A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites

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SUMMARY

We examined the relative merits of mitochondrial DNA loci and ribosomal DNA internal transcribed spacers for their use in prospecting for cryptic species of platyhelminth parasites. Sequence divergence at ITS1 and ITS2 was compared with divergence at 2 mtDNA loci (NADH dehydrogenase-1 and cytochrome *c* oxidase I) between closely related species of trematodes and cestodes. Both spacers accumulated substitutions substantially more slowly than mtDNA, which clearly shows a higher level of divergence among species relative to intra-specific variation. Besides a slow rate of substitution, other caveats that may be encountered when using ITS sequences as a prospecting marker are discussed. In particular, we note recent studies that suggest the existence of substantial levels of intra-individual variation in ITS sequences of flatworms. Because it is likely that closely related species share this phenomenon, it may confound the detection of cryptic species, especially if small sample sizes are studied. Although potential limitations of mtDNA are also recognized, the higher rate of evolution and smaller effective population size of this marker increases the probability of detecting diagnostic characters between cryptic species.

Key words: cytochrome *c* oxidase I, NADH dehydrogenase-1, rDNA internal transcribed spacer, platyhelminth parasites, cryptic species, molecular prospecting.

INTRODUCTION

The application of DNA methods to studies on the systematics and population genetics of platyhelminth parasites has become widespread in recent years. These molecular studies have led to the chance discovery of many genetically distinct but morphologically very similar species (Jousson, Bartoli and Pawlowski, 2000; Macnish *et al*. 2002; Criscione and Blouin, 2004; Xiao *et al*. 2005). What is not commonly done is to use a DNA marker to deliberately search for evidence of cryptic species (e.g., Jousson and Bartoli, 2000; Haukisalmi *et al*. 2001; Hu *et al*. 2005; Wu *et al*. 2005), an approach sometimes referred to as molecular prospecting (Blouin, 2002; Criscione, Poulin and Blouin, 2005). Molecular prospecting is usually initiated when one suspects the presence of cryptic species or when one needs to be sure they are dealing with a single species or population. For example, if a species occurs in multiple hosts or habitats it may be prudent to check for evidence of cryptic species. If one uses DNA sequence data, then cryptic species might be indicated if one finds unusually large genetic distances between certain individuals (distances greater than typically found within species of that taxon), or if one finds evidence of reciprocally monophyletic groups in large population samples. Note that prospecting is simply a first pass to look for evidence that species may be present. Given such evidence (e.g., a few individuals having unusually large genetic distance), the next step would be to formally test the hypothesis of absence of gene flow or lineage exclusivity by collecting more samples and applying multiple markers. This second step can be called species delimitation. In that sense prospecting is a form of exploratory data analysis that generates hypotheses, and delimitation is the next step of hypothesis testing. Finally, we of course recognize that using an arbitrary genetic distance yardstick is not a sound way to delimit species. But it *is* a rational approach in prospecting.

An ideal molecular marker for prospecting would be easy to obtain, provide unambiguous data, and have a rapid evolutionary rate. Mitochondrial DNA and ITS are commonly used on platyhelminths for phylogenetics and species identification (distinguishing between previously identified, but morphologically similar, species; Morgan and Blair, 1995, 1998; León-Règagnon, Brooks and Pérez-Ponce de León, 1999; Tkach, Pawlowski and Sharpilo, 2000; Kostadinova *et al*. 2003; Scholz *et al*. 2004). Given that cryptic species of platyhelminth parasites are probably common, it is useful to review the relative merits of mtDNA and ITS for their use

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Table 1. Species and GenBank accessions corresponding to the sequences used in this study

