Phylogeny of *Dirofilaria immitis* and related nematodes inferred by maximum likelihood (MP) and Bayesian methods using the complete sequences of mitochondrial (A) 16S and (B) ND1 rDNA genes. Values on the left tree are the MP bootstrap *P* values (%), and Bayesian posterior probabilities are shown on the right.

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