Making the most of mitochondrial genomes – Markers for phylogeny, molecular ecology and barcodes in *Schistosoma* (Platyhelminthes: Digenea)

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Abstract

An increasing number of complete sequences of mitochondrial (mt) genomes provides the opportunity to optimise the choice of molecular markers for phylogenetic and ecological studies. This is particularly the case where mt genomes from closely related taxa have been sequenced; e.g., within *Schistosoma*. These blood flukes include species that are the causative agents of schistosomiasis, where there has been a need to optimise markers for species and strain recognition. For many phylogenetic and population genetic studies, the choice of nucleotide sequences depends primarily on suitable PCR primers. Complete mt genomes allow individual gene or other mt markers to be assessed relative to one another for potential information content, prior to broad-scale sampling. We assess the phylogenetic utility of individual genes and identify regions that contain the greatest interspecific variation for molecular ecological and diagnostic markers. We show that variable characters are not randomly distributed along the genome and there is a positive correlation between polymorphism and divergence. The mt genomes of African and Asian schistosomes were compared with the available intraspecific dataset of *Schistosoma mansoni* through sliding window analyses, in order to assess whether the observed polymorphism was at a level predicted from interspecific comparisons. We found a positive correlation except for the two genes (*cox1* and *nad1*) adjoining the putative control region in *S. mansoni*. The genes *nad1*, *nad4*, *nad5*, *cox1* and *cox3* resolved phylogenies that were consistent with a benchmark phylogeny and in general, longer genes performed better in phylogenetic reconstruction. Considering the information content of entire mt genome sequences, partial *cox1* would not be the ideal marker for either species identification (barcoding) or population studies with *Schistosoma* species. Instead, we suggest the use of *cox3* and *nad5* for both phylogenetic and population studies. Five primer pairs designed against *Schistosoma mekongi* and *Schistosoma malayensis* were tested successfully against *Schistosoma japonicum*. In combination, these fragments encompass 20–27% of the variation amongst the genomes (average total length ~14,000 bp), thus providing an efficient means of encapsulating the greatest amount of variation within the shortest sequence. Comparative mitogenomics provides the basis of a rational approach to molecular marker selection and optimisation.

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1. Introduction

The family Schistosomatidae (Platyhelminthes: Digenea) includes 14 genera and some 100 species of blood fluke (Basch, 1991; Khalil, 2002). All are obligate parasites of

birds or mammals and use freshwater gastropods as intermediate hosts to complete their life cycle. The schistosomes include a number of species of significant medical, veterinary and economic importance. Schistosomiasis, the disease caused by the parasite when it parasitises its definitive host, is particularly important in humans, with over 200 million people recorded as having infection per year (WHO, 2001). This disease is endemic in 74 tropical

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developing countries with some 600 million people at risk. There are six species of *Schistosoma* known to parasitise humans as definitive hosts including, *Schistosoma japonicum*, *Schistosoma malayensis*, *Schistosoma mekongi*, *Schistosoma mansoni*, *Schistosoma guineensis*, *Schistosoma intercalatum* and, to a much lesser extent with only isolated reports, *Schistosoma mattheei* (more commonly found in cattle); it is possible that reports of *S. mattheei* are in fact hybrids between *S. mattheei* and *Schistosoma haematobium* (Kruger and Evans, 1990). Understanding and controlling schistosomiasis requires an understanding of evolutionary history, host–parasite interactions and epidemiology. Accurate species and strain identification is key to successful epidemiological studies, monitoring and control. Increasingly, molecular-based methods are being developed and implemented for accurate, rapid and high-throughput identification, but to date such methods have relied on a poorly informed approach to the selection of appropriate molecular markers.

In general, the choice of molecular markers is governed by balancing a suite of factors (including cost, proven utility and likely efficacy) matched to the desired purpose for which they have been selected. It is at best dictated by preliminary empirical studies, but usually proceeds through an assessment of results as they accumulate, with the disadvantage of ambiguous or uninformative markers being recorded simultaneously (Morgan et al., 2005). There is a certain degree of conservatism in the approach; researchers tend to re-use PCR primers from authors who have worked on the same or similar taxa. Thus, such primers become widely used and are sometimes chosen even if they do not represent the most suitable marker. The pursuit of a universal DNA ‘barcoding’ region (Hebert et al., 2003a,b; Hebert and Gregory, 2005) has contributed to this conservatism, and large projects for species identification and phylogeny are encouraged to rely primarily (or even exclusively) on partial mitochondrial (mt) cytochrome *c* oxidase I (*coxI*), even though this marker may not be the most informative or most suitable marker for all taxa (Moritz and Cicero, 2004; Will et al., 2005).

Among flatworms there is no established precedent as to which molecular markers should be used or for what purpose. Popular nucleotide sequences for phylogenetic assessment have included the complete small (SSU) and variable domains D1–D3 of the large (LSU) nuclear subunit rRNA genes (e.g., Littlewood and Olson, 2001; Lockyer et al., 2003a; Littlewood, 2006) and, to a much lesser extent, *coxI* (e.g., Olson and Tkach, 2005). This is also the case for schistosome phylogenetics (Lockyer et al., 2003b; Morgan et al., 2003). In contrast, phylogeographic studies have relied almost exclusively on partial *coxI* (e.g., Iwagami et al., 2003; Pongratz et al., 2003; Wickström et al., 2003) and, to a lesser extent, on ribosomal DNA (Attwood et al., 2002). Population genetic studies of flatworms are few, and have focussed on the accumulation of data from random amplified polymorphic DNA (RAPD) analyses (e.g., Semyanova et al., 2003; Vilas et al., 2003; Theron

et al., 2004). Targeted gene approaches have relied heavily on mt markers, including *coxI* (e.g., Wickström et al., 2003), *nadI* (Criscione and Blouin, 2004) and/or multiple mt fragments (Morgan et al., 2005).