Species	ITS1	ITS2	ND1	COI
TREMATODA				
Deropegus aspina A	AY269443	–	AY269445–AY269466	–
Deropegus aspina B	AY269444	–	AY269511–AY2969532	–
Echinostoma caproni	U58098	U58098	AF025837	–
Echinostoma paraensei	U58100	U58100	AF025834	–
Echinostoma revolutum	AY168930	AY168930	AY168933	–
Echinostoma robustum	U58102	U58102	AF025832	–
Echinostoma trivolvis	U58097	U58097	AF025831	–
Fasciola hepatica	–	AJ557568	NC002546	–
Fasciola gigantica	–	AJ557569	AF286338	–
Glypthelmins brownorumae	–	AY278062	–	AY278055
Glypthelmins facioi	–	AY278060	–	AY278053
Glypthelmins tuxtlasensis	–	AY278061	–	AY278054
*Lecithochirium rufoviride**	Unpubl.	–	Unpubl.	–
Lecithochirium furcolabiatum A	Unpubl.	–	Unpubl.	–
Lecithochirium furcolabiatum B	Unpubl.	–	Unpubl.	–
Paragonimus hokuoensis	–	AY618756	–	AY618837
Paragonimus miyazaki	–	AY618757	–	U97215
Paragonimus skrjabini	–	AY618752	–	U97216
Paragonimus westermani (Jap.)	AF040942	U96907	–	U97208
Paragonimus westermani (Mal.)	AF071426	U96909	–	U97211
Paragonimus westermani (Phil.)	AF040943	U96910	–	U97213
Paragonimus westermani (Thail.)	AB094992	–	–	U97212
Schistosoma bovis	–	AF146035	–	AY157212
Schistosoma edwardiense	AY197344	AY197344	–	AY197347
Schistosoma haematobium	Z21716	Z21716	–	AY157209
Schistosoma hippopotami	AY197343	AY197343	–	AY197346
Schistosoma intercalatum	Z21717	Z21717	–	AY157208
Schistosoma japonicum	–	U22167	NC002544	U82264
Schistosoma malayensis	–	U82398	AF295106	U82262
Schistosoma mansoni	AF029309	U22168	–	NC002545
Schistosoma matheei	Z21718	Z21718	–	AY157211
Schistosoma mekongi	U89871	U22169	NC002529	U82263
Schistosoma rodhaini	AF531312	AF531312	–	AY157202
CESTODA				
Echinococcus equinus	AJ237773	–	AF346403	AF346403
Echinococcus granulosus G1	AJ237777	–	AF297617	AF297617
Echinococcus granulosus G7	AJ237821	–	AJ237638	AF458876
Echinococcus granulosus G10	AY185199	–	AF525297	AF525457
Echinococcus multilocularis	AJ237778	–	NC000928	NC000928
Echinococccus ortleppi	AJ237774	–	AJ237636	M84665
Paranoplocephala alternata	AY299557	–	–	AY181502
Paranoplocephala arctica	AY752661	–	–	AY181509
Paranoplocephala fellmani	AY752655	–	–	AY586612
Paranoplocephala kalelai	AY752660	–	–	AY189959
Paranoplocephala macrocephala	AY752658	–	–	AY181518
Paranoplocephala primordialis	AY752662	–	–	AY568218
Paranoplocephala serrata	AF314414	–	–	AY568220
Taenia asiatica	AY606272	–	NC004826	NC004826
Taenia saginata	AY392045	–	AJ239106	AB107242

* Unpublished data for *Lecithochirium* species.

in the molecular prospecting in this group of organisms. Little is known about the relative divergence rates of nuclear versus mtDNA in flatworms (Morgan and Blair, 1998), so here we first establish the relative levels of intra-versus inter-specific variation typical of the two types of markers in flatworms. Then we review data on molecular evolution of each marker and discuss caveats that may be encountered when using either mtDNA and ITS as a prospecting marker in platyhelminths.

MATERIALS AND METHODS

We obtained DNA sequences from GenBank for pairs of congeners for which both mitochondrial sequence data and ITS data were available (Table 1).

