

Invited Review

# Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer

Michael S. Blouin\*

Department of Zoology, Oregon State University, Corvallis, OR 97331, USA

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## Abstract

DNA sequence divergence at internal transcribed spacer regions (ITS-1 and ITS-2) was compared with divergence at mitochondrial *cox1* or *nad4* loci in pairs of congeneric nematode species. Mitochondrial sequences accumulate substitutions much more quickly than internal transcribed spacer, the difference being most striking in the most closely related species pairs. Thus, mitochondrial DNA may be the best choice for applications in which one is using sequence data on small numbers of individuals to search for potential cryptic species. On the other hand, internal transcribed spacer remains an excellent tool for DNA diagnostics (quickly distinguishing between known species) owing to its lower level of intraspecific polymorphism. © 2002 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

**Keywords:** Mitochondrial DNA; Internal transcribed spacer; Diagnostics; Species identification; Nematoda

## 1. Introduction

Nematodes tend to be very conserved in gross morphology and molecular techniques have recently shown that many presumed monospecific species actually consist of several cryptic species (Anderson et al., 1998). However, which molecular methods will be most useful in the search for cryptic species of nematodes is not yet clear. Ribosomal internal transcribed spacer (ITS-1 and ITS-2) sequences are the markers used most commonly to discriminate among nematode species (Powers et al., 1997; Gasser and Newton, 2000). ITS sequences have been a popular choice because ITS regions are one of the most variable nuclear loci and because of the ready availability of universal primers that work with most nematodes. Relatively little attention has been paid to mitochondrial DNA (mtDNA) as a source of species-specific markers, even though mtDNA evolves very quickly in nematodes (Denver et al., 2000; Blouin et al., 1998), and quickly reaches reciprocal monophyly between even very closely related species (Blouin et al., 1997; Hoberg et al., 1999). mtDNA sequence variation among individuals of the same species averages a fraction of a percent up to 2% and the maximum difference ever observed between a pair of individuals that were clearly

members of the same interbreeding population was 6% (*Ostertagia ostertagi*) (Blouin et al., 1998). mtDNA sequence difference between closely related species is typically in the 10–20% range, so if two individuals differ by, say 10% or more, one might question whether they really are conspecific (Blouin et al., 1998). The level of ITS sequence variation observed among individuals of the same species is about the same as that observed among ITS repeats within individuals, typically  $\leq 1\%$  (Stevenson et al., 1995, 1996; Gasser et al., 1998; Heise et al., 1999; Hugall et al., 1999; Dallas et al., 2000; Nadler et al., 2000). This is also the level of fixed sequence difference seen between some closely related nematode species (Stevenson et al., 1995; Nadler et al., 2000). Therefore, ITS sequence data may not be as useful as mtDNA data for identifying potential cryptic species from small numbers of individuals. Furthermore, in a phylogenetic analysis of trematode (*Echinostoma*) species using both mtDNA and ITS sequences, Morgan and Blair (1998) showed that mtDNA evolves much more quickly than ITS, and is more useful for distinguishing among closely related species. mtDNA in trematodes appears to evolve in a manner very similar to that of nematodes (Morgan and Blair, 1998; Blouin et al., 1998; McDonnell et al., 2000; Nadler and Hudspeth, 2000), so it is likely that the difference between ITS and mtDNA observed in trematodes also holds for nematodes. In this study I used paired ITS and mtDNA sequences from closely

\* Tel.: +1-541-737-2362; fax: +1-541-737-0501.

E-mail address: blouinm@bcc.orst.edu (M.S. Blouin).