New and potentially informative molecular data have been accumulated for species of *Schistosoma* from genome projects (e.g., El-Sayed et al., 2004), expressed sequence tag (EST) projects (e.g., Franco et al., 1995), phylogenetic studies (e.g., Lockyer et al., 2003b) and post-genomic research programmes focusing on gene function (e.g., Oliveira et al., 2004). Genomic data have been examined for repeat motifs for the identification of and characterisation of microsatellite markers (Rodrigues et al., 2002, 2007). Importantly, another potential source of molecular markers of schistosomes has emerged recently. There now exist completely characterised mt genomes of five species (*S. mansoni*, *S. japonicum*, *S. mekongi*, *S. haematobium* and *Schistosoma spindale*) and an almost complete genome for *S. malayensis*; four of these are human parasites. These data have arisen through phylogenetic and evolutionary studies and the desire to explore the extent to which a novel mt gene order is present within the genus (Le et al., 2000a, 2001; Littlewood et al., 2006). A comparative mitogenomic approach to identify diagnostic and highly variable molecular markers for population studies offers perhaps the best opportunity to assess existing mt markers and to identify regions of the genomes with potential. Few authors have considered mt sequences as potential markers for molecular ecological studies of schistosomiasis and yet their potential is well recognised (Curtis and Minchella, 2000). The ability to provide diagnostic markers for individual subpopulations may be within reach with full mt genome sequences. The most extensive population genetic study of schistosomes with mt markers is that of Morgan et al. (2005) and covers ~2535 bp from four marker regions on the mt genome for 143 specimens of *S. mansoni* from its entire geographical range. In the same study, nuclear (ribosomal internal transcribed spacers ITS-1 and ITS-2) were also characterised, but very limited variation was detected (three variable positions in ITS-1).

Mitochondrial genomes are involved in the maintenance of the mitochondrion and its various functions in the eukaryotic cell. Many factors influence the evolutionary dynamics of nucleotide change within mt genomes, and these may act within individuals, populations and species lineages during phylogenesis (Ballard, 2000a,b); differential rates of genome replication and DNA repair, biases in base substitution, adaptive evolution and differential rates of heterogeneity are but some of the processes that contribute to mitochondrial sequence variation. Use of this variation within and between species has been exploited for a diversity of purposes in molecular ecology and phylogeography (Avice, 2004; Ballard and Whitlock, 2004). Mitochondrial genomes of flatworms include two ribosomal genes, 12 protein-coding (they lack *atp8*) and 22 transfer RNA (tRNA), and are 13,503–16,901 bp in length. An earlier review of mt genomes of schistosomes and their potential as a source of

markers relied on the few *Schistosoma* species available at the time (Le et al., 2000b); the study lacked the *cox3*, *nad1*, *nad4L*, *nad5* and 10 tRNA genes and included incomplete sequences for *cox1*, *nad2* and *cob*. Many mt genomes are not fully sequenced as some regions, particularly non-coding repeat regions, are difficult to amplify or sequence; many ‘completed’ mt genomes have at least all the coding regions fully sequenced and characterised. There are very few genera for which more than one complete mt genome has been sequenced; exceptions include *Homo* (e.g., Torroni et al., 2006) and *Drosophila* (e.g., Ballard, 2000a). The data for *Schistosoma* offer an additional useful model system.

In this paper, complete mt genomes are assessed for their potential information content for research on species of *Schistosoma*. (i) The extent and magnitude of the phylogenetic information content in mt genomes is investigated; the performance of nucleotides and inferred amino acid sequences are each compared; (ii) the distribution of polymorphic sites (differences within a species) and divergent sites (differences between species) along the mt sequences is described and correlations sought; finally, (iii) the utility of comparing complete mt genomes of closely related species in identifying suitable markers for molecular phylogenetic and ecological studies is discussed. We chose to assess phylogenetic information content and nucleotide variability per unit length, allowing us to address two central questions: (i) how useful are individual genes at recovering species phylogenies, and (ii) which are the least and most variable regions of the schistosome mt genomes? Addressing the latter question allowed primer design for population genetic purposes.

2. Materials and methods

2.1. Sequences utilised

The mt genomes of *S. mansoni* (Accession No. NC_002545), *S. japonicum* (NC_002544), *S. mekongi* (NC_002529), *S. haematobium* (DQ157222) and *S. spindale* (DQ157223), and one almost complete genome for *S. malayensis* (AF29516) were used for this study. Additionally, for the phylogenetic analyses, we used the closest related outgroups with completely characterised mt genomes: the digeneans *Fasciola hepatica* (NC_002547) and *Paragonimus westermani* (NC_002354). Mitochondrial sequences of *S. mansoni* from multiple geographic isolates, used for assessing intraspecific variation, were taken from sequences detailed in Morgan et al. (2005).

2.2. Alignments for phylogeny

As a result of gene rearrangements in the mt genomes, entire genome sequences could not be aligned. Instead, individual rRNA and protein-coding genes were chosen for alignment. Transfer RNA genes were excluded as they may not be truly homologous based on their anticodon

identity alone and are known in some taxa to remold and change through time, rendering them paralogous (Rawlings et al., 2003). Non-coding regions are unlikely to be homologous and were thus also excluded. Nucleotides and amino acids for individual genes were each aligned using ClustalW (Thompson et al., 1994) with default settings. The alignments were then inspected by eye and adjusted using MacClade 4.06 (Maddison and Maddison, 2000). Nucleotide sequences from protein-coding genes were aligned in frame with reference to their amino acid translation and the amino acid alignments. Ribosomal RNA genes were aligned with ClustalW and regions with ambiguous base positional homology were excluded. Alignments are available from the authors upon request.