When available, previous phylogenetic information was used to ensure that comparisons were among closely related or sister species. Our goal is not to use this information to identify a minimum genetic distance for defining species, but to compare the relative rates of evolution of the two markers, and evaluate which is more useful in molecular prospecting for cryptic species. Because certain studies suggest that ITS1 appears to accumulate substitution at higher rate than ITS2 (Morgan and Blair, 1995; van Herwerden, Blair and Agatsuma, 1999; Tkach *et al*. 2000), we included data from both loci separately. Although the mitochondrial genome contains different functional units (genes), the whole molecule is a single genealogical unit due to a lack of recombination. However, the rate of substitution among mitochondrial genes is variable. Thus, we restricted the data set to species pairs for which nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) and cytochrome *c* oxidase subunit 1 (COI) sequences were available. These are the most commonly used mitochondrial markers in molecular systematics of flatworms. According to the identification given in Kostadinova *et al*. (2003), the *Echinostoma robustum* sequences used in this study were those provided by Morgan and Blair (1998) as a German isolate of *Echinostoma revolutum*. Several strains of *Echinococcus granulosus* that could represent different species were included in the comparative analyses. We followed the recent proposition of species status for 2 'strains' (G4 and G5) of *Echinococcus granulosus*, which were named *E. equinus* and *E. ortleppi*, respectively (see Thompson and McManus, 2002). We also compared specimens identified as *Paragonimus westermani* from different localities that probably constitute cryptic species (Blair *et al*. 1997). Recently, the reduction of *Paragonimus skrjabini* and *P. miyazakii* to subspecific status has been suggested (Blair *et al*. 2005); however, we retain the original designation. Species of *Lecithochirium* show both repetitive elements within ITS1 and length variation for this spacer within a single individual (unpublished observation). We assumed that intra-individual ITS1 length variation found within *Lecithochirium* species was due to variation in the number of repeats and not owing to the presence of paralogues in other rDNA loci. Because of the size of the repetitive region, only the 5′ end was compared among species. Similarly, a repeat region present in ITS1 of some species of *Schistosoma* was not included in the comparisons between *S. mekongi* and *S. malayensis*, and species of the *S. haematobium* group. Repeats in the ITS were not observed for other species in our data set. The mtDNA sequences aligned unambiguously with no gaps. However, because ITS is a non-coding sequence, frequent insertions or deletions (indels) were present in the comparison of different taxa, making the alignment more complicated than in the case of mtDNA genes. In order to be consistent we aligned each species pair using Clustal W, applying the same algorithm in all cases. The percentage of sequence difference was computed as the proportion of nucleotide sites at which 2 sequences are different (uncorrected *p*). No correction is necessary for multiple changes because the sequences of closely related species exhibit little divergence. Thus, uncorrected *p* is an estimate of the accumulated number of nucleotide substitutions per site. Because ND1 sequences of multiple individuals were available for *Lecithochirium* and *Deropegus* species, we computed average distances between sibling species in each case. MEGA 2.0 (Kumar *et al*. 2001) was used to make all calculations.

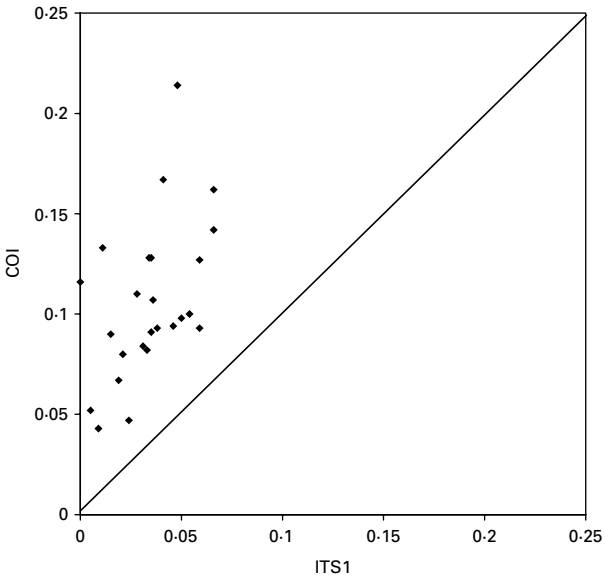

Fig. 1. Cytochrome *c* oxidase I uncorrected *p* distance versus ribosomal DNA internal transcribed spacer1 uncorrected *p* distance for each species pair. The diagonal line indicates where equal distances for both markers would lie.