Table 1  
Species used for comparing mtDNA distance with internal transcribed spacer distance

Species	Abbreviation <sup>a</sup>	ITS source <sup>b</sup>	mtDNA source <sup>b</sup>
<i>Ancylostoma</i>			
<i>A. caninum</i>	Acan	Y19181, AJ001591	J. Hawdon
<i>A. duodenale</i>	Aduo	AJ001679, AJ001594	J. Hawdon
<i>A. ceylanicum</i>	Acey	Y19183, AJ001593	J. Hawdon
<i>A. tubaeforme</i>	Atub	Y19182, AJ001592	J. Hawdon
<i>Cylicocycylus</i>			
<i>C. ashworthi</i>	Cash	Y08586	AF268479
<i>C. insignis</i>	Cins	Y08588	AF263477
<i>C. nassatus</i>	Cnas	Y08585	AF263481
<i>Cylicostephanus</i>			
<i>C. longibursatus</i>	Clon	AJ228240, AF263495	AF263478
<i>C. goldi</i>	Cgol	AJ228239, AF263494	AF263475
<i>Heterorhabditis</i>			
<i>H. bacteriophora</i>	Hbac	AF029708	AF066888
<i>H. marelatus</i>	Hmar	AF029713	AF066881
<i>H. megidis</i>	Hmeg	AF029711	B. Adams <sup>c</sup>
<i>Teladorsagia</i>			
<i>T. boreoarcticus</i>	Tbor	V. Leignel	AF144551–AF144561
<i>T. sp.</i> (goat strain)	Tgoa	V. Leignel	V. Leignel
<i>T. circumcincta</i>	Tcir	X86026, V. Leignel	AF070877–AF070916
<i>Ostertagi</i>			
<i>O. ostertagi</i>	Oost	AF044934, X86027	AF044941
<i>O. mossi</i>	Omos	AF044932	AF044940
<i>O. leptospicularis</i>	Olep	AF044931, X86025	AF044939
<i>Haemonchus</i>			
<i>H. contortus</i>	Hcon	AF044927	AF044935, AF070736–AF070785
<i>H. placei</i>	Hpla	AF044929	AF044937, AF070786–AF070825

<sup>a</sup> Abbreviations used in Table 2.

<sup>b</sup> GenBank Accession numbers or person providing unpublished data.

<sup>c</sup> Liu et al. (1999) also report *nad4* sequence from *H. megidis*, but it appears that they sequenced an isolate that is not the same species as the isolate sequenced for both ITS and *nad4* by Adams (Adams et al., 1998; Adams, pers. commun.).

related species from a variety of nematode taxa in order to compare the relative rates of evolution of the two markers, and to assess their utility for identifying cryptic species.

## 2. Materials and methods

I obtained DNA sequences from pairs of congeners for which both mitochondrial sequence data and ITS sequence data were available (Table 1). ITS-1 and ITS-2 appear to accumulate substitutions at the same rate (data not shown), so I included species pairs for which either or both ITS loci were available. Because the rate of substitution varies among mitochondrial loci, I limited the dataset to only species pairs for which NADH dehydrogenase subunit 4 (*nad4*) sequences (10 pairs) or cytochrome oxidase I (*cox1*) sequences (11 pairs) were available. These are the

mitochondrial loci that are most commonly sequenced by nematologists and they represent loci from mitochondrial protein groups that are considered to show relatively low conservation (NADH dehydrogenase group) or high conservation (cytochromes). Data from both loci were available for the *Haemonchus placei*–*Haemonchus contortus* pair, so I included both loci in the dataset for that species pair.

The mitochondrial sequences aligned unambiguously with no gaps. I computed the total percentage of sequence difference (p distance) for each pair. ITS sequences often contain many insertions/deletions (indels) and the number of gaps will obviously be sensitive to the alignment algorithm used. In order to be consistent, I aligned the ITS of each species pair using the same algorithm (ClustalW 1.8 pairwise, gap penalty 5). I then computed the percentage of sequence difference two ways: (1) total difference, which includes gaps (e.g. a 3 bp gap is counted as three substitu-

Table 2  
Mitochondrial and internal transcribed spacer distances for each species pair