2.3. Alignments for sliding window analysis

Using the same method as described in Section 2.2, complete genome alignments were made between closely related species (for which no gene order differences occurred), including tRNAs and all intergenic (non-coding) spaces. Pairwise alignments of complete genomes were made between the most closely related species, *S. mekongi* and *S. malayensis*, and *S. haematobium* and *S. spindale*. The complete genomes of the African *S. mansoni*, *S. haematobium*, and the Indian *S. spindale* were also aligned; hereafter referred to as the African-Indian group. In order to test the co-variation of polymorphism on different scales and different levels of phylogeny, mt data from Morgan et al. (2005) were aligned to each other and the corresponding region in the alignment of the complete mt genome sequences of the African-Indian group.

2.4. Phylogenetic information content

Three criteria were chosen to evaluate phylogenetic information content, (i) length variance and information content of individual genes, (ii) topological congruence and nodal support of phylogenies estimated from individual genes, and (iii) estimates of signal and noise in the individual data sets.

Phylogenies were constructed for each gene. Throughout the tests, all data were weighted equally (Baker and DeSalle, 1997; Wenzel and Siddall, 1999; Baker et al., 2001). For protein-coding genes, all codon positions were included, since the more variable third base positions have been demonstrated to give the inferred phylogeny higher resolution than if they are excluded (Källersjö et al., 1999; Baker et al., 2001). The test of substitution saturation (Xia et al., 2003) available in DAMBE (Xia and Xie, 2001) showed signs of saturation at the level of ingroup–outgroup comparison. Plotting transitions and transversions against Tamura–Nei distances showed a linear relationship only when third positions were excluded (Fig. 1). Protein-coding and ribosomal genes were analysed individually and as concatenated gene alignments in a single file.

Fig. 1. Graphical plot of transitions (Ts) and transversions (Tv) against Tamura–Nei distances using DAMBE (Xia and Xie, 2001) on the complete dataset (a) and when the third base position is excluded (b).

Phylogenies were inferred using maximum parsimony (MP) and Bayesian inference (BI). MP analyses were performed in PAUP* v 4.0b10 (Swofford, 2002), using the heuristic search method (10 search replicates) and tree-bisection–reconnection (TBR) branch-swapping algorithm; all characters were unordered and were weighted equally, gaps were treated as missing data and majority consensus trees were constructed from nonparametric bootstrap replicates (Felsenstein, 1985). Bayesian analysis was performed with MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). Models for sequence evolution were calculated using MrModeltest 2.2 (Nylander, J.A.A., 2004; Evolutionary Biology Centre, Uppsala University) and ProtTest 1.2.6 (Abascal et al., 2005), using the AIC criterion (Sakamoto et al., 1986), and included in the command block. For the concatenated nucleotide analyses, the genes were partitioned, and the parameters were allowed to vary independently. For the concatenated protein analysis, a single substitution model JTT + G (Jones et al., 1992) was employed; this was the most frequently optimal model chosen for the individual proteins with MrModeltest. However, the parameters were allowed to vary independently between genes. Temperatures were set individually for each partition in BI to ensure chain switching at a suitable rate.

The phylogeny proposed by Lockyer et al. (2003b), based on complete SSU ribosomal DNA (rDNA), complete LSU rDNA and partial mt *cox1* sequences, was used as a reference topology against which individual and combined gene phylogenetic analyses were compared; outgroup ((*S. japonicum* (*S. malayensis*, *S. mekongi*)), (*S. mansoni* (*S. spindale*, *S. haematobium*))). For the taxa used in the present study, the topology of the tree presented by Lockyer et al. (2003b) has been supported by numerous molecular studies (Littlewood and Johnston, 1995; Barker and Blair, 1996; Morgan et al., 2003) and is designated herein as the ‘benchmark topology’.

Between-partition-differences were assessed with partitioned Bremer support (Baker and DeSalle, 1997). These and Bremer support values (Bremer, 1994) were each calculated using TreeRot v. 2.0 (Sorenson, M.D., 1999, Boston University, Boston, MA) to generate commands

for PAUP* and were executed with 100 repetitions. Partitioned Bremer support (Baker and DeSalle, 1997; Baker et al., 1998, 2001) assesses the extent to which a particular data set (each of the mt genes) contributes to the resolution of each node. The partitioned Bremer support was rescaled in order to be comparable between genes, and was expressed as how much longer (as a percentage) the node is without the optimal clade. Permutation tail probability (PTP) distances were calculated in PAUP* for all resultant topologies, in order to test whether the topologies were significantly different. The topology-dependent cladistic permutation tail probability (T-PTP) test, as implemented in PAUP*, was used to compare the resultant topologies to the benchmark topology. An incongruence length difference (ILD) test (Farris et al., 1995), as implemented in PAUP*, was used to evaluate the null hypothesis that the characters that make up data partitions are drawn at random from a single population of characters, a single phylogeny and a single set of evolutionary processes (Farris et al., 1995; Hipp et al., 2004). The test is sensitive to between-partition differences in among-site variation (Hipp et al., 2004) and the size of the data partitions being tested (Dowton and Austin, 2002). Therefore, to avoid these problems, all phylogenetically uninformative characters were removed from the dataset (cf. Lee, 2001).