RESULTS AND DISCUSSION

Comparisons of genetic distances from pairs of congeners for both mtDNA and ITS sequences clearly demonstrated that mtDNA sequences of platyhelminths accumulate nucleotide substitutions at a much higher rate than ITS (Fig. 1). It is notable that several pairs of congeners showed up to 10% divergence at the mtDNA, but about 1% at the ITS (Table 2). Species pairs of platyhelminths that have approximately 1% divergence between ITS sequences are relatively frequent (Després *et al*. 1992; Kane and Rollinson, 1994; Anderson and Barker, 1998; Criscione and Blouin, 2004). This degree of variation in the ITS was also typical of the variation within and among individuals of the same species (e.g., van Herwerden *et al*. 1999; Macnish *et al*. 2002). In contrast, large population samples from

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

	ITS1	ITS2	ND1	COI
TREMATODA				
Deropegus				
D. aspinaA-D. aspinaB	0·018	–	0·101*	–
Echinostoma				
E. caproni-E. paraensei	0·022	0·019	0·150	–
E. caproni-E. robustum	0·010	0·026	0·147	–
E. caproni-E. trivolvis	0·022	0·024	0·150	–
E. caproni-E. revolutum	0·032	0·038	0·166	–
E. paraensei-E. robustum	0·015	0·007	0·161	–
E. paraensei-E. revolutum	0·035	0·019	0·162	–
E. paraensei-E. trivolvis	0·017	0·005	0·138	–
E. revolutum-E. trivolvis	0·044	0·024	0·130	–
E. revolutum-E. robustum	0·024	0·026	0·119	–
E. trivolvis-E. robustum	0·021	0·124	0·130	–
Fasciola				
F. hepatica-F. gigantica	–	0·014	0·103	–
Glypthelmins				
G. brownorumae-G. facioi	–	0·010	–	0·126
G. brownorumae-G. tuxtlasensis	–	0·014	–	0·119
G. facioi-G. tuxtlasensis	–	0·007	–	0·119
Lecithochirium				
L. rufoviride-L. furcolabiatumA	0·026	–	0·118*	–
L. rufoviride-L. furcolabiatumB	0·026	–	0·121*	–
L. furcolabiatumA-L. furcolab.B	0·026	–	0·076*	–
Paragonimus				
P. skrjabini-P. miyazaki	–	0·003	–	0·097
P. skjrabini-P. hokuoensis	–	0·003	–	0·063
P. hokuoensis-miyazaki	–	0·006	–	0·097
P. westermani Malaysia-Japan	0·036	0·017	–	0·107
P. westermani Malaysia-Philippines	0·021	0·003	–	0·087
P. westermani Malaysia-Thailand	0·015	–	–	0·090
P. westermani Japan-Philippines	0·028	0·020	–	0·110
P. westermani Japan-Thailand	0·035	–	–	0·128
P. westermani Philippines-Thailand	0·019	–	–	0·067
Schistosoma				
S. mekongi-S. malayensis	0·046	0·015	0·108	0·094
S. mekongi-S. japonicum	–	0·060	0·347	0·158
S. malayensis-S. japonicum	–	0·080	0·354	0·164
S. haematobium-S. intercalatum	0·000	0·013	–	0·116
S. haematobium-S. mattheei	0·066	0·029	–	0·162
S. intercalatum-S. mattheei	0·066	0·016	–	0·142
S. bovis-S. mattheei	–	0·016	–	0·138
S. bovis-S. intercalatum	–	0·000	–	0·065
S. bovis-S. haematobium	–	0·013	–	0·113
S. hippopotami-S. edwardiense	0·048	0·019	–	0·214
S. rodhaini-S. mansoni	0·011	0·010	–	0·133
CESTODA				
Echinococcus				
E. granulosus G1-*E. granulosus* G7	0·050	–	0·155	0·098
E. granulosus G10-*E. equinus*	0·033	–	0·129	0·082
E. granulosus G10-*E. ortleppi*	0·009	–	0·068	0·043
E. granulosus G10-*E. multilocularis*	0·054	–	0·158	0·100
E. equinus-E. ortleppi	0·038	–	0·122	0·093
E. equinus-E. multilocularis	0·035	–	0·145	0·091
E. ortleppi-E. multilocularis	0·059	–	0·157	0·093
Paranoplocephala				
P. arctica-P. alternata	0·005	–	–	0·052
P. arctica-P. serrata	0·034	–	–	0·128
P. serrata-P. alternata	0·059	–	–	0·127
P. macrocephala-P. kalelai	0·031	–	–	0·084
P. primordialis-P. fellmani	0·041	–	–	0·167
Taenia				
T. saginata-T. asiatica	0·024	–	0·053	0·047