Species pair <sup>a</sup>	Mt locus <sup>b</sup>	Mt dist <sup>c</sup>	ITS locus <sup>d</sup>	ITS shared site dist <sup>e</sup>	ITS total dist <sup>f</sup>
Acan–Aduo	<i>cox1</i>	0.093	1, 2	0.015	0.019
Acan–Acey	<i>cox1</i>	0.094	1, 2	0.091	0.110
Acan–Atub	<i>cox1</i>	0.096	1, 2	0.022	0.024
Aduo–Acey	<i>cox1</i>	0.107	1, 2	0.072	0.098
Aduo–Atub	<i>cox1</i>	0.069	1, 2	0.014	0.019
Acey–Atub	<i>cox1</i>	0.090	1, 2	0.085	0.110
Cash–Cins	<i>cox1</i>	0.130	1, 2	0.035	0.051
Cash–Cnas	<i>cox1</i>	0.110	1, 2	0.021	0.035
Cins–Cnas	<i>cox1</i>	0.110	1, 2	0.035	0.036
Clon–Cgol	<i>cox1</i>	0.092	1, 2	0.016	0.022
Hcon–Hpla	<i>cox1</i>	0.130	1, 2	0.008	0.008
Hcon–Hpla	<i>nad4</i>	0.160	1, 2	0.008	0.008
Hbac–Hmar	<i>nad4</i>	0.130	1	0.128	0.180
Hbac–Hmeg	<i>nad4</i>	0.130	1	0.150	0.230
Hmar–Hmeg	<i>nad4</i>	0.130	1	0.079	0.130
Tbor–Tgoa	<i>nad4</i>	0.160	2	0.011	0.022
Tbor–Tcir	<i>nad4</i>	0.120	2	0.012	0.035
Tgoa–Tcir	<i>nad4</i>	0.150	2	0.012	0.031
Oost–Omos	<i>nad4</i>	0.140	1	0.077	0.110
Omos–Olep	<i>nad4</i>	0.160	1	0.058	0.100
Oost–Olep	<i>nad4</i>	0.130	1, 2	0.069	0.100

<sup>a</sup> See Table 1 for abbreviations.

<sup>b</sup> mtDNA locus on which the distance is based.

<sup>c</sup> mtDNA distance.

<sup>d</sup> ITS locus used; 1, ITS-1; 2, ITS-2.

<sup>e</sup> ITS distance excluding indels.

<sup>f</sup> ITS distance including indels.

tions); and (2) shared-site difference, which is the percentage of difference only at sites shared by the two sequences (Table 2).

### 3. Results and discussion

Fig. 1 shows that the mtDNA sequences accumulate substitutions much more quickly than ITS sequences. More importantly, interspecific mtDNA distances among the most closely related species are well outside the typical intraspecific range, whereas the same is not true for ITS. Consequently, given samples of just a few individuals, one would be more likely to be alerted to the presence of cryptic species using mtDNA than ITS. As expected, *cox1* distances are consistently lower than *nad4* distances (averages of 10 versus 14%), a result very similar to that observed by Morgan and Blair (1998) for *nd1* versus *cox1* in trematodes.

In comparing the utility of mtDNA versus ITS, it is important to distinguish between two applications, diagnostics and prospecting. Diagnostics uses DNA methods to identify species that are already known to be different. For example, one may wish to distinguish among eggs or other difficult-to-identify stages of otherwise well defined species (e.g. Romstad et al., 1997; Monti et al., 1998). Prospecting uses sequence data and some genetic distance yardstick to search for individuals that might be cryptic species. ITS

sequences remain an excellent diagnostic tool. Fixed differences at indels are a particularly useful feature of ITS. Indeed, the high substitution rate of mtDNA sequences might make them a less suitable target than ITS for quick diagnostic tests such as via allele-specific PCR, allele-specific hybridisation or single strand conformation polymorphism analysis, because of the high possibility of intraspecific polymorphism. On the other hand, if the goal is to use DNA sequence data to prospect for new species, especially given small sample sizes, then mtDNA sequences appear to be a better choice than ITS. Incidentally, note that I am not promoting any genetic yardstick as a sound way to define species. Here the yardstick is useful only for suggesting the possibility that cryptic species may exist. The next step would be to obtain sequence from more individuals and to conduct further studies on morphology and other molecular characters.