2.5. Sliding window analysis to detect regions of high sequence variability

In order to study the distribution of variation along the mt genome and to detect regions of highest variability, two pairs of closely related species were used: *S. malayensis* and *S. mekongi* (9% *p*-distance), and *S. spindale* and *S. haematobium* (29% *p*-distance). Pairwise comparisons of closely related species are less likely to reveal convergent similarities (incorrectly identified invariant positions) at third base positions in codons than comparisons between more distantly related taxa. A sliding window analysis, using DnaSP (Rokas et al., 2003), was employed for pairwise comparisons to detect variable nucleotide sites; DnaSP cannot analyse gaps as

a fifth state, but excludes gapped sites. Sliding window analyses were performed with window/step sizes of 300/10, 24/3 and 1/1. Results were evaluated with Microsoft Excel according to requirements (e.g., size, proportion of variable sites) for particular molecular markers. Sliding window analyses were also conducted to compare intraspecific sequence polymorphisms using the dataset of Morgan et al. (2005). Correlation and regression analyses were conducted using SPSS v. 13.0 (SPSS, 2004). Finally, the position of the *cox1* barcode, as defined by the “Barcoding of Life” consortium (<http://www.barcoding-life.com>), was plotted on the sliding windows to compare and contrast sequence variability in this region against the entire mt genomes.

2.6. Primer design

The selection of molecular markers is principally dictated by a set of methodological constraints. For example, regions useful for species identification need to be bordered by regions of high sequence conservation, so that PCR-based methods can rely on a limited set of primers, and the length of resultant PCR products may need to fall within a particular size range, facilitating subsequent sequence or single-strand conformation polymorphism (SSCP) analysis. This is typically 300–600 bp in molecular ecological studies (cf. DeSalle and Schierwater, 1998). The alignment of *S. malayensis* and *S. mekongi* was used as a basis for primer design in order to demonstrate the principle of sliding window analysis in identifying suitable regions for PCR-based studies. Although this alignment was incomplete, it offered the chance to compare the two most closely related taxa in order to identify regions of high nucleotide variability. Sliding window analyses of 300 bp were run to indicate the most variable regions, whereas a window size of 20 bp was used to identify the relatively conserved regions for primer design. By doing so, PCR primers were placed as efficiently as possible; i.e., capturing many variable positions in a short stretch. Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and tested in silico on the *S. japonicum* mt genome using MacVector® 7.2.3 (Accelrys). These same primer pairs were then tested against genomic DNA from *S. japonicum*, thus verifying their potential utility across species of southeast (SE) Asian schistosomes. DNA extracts from male worms were kindly provided by Dr David Johnston (NHM). The PCR was performed in a volume of 25 µl using Ready-to-go PCR Beads (Amersham Pharmacia Biotech) each containing 1.5 U DNA Taq Polymerase, 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each deoxyribonucleotide triphosphate (dNTP) and stabilizers including BSA, 0.4 µM of each primer and 2 µl of DNA (~10 ng). Cycling was performed in a Perkin Elmer 9600 Thermal Cycler as follows: denaturation at 94 °C for 2 min, followed by 40 cycles of 1 min at 94 °C, 30 s at 52 °C, 2 min at 72 °C; followed by a final extension of 7 min at 72 °C. Amplicons were detected by loading 3 µl

of each PCR on 1% TAE (Tris–acetate–EDTA) agarose gels.

3. Results

3.1. Phylogenetic information content

Results from each of the three criteria to determine phylogenetic information content are considered.

3.1.1. Length variance and information content

The mt genomes used in this study were 14–15 kb long, excluding the control region, with ~12 kb unambiguously alignable. The base composition was heavily skewed (A = 23%, C = 8%, G = 23% and T = 46%) and homogeneous for the ingroup species when the protein-coding genes alone were considered. The length and length variance of individual genes, as determined for nucleotides, is shown in Table 1. The relative lengths of mt genes were essentially the same among flatworm mt genes except in the cases of *cox1* and *cox2*, which were the most variable in length; for *cox1*, gene length varied between 1494 and 1830 bp amongst digeneans, and *cox2* differed by more than 100 bp in length between *S. mansoni* and *S. mekongi*. Length variance has implications in phylogenetic analysis when dealing with indels in alignments, and in seeking unique diagnostic markers that incorporate or make use of indel signatures. The gene sequence datasets, excluding tRNAs, contained 45% phylogenetically informative characters (coded as nucleotides and including ribosomal genes, under the principles of parsimony). When conceptually translated as amino acids (protein-coding genes only), 54% of the characters were phylogenetically informative. Maximum and minimum proportions of phylogenetically informative positions with nucleotides were 57% for *nad5* and 30% for the *rrnS* datasets, respectively. For proteins, the maximum was 71% informative characters in *nad4L* and the minimum was 25% in *cox1* (Table 2).

3.1.2. Phylogenetic analysis and topological congruence

The resulting topologies from BI, with posterior probability support values for all analysed datasets, are listed in Table 3. Almost identical results and relative levels of support were obtained with MP; differences are shown in the table. Support values were generally very high, with the most frequent posterior probability of 100%, reflecting general agreement with the benchmark phylogeny. Less congruent topologies generally had lower support values. The partitioned Bremer support showed that the optimal tree for a partition without the node from the hypothesis was 1–5% longer in tree length (data not shown). Most support was given to the outgroup clade *F. hepatica* + *P. westermani*. The least support was given to the *S. spindale* + *S. haematobium* clade, where there were a few negative values, indicating that another topology had a more optimal solution for those particular datasets.