* Average distances between sibling species.

Table 3. ND1 average and maximum percentage of sequence differences observed between individuals within populations of trematodes

(*N* is the number of individuals studied.)

Species	Average	Maximum	*N*
Deropegus aspina A*	0·5	1·6	66
Deropegus aspina B*	1·1	2·0	89
Lecithochirium furcolabiatum	1·3	2·2	19
Lecithochirium fusiforme	1·1	2·3	27
Lecithochirium musculus	0·03	0·3	19
Lecithochirium rufoviride	0·5	1·6	22
*Nanophyetus salmicola**	0·8	2·2	91
*Plagioporus shawi**	0·8	2·2	52

* Data over 3 or 4 geographical populations.

species of trematodes show maximum intraspecific divergences ranging from 0·3 to 2·2% at mtDNA (Table 3). These values do not overlap with divergences between species (Table 2). Thus, for example, if one has a sample of individuals that are putatively from a single population, then the presence of individuals differing by more than say, 5% at mtDNA should raise a red flag and motivate further investigation.

In accordance with previous data on flatworms (Gasser, Zhu, and McManus, 1999; Obwaller *et al*. 2004), our results suggest that COI exhibits less divergence between pairs than ND1 (Table 2). Nevertheless, even COI accumulates substitutions substantially more quickly than ITS1 (Fig. 1), which is considered more variable than ITS2. Interestingly, comparisons between ITS1 and ITS2 did not reveal a consistent pattern in terms of which region had a higher nucleotide substitution rate. This inconsistent pattern is in contrast to previous studies in flatworms that suggest ITS1 is more variable than ITS2 (Luton, Walker and Blair, 1992; Morgan and Blair, 1995; van Herwerden *et al*. 1999; Tkach *et al*. 2000; Galazzo *et al*. 2002).