Protein coding loci in the NADH dehydrogenase group such as *nad4* appear to be a better choice for prospecting than cytochrome oxidases because the strong amino acid conservation observed in CO genes limits most of the useful variation to silent sites (Fig. 1) (McDonnell et al., 2000). There are no universal primers currently available for NADH genes in nematodes as there are for ITS loci. It is unlikely that primers designed from any one nematode taxon will work in all others (especially given the apparently frequent rearrangement of gene positions in nematode

mtDNA; Lavrov and Brown, 2001). However, given the large amount of sequence information now available for nematode mtDNA, including the entire mtDNA sequences of four species (Okimoto et al., 1994; Keddie et al., 1998; Lavrov and Brown, 2001), it should take relatively little effort to design primers that amplify NADH genes from almost any nematode species of interest.

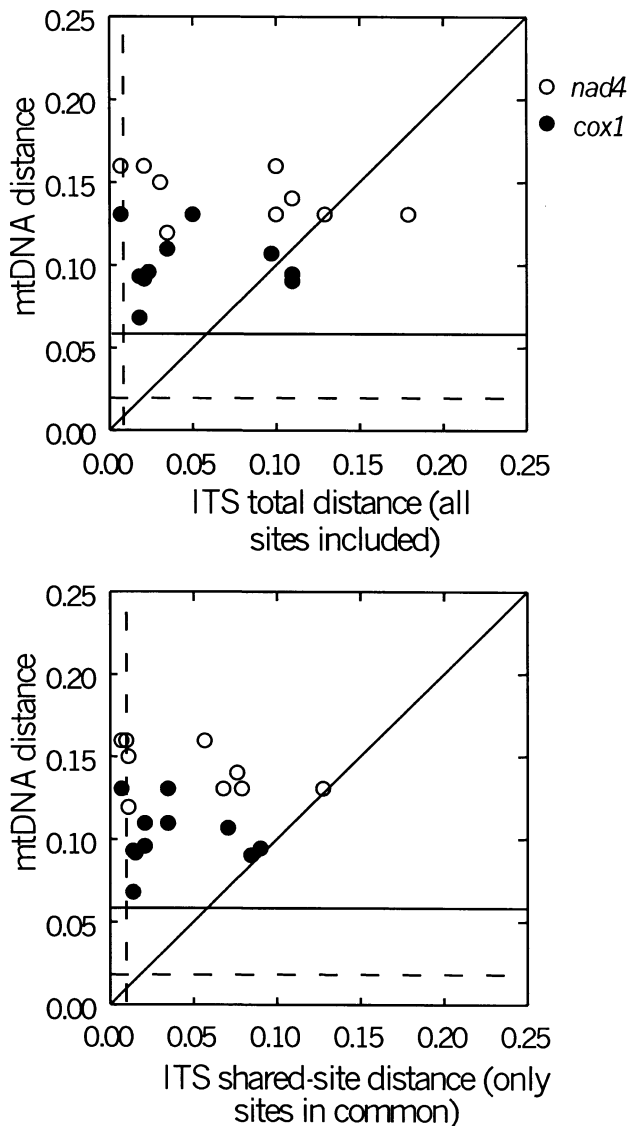


Fig. 1. Mitochondrial p distance (percentage of sequence difference) versus internal transcribed spacer p distance for each species pair. (Top) Internal transcribed spacer distance including indels. (Bottom) Internal transcribed spacer distance calculated excluding indels. A solid diagonal line indicates where equal mtDNA and internal transcribed spacer distances would lie. Filled circles indicate *cox1* sequence data. Open circles indicate *nad4* sequence data. A dashed vertical line indicates the level of internal transcribed spacer divergence typically observed within and among individuals of the same species (about 1%). A dashed horizontal line indicates the average level of *nad4* divergence between conspecifics in highly diverse species (around 2%). A solid horizontal line indicates the largest distance ever observed between a pair of conspecific nematodes (6%; estimated from restriction fragment length polymorphism of entire mtDNA).

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