Table 1
Properties of the mitochondrial genes

	Length of individual genes (bp)												No. amino acids
	<i>atp6</i>	<i>cox1</i>	<i>cox2</i>	<i>cox3</i>	<i>cob</i>	<i>nad1</i>	<i>nad2</i>	<i>nad3</i>	<i>nad4</i>	<i>nad4L</i>	<i>nad5</i>	<i>nad6</i>	
<i>Fasciola hepatica</i>	516	1533	603	642	1113	903	867	357	1272	273	1569	453	3367
<i>Paragonimus westermani</i>	513	1494	600	645	1119	891	867	357	1206	258	1584	453	3329
<i>Schistosoma haematobium</i>	525	1806	597	666	1116	882	840	369	1266	261	1584	474	3462
<i>Schistosoma japonicum</i>	519	1644	609	650	1116	891	855	360	1275	264	1587	459	3410
<i>Schistosoma malayensis</i>	522	<u>1209</u>	^a	651	1119	894	852	363	1272	264	<u>999</u>	^a	1979
<i>Schistosoma mansoni</i>	522	1830	594	654	1095	891	840	363	1260	261	1584	450	3448
<i>Schistosoma mekongi</i>	522	1650	699	651	1119	888	852	363	1272	264	1593	462	3445
<i>Schistosoma spindale</i>	522	1764	642	666	1095	876	840	369	1263	255	1587	468	3449
Average length (bp)	520	1674	621	653	1112	890	852	363	1261	263	1584	460	
Length variance ^b	15	17,269	1456	77	108	64	127	22	516	28	54	77	
Rescaled variance ^c	0.0	10.3	2.3	0.1	0.1	0.1	0.1	0.1	0.4	0.1	0.0	0.2	

The length of each inferred gene and the total number of amino acids coded within each mitochondrial genome is given for each taxon. Underscored figures represent partial genes and were not used to calculate the average gene length or length variance.

^a Indicates no data available.

^b Length variance is a measure of how much the length of each sample deviates from the mean.

^c Rescaled variance is the variance/bp.

Table 2
Information content for each mitochondrial gene calculated for all taxa

	Gene													
	<i>atp6</i>	<i>cox1</i>	<i>cox2</i>	<i>cox3</i>	<i>cob</i>	<i>nad1</i>	<i>nad2</i>	<i>nad3</i>	<i>nad4</i>	<i>nad4L</i>	<i>nad5</i>	<i>nad6</i>	<i>rrnS</i>	<i>rrnL</i>
<i>Nucleotides</i>														
Number of characters	528	1524	597	651	1104	891	882	369	1275	258	1617	471	782	1068
Constant characters	144	757	191	147	405	278	212	106	279	67	363	104	332	399
Variable characters	384	767	406	504	699	613	670	263	996	191	1254	367	450	669
Variable uninformative	95	291	137	149	195	160	223	71	286	46	334	109	219	301
Informative characters	289	476	269	355	504	453	447	192	710	145	920	258	231	368
% variable	73%	50%	68%	77%	63%	69%	76%	71%	78%	74%	78%	78%	58%	63%
% informative	55%	31%	45%	55%	46%	51%	51%	52%	56%	56%	57%	55%	30%	34%
Phylogenetic accuracy	High	High	None	High	Low	High	High	Low	High	Low	Low	None	None	None
<i>Amino acids</i>														
Number of characters	176	508	199	217	364	297	294	123	425	86	539	157		
Constant characters	39	292	60	34	125	77	49	32	76	13	101	24		
Variable characters	137	216	139	183	239	220	245	91	349	73	438	133		
Variable uninformative	36	88	68	51	61	54	77	17	96	12	121	44		
Informative characters	101	128	71	132	178	166	168	74	253	61	317	89		
% variable	78%	43%	70%	84%	66%	74%	83%	74%	82%	85%	81%	85%		
% informative	57%	25%	36%	61%	49%	56%	57%	60%	60%	71%	59%	57%		
Phylogenetic accuracy	None	High	None	High	Low	High	Low	High	High	Low	Low	None		

Phylogenetic accuracy is judged on the basis of T-PTP test results comparing individual gene topologies from different analytical methods against the benchmark hypothesis of Lockyer et al. (2003b); see text for further details.

3.1.3. Signal and noise in the molecular data

The partition homogeneity test (ILD) showed that the various genes, using the concatenated protein dataset, did not contain conflicting phylogenetic signal ($P = 0.48$). The same was true in the rRNA sequence dataset ($P = 0.93$), suggesting that both datasets were homogeneous. In contrast, the tRNAs were not found to be homogeneous ($P = 0.01$). Suboptimal phylogenies were ranked as low, with the aid of the T-PTP test results; phylogenies entirely incongruent with the benchmark were scored as having no phylogenetic accuracy (data not shown). Table 4 provides a summary of the phylogenetic utility of the mt genes based on length variance, information content, nodal support and topological congruence.

3.2. Comparison of pairwise alignments

Two groups of schistosomes were chosen for sliding window analysis: a pair of closely related sister taxa (*S. mekongi* and *S. malayensis*; Fig. 2) and two more distantly related taxa but with the same gene order (*S. haematobium* and *S. spindale*; Fig. 6). The alignment of *S. mekongi* and *S. malayensis* (*mekongi-malayensis*) was 9598 bp long, as the *S. malayensis* sequence was incomplete. It contained 9.6% variable ungapped characters with the smallest gap between two variable characters being 1 bp and the longest 80 bp. The uncorrected pairwise distance between this pair of taxa, protein-coding genes only, was 9%. The alignment of *S. haematobium* and