A prospecting marker should exhibit relatively low intra-specific variation and a high level of divergence between closely related species. Moreover, it is important that the marker lacks intra-individual variation because one may not be able to determine which variant should be compared among samples. Low intra-individual and intra-specific variation for ITS markers is traditionally assumed due to the action of molecular turnover mechanisms (Dover, 1982). However, the efficiency of concerted evolution depends on whether rDNA variants are on nonhomologous chromosomes (Polanco, González and Dover, 1998; Parkin and Butlin, 2004), on the speciation rate (Vogler and DeSalle, 1994; Harris and Crandall, 2000), and on the frequency of chiasmata (the sites of crossing over), which may be highly variable in trematodes (van Herwerden *et al*. 1999). Under circumstances of slow concerted evolution, processes such as hybridization, incomplete lineage sorting or retention of an ancestral polymorphism could cause the sharing of different ITS types among species of flatworms, and could therefore confound a prospecting study by using this marker (e.g., Rollinson *et al*. 1990; Huang *et al*. 2004). Hybrids having mtDNA and ITS sequences from different species were recently reported for *Schistosoma* (Morgan *et al*. 2003). These potential problems are generally recognized for mtDNA (Ballard and Whitlock, 2004). However, the importance of hybridization and lineage sorting for ITS probably has been overlooked because of the assumption of concerted evolution. There are several observations that indicate concerted evolution may not be efficient in platyhelminths. For example, 2 distinct ITS1 types were found in a single isolate of *Echinococcus granulosus* (see Bowles, Blair and McManus, 1995). Intra-individual variation in ITS of trematodes and cestodes may result from a variable copy number of tandem repeats within the spacer (van Herwerden, Blair and Agatsuma, 1998, 1999; Luo *et al*. 2002). Such repetitive elements are common in ITS of flukes (Luton *et al*. 1992; Kane and Rollinson, 1994; Kane *et al*. 1996; van Herwerden *et al*. 1998; León-Regàgnon *et al*. 1999; van Herwerden *et al*. 1999; Jousson and Bartoli, 2000; Bell and Sommerville, 2002; Dvorák *et al*. 2002). Indeed, the evolution by internal repetition in the ITS may be a general process in a variety of taxa (von der Schulenburg *et al*. 2001).

Intra-individual variation for mtDNA may be explained by heteroplasmy or the presence of pseudogenes. Heteroplasmy has been described for flatworms (Pena *et al*. 1995), and can result from somatic mutations, introgression or bi-parental inheritance (Avise, 2000). The rapid sorting of mtDNA molecules in cell lineages should maintain identical or very similar sequences within individuals (Avise, 2000). Thus, heteroplasmy by somatic mutations should not present a problem for molecular prospecting. However, mtDNA may introgress faster than nuclear genes (Ballard and Whitlock, 2004; Chan and Levin, 2005), and both introgression and bi-parental inheritance can lead to divergent mtDNA lineages within individuals. Bi-parental inheritance was reported for *Schistosoma mansoni* (Janotti-Passos *et al*. 2001, but see Bieberich and Minchella, 2001 for an alternative explanation). Although more work is needed to determine if mtDNA heteroplasmy is common among parasitic flatworms, the typically maternal inheritance of animal mtDNA suggests that the influence of heteroplasmy in molecular prospecting for cryptic species is probably of little importance. Large divergence between samples may also be falsely concluded if a comparison is made between a pseudogene and a functional gene. Both mitochondrial and ITS pseudogenes have been found in parasitic

helminths (Morgan and Blair, 1998; Hugall, Stanton and Moritz, 1999; van Herwerden, Blair and Agatsuma, 2000; Obwaller *et al*. 2004). Because frame-shift mutations or premature stop codons are common in pseudogenes, protein-coding genes of the mtDNA can be error checked by translating the nucleotide sequence (Benasson *et al*. 2001). However, detection of ITS pseudogenes might be more difficult because they are not protein-coding genes.

Undetected paralogy, incomplete lineage sorting and introgressive hybridization could mislead a prospecting study using either ITS or mitochondrial sequences. However, a rapid rate of evolution is a key requirement for a prospecting marker, and the results obtained here indicate mtDNA of flukes evolves much faster than ITS. Furthermore, mtDNA generally has a smaller effective population size than nuclear genes. Given that small populations reach a state of reciprocal monophyly faster than large populations, the probability of detecting diagnostic characters between cryptic species is increased by the use of mtDNA. Thus, despite limitations, mtDNA is preferable to ITS to prospect for cryptic species of parasitic platyhelminths owing to its higher sensitivity to reveal species with recent ancestry.

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