Table 3

Results of Bayesian inference (BI) and maximum parsimony (MP) analyses showing: (a) all partitions supported by analyses, (b) benchmark hypothesis of Lockyer et al. (2003) with bipartitions 1–5, (c) BI (posterior probability) and MP (% bootstrap) nodal support values given to different nodes in analyses of partitioned datasets (genes) of both nucleotides (nuc) and amino acids (aa, of protein coding genes); RNA genes were analysed as nucleotides

(a)		Bipartition (node) number												(b)	
		1	2	3	4	5	6	7	8	9	10	11	12		
<i>Schistosoma spindale</i>		–	+	+	–	–	+	–	–	+	+	+	–
<i>Schistosoma haematobium</i>		–	+	+	–	–	–	–	+	+	+	+	+		
<i>Schistosoma mansoni</i>		–	–	+	–	–	+	+	+	–	+	+	–		
<i>Schistosoma mekongi</i>		–	–	–	+	+	–	+	–	+	+	+	+		
<i>Schistosoma japonicum</i>		–	–	–	–	+	–	–	–	+	–	–	+		
<i>S. malayensis</i>		–	–	–	+	+	–	+	–	+	+	–	–		
<i>Fasciola</i>		+	–	–	–	–	–	–	–	–	–	–	–		
<i>Paragonimus</i>		+	–	–	–	–	–	–	–	–	–	–	–		
Gene		Data	Bipartition number and nodal support (BI/MP)												Inclusiveness (%)
			1	2	3	4	5	6	7	8	9	10	11	12	
(c)															
<i>atp6</i>	nuc	1.00	0.53	0.99	1.00	0.98									90
		100	65	98	100	100									93
	aa	1.00		1.00	1.00	1.00				0.77					95
		100		90	100	99				58					89
<i>cox1</i>	nuc	1.00	0.92	0.73	1.00	1.00									93
		100	95	92	100	100									97
	aa	1.00	1.00	1.00	1.00	1.00									100
		100	94	96	100	98									98
<i>cox2</i>	nuc	1.00		1.00						0.52			0.51		76
		100		90		55				41			*		72
	aa	1.00		1.00		0.99	0.80								95
		100				91	45						41	*	69
<i>cox3</i>	nuc	1.00	1.00	0.99	0.91	0.99									98
		100	100	95	100	100									99
	aa	1.00	1.00	1.00	1.00	1.00									100
		100	96	96	100	100									98
<i>cytb</i>	nuc	1.00		1.00	1.00	0.95	0.99								99
		100	62	98	100	99									92
	aa	1.00	0.92	1.00	1.00	1.00									98
		100	67	98	100	100									93
<i>nad1</i>	nuc	1.00	0.99	1.00	1.00	0.82									96
		100	93	99	100	99									98
	aa	1.00	1.00	1.00	1.00	1.00									100
		100	67	99	100	96									92
<i>nad2</i>	nuc	1.00	0.83	1.00	0.99	0.79									92
		100	97	100	100	100									99
	aa	1.00	0.83	1.00	1.00	1.00									98
		98		94	100	98			59						90
<i>nad3</i>	nuc	1.00	0.91	1.00	0.85						0.67				90
		100	81	100	100	89									94
	aa	1.00	0.91	1.00	0.90	0.68									91
		100	78	95	95	99									93
<i>nad4</i>	nuc	1.00	0.97	1.00	1.00	0.99									100
		100	98	98	100	100									99
	aa	1.00	0.99	1.00	1.00	1.00									100
		100	74	97	100	100									94
<i>nad4L</i>	nuc	1.00	0.99		0.52	0.97					0.61				82
		100	97	58	96	86									87
	aa	1.00	1.00	0.97	0.98	0.84									96
		100	83	44	86	96									82

(continued on next page)

Table 3 (continued)

Gene	Data	Bipartition number and nodal support (BI/MP)												Inclusiveness (%)
		1	2	3	4	5	6	7	8	9	10	11	12	
<i>nad5</i>	nuc	1.00	1.00	1.00	1.00	1.00								99
		100	76	100	100	100								95
	aa	1.00	0.96	1.00	1.00	1.00								100
		100	40	83	100	100								85
<i>nad6</i>	nuc	1.00		1.00		0.98	0.83		0.57					88
		100	79	86		100						*		91
	aa	1.00		1.00		1.00								96
		100		77		100	58					*		84
<i>rrnS</i>	nuc	1.00	1.00	1.00		1.00								100
		100	88	100		99						*		97
<i>rrnL</i>	nuc	1.00	1.00	1.00		0.73								89
		100	77	100		73						*		88
<i>trns</i>	nuc	1.00		1.00	1.00	1.00								92
		100	80	100	100	100								96
All coding	nuc	1.00	1.00	1.00	1.00	1.00								100
		100	100	100	100	100								100
All data	nuc	1.00	1.00	1.00	1.00	1.00								100
		100	100	100	100	100								100
All coding	aa	1.00	1.00	1.00	1.00	1.00								100
		100	100	100	100	100								100
<i>N</i>		34	29	33	25	34	2	0	3	0	0	0	1	
Average score		1.00	0.94	0.99	0.97	0.95	0.77		0.62	0.61	0.67	0.51		
		100	81	93	99	96	52		53				41	

Bipartitions 1–5 are those nodes corresponding to the phylogenetic hypothesis of Lockyer et al. (2003). An asterisk at the end of a line indicates that data for *Schistosoma malayensis* was not available. Percent inclusiveness indicates how much of the topology is explained by the best-supported nodes. At the bottom of the table the number of occurrences of the node is given, along with the average score.

S. spindale (*haematobium*–*spindale*) was 17,456 bp long and contained 3916 variable ungapped characters. The mean genetic distance between this pair of taxa was 29% (protein-coding genes only).

Between the pairs of alignments there were several changes in gene order (Le et al., 2000a). The longest conserved fragment includes *nad5*, *cox3*, *cob*, *nad4L* and *nad4*. A few features were common between the alignments of *mekongi*–*malayensis* and *spindale*–*haematobium*. One was that the 3' end of *cox1*, encompassing the region chosen as a DNA barcode (Hebert et al., 2003b), was the most conserved longer region (Figs. 2 and 6). A distinct difference was that the very beginning of *cox1* had the highest nucleotide diversity in the *spindale*–*haematobium* alignment, but very low nucleotide diversity in the *mekongi*–*malayensis* alignment. This might have been due to the gene rearrangement that moved *cox1* to a position adjoining the large non-coding region in *S. spindale* and *S. haematobium*. The beginning of *cox1* was unalignable for these taxa. Both pairwise alignments had greatest nucleotide diversity at the 3' end of *nad5*. The profile of *atp6* and *nad2* was similar, with three maxima and three minima of average nucleotide diversity. Genes *nad4L* and *nad4* showed contrasting patterns of divergence; in the *spindale*–*haematobium* alignment it was most conserved in the middle, but in the *mekongi*–

malayensis alignment, this middle region was the most variable. Generally, the divergence seemed to be highest towards the ends of a protein.

3.3. Distribution and co-variation of divergence and polymorphisms over different scales

The distribution of divergent sites between *S. mekongi* and *S. malayensis* is shown in Fig. 2. It was unevenly distributed, confirmed by frequency distributions of divergence (Fig. 3a and b) and plots of the distance between them (Fig. 4). The random distribution (grey bars) was generated in Excel (Microsoft) with the same number of divergent sites as in the original dataset. Fig. 4 shows that the majority of divergent sites were clustered together, with conserved stretches of up to 81 bp. Every third position contained more divergent sites, confirming that third codon positions evolve more rapidly.

In order to establish whether the distribution of divergent characters was conserved between different phylogenetic levels, we used the alignment of *S. mekongi* and *S. malayensis*, and the alignment of *S. haematobium* and *S. spindale* combined with the intraspecific data of *S. mansoni* from Morgan et al. (2005). This latter dataset consists of *cox1*, *rrnL*–*rrnS*, *cytb*–*nad4L*–*nad4* and *nad1*.

Table 4

Overview of phylogenetic utility of individual mitochondrial genes in providing sufficient signal and resolving accurately the benchmark topology of Lockyer et al. (2003b), with strong nodal support across and in relation to relative length of genes

Criteria for gene utility of phylogenetic inference	Best	Worst
1. <i>Data features</i>	<i>atp6</i>	<i>cox1</i>
Datasets that have the lowest amount of length variance and the highest information content have been considered good	<i>cox3</i>	<i>cox2</i>
	<i>nad1</i>	<i>nad4</i>
	<i>nad2</i>	<i>rrnL</i>
	<i>nad3</i>	<i>rrnS</i>
	<i>nad4L</i>	
	<i>nad5</i>	
	<i>nad6</i>	
2. <i>Support values, resolution</i>	<i>cox1</i>	<i>cox2</i>
Datasets with high bootstrap support and posterior probabilities have been considered good. Several datasets not listed have good support values in either, but not in both	<i>cox3</i>	<i>nad4L</i>
	<i>nad4</i>	<i>rrnL</i>
	<i>rrnS</i>	
3. <i>Topological congruence^a</i>	<i>cox1</i>	<i>cox2</i>
The best genes give a phylogenetic signal that is congruent to the benchmark hypothesis no matter which method is used	<i>cox3</i>	<i>nad6</i>
	<i>nad1</i>	<i>rrnL</i>
	<i>nad4</i>	<i>rrnS</i>
	<i>nad5</i>	

^a With reference to benchmark hypothesis of Lockyer et al. (2003b).

For each of these gene fragments, we tested the correlation between intraspecific and interspecific variation (Pearson's correlation, window size of 24 bp and step size 3). Table 5 shows that the level of polymorphism in *S. mansoni* was always correlated with the level of divergence between *S. spindale* and *S. haematobium*, but only correlated with the divergence in *S. malayensis* and *S. mekongi* for *nad4* and *rrnL-rrnS*. The levels of divergence in the pairs *S. malayensis* and *S. mekongi*, and *S. spindale* and *S. haematobium* were always correlated except for the *nad1* dataset. Applying a sliding window of 1 bp and step size 1 resulted in positive correlations between all comparisons. This is also shown in Fig. 5a and b where the average nucleotide diversity (π) among the three groups of sequences is plotted for the *nad1* and *rrnL-rrnS* datasets. Thus, if there is interspecific variation at a particular site there is also likely to be intraspecific variation at that site.

3.4. Sliding window analysis and the identification of potential population markers

Fig. 3 shows the frequency distribution of variable positions in any stretch of 300 bp in the *S. mekongi* and *S. malayensis* alignment (black bars). The randomly generated dataset (grey bars) had a normal distribution whereas

the variation in the real dataset appeared to be non-randomly distributed. Also, the random dataset had a lower maximum number of variable sites per 300 bp than the real dataset (41 compared with a maximum of 52 sites). A 300 bp sequence in the *S. mekongi* and *S. malayensis* alignment contained between 12 and 52 nucleotide changes (see Fig. 3). The sliding window analysis of the nucleotide diversity is shown in Fig. 2 (window size 300; step size 10). Using this alignment and sliding window results, PCR primers were designed in flanking sequences to allow the capture of as much of the sequence variability as possible. The regions that these primers amplify make up 20% of the total alignment, but contain 27% of the total number of nucleotide changes (Table 6). These primers were designed for *S. mekongi* and *S. malayensis* and should be applicable to species closely related to them; they were tested successfully using genomic DNA from *S. japonicum* (Fig. 7).

4. Discussion

With complete mt genomes available for a number of closely related species, we can test empirically the phylogenetic utility of individual genes and determine regions of the genomes that contain the greatest interspecific variation for ecological and diagnostic markers. By doing so, we can make optimal use of mt genomes. The utility of existing mt markers can also be assessed in the context of entire genomes through comparisons of sequence variation within and between species. In this way, existing primers (and gene regions) can be assessed and new primers can be designed and optimised, prior to extensive sampling. We present a comparative mitogenomic assessment of available data from *Schistosoma*, but the general methodology can be applied to other organisms.

The simplest criterion to judge the phylogenetic utility of any individual gene includes a determination of whether the gene recovers the “true phylogeny”, with a measurement as to how robust that recovered phylogeny is. The “true phylogeny” is rarely available. For this study, genes yielding topologies identical to the benchmark hypothesis of Lockyer et al. (2003b) with full nodal support throughout the trees and full congruence among methods of analysis and between both nucleotide and amino acid data, were judged as most useful.

There are limits to the usefulness of the present dataset; e.g., it is an incomplete clade with only six ingroup taxa, providing 945 distinct tree topologies (Felsenstein, 1982). In spite of this low level of sampling, both closely related and less closely related species are included; hence no particular level of the phylogeny was excluded. Also, genes were used as units to compare their usefulness for phylogenetic reconstruction, for convenience and because gene order changes between the African-Indian and SE Asian clades obscure base positional homology in longer alignments spanning genes that have moved. Further studies with longer fragments of mtDNA, spanning different gene boundaries are possible. However, researchers are

Fig. 2. Sliding window analyses of the alignment between *Schistosoma mekongi* and *Schistosoma malayensis*. The line shows the value of nucleotide diversity (π) in a sliding window analysis of window size 300 bp with step size 10, the value is inserted at its mid-point. The horizontal grey dashed line is a grid-line for reference. Gene boundaries are indicated; *nad4L* and *nad4* are overlapping. Grey boxes indicate the fragments amplified by five primer pairs (p1–p5) designed from this alignment (see Table 6 and Fig. 7).

Fig. 3. (a) Distribution of polymorphic sites in the alignment of *Schistosoma mekongi* and *Schistosoma malayensis* using the same dataset as in Fig. 2. Each black bar is a polymorphism; polymorphisms were calculated by DnaSP and do not include gaps in the alignment. (b) Frequency distribution of the number of polymorphisms (x-axis [bp]) in each possible region of 300 bp, along the alignment of the whole pairwise mitochondrial sequence of *S. mekongi* and *S. malayensis*; black bars show the real distribution, and grey bars are from a random distribution.

Fig. 4. Frequency distribution of distance between polymorphic sites in the *Schistosoma mekongi* and *Schistosoma malayensis* alignment (black bars), and values from a random distribution (grey).

Table 5
Summary of results of sliding window analyses, correlating intraspecific (*Schistosoma mansoni*) and interspecific variation

Gene	Sliding window length_step size (bp)	Level of polymorphism (π) within <i>S. mansoni</i> correlated with divergence (π) between :		
		<i>spindale</i> – <i>haematobium</i>	<i>malayensis</i> – <i>mekongi</i>	<i>mekongi</i> – <i>japonicum</i>
<i>cox1</i>	24_3	0.216**	–0.130	0.138
	1_1	0.167**	0.119**	0.014
<i>nad4</i>	24_3	0.249**	0.269**	n.a.
	1_1	0.086*	0.090**	n.a.
<i>rrnS</i> – <i>rrnL</i>	24_3	0.480**	n.a.	0.555**
	1_1	0.222**	n.a.	0.152**
<i>nad1</i>	24_3	0.462**	0.022	n.a.
	1_1	0.093*	0.070	n.a.

Note: *cox1* in the *malayensis*–*mekongi* alignment was not complete. n.a. – data not available due to partial *Schistosoma malayensis* sequence.

* $P < 0.05$.

** $P < 0.001$.

primarily interested in amplifying and targeting specific genes or gene fragments; spanning gene boundaries is not of primary interest unless genes of interest are adjoining and can be amplified together.

Taking the topology of Lockyer et al. (2003b) as a benchmark, two genes were shown to be unsuitable as phylogenetic markers: *cox2* and *nad6*. Both of these genes

resolved phylogenies that were inconsistent with the benchmark, and with each other, no matter which method of analysis was used. The value of these genes for constructing phylogenies of schistosomes is deemed low. In contrast, genes *nad1*, *nad4*, *nad5*, *cox1* and *cox3* gave results that are consistent with the hypothesis, no matter which method was used and whether data were treated as nucleotides or amino acids (Tables 3 and 4). Their value for phylogenetic analyses in schistosomes is therefore considered to be high. Other protein-coding genes gave results that were inconsistent, depending on which method was used. However, longer genes generally contain more variable characters with proportionally more signal, and hence offer better resolution than shorter ones. Genes comprising more than 1000 bp gave an unambiguous phylogenetic signal. Comparing all individual gene datasets, they are congruent in most cases and resolve the expected phylogeny (Table 3). As a function of size of gene, lowest length variance and highest information content, the genes predicted to be best include *cox3*, *nad1* and *nad5*. Amongst the preferred genes, *cox1*, *cox3* and *nad4* resolved benchmark trees with consistently high nodal support throughout. There is a clear divergence between the SE Asian schistosome species and the other taxa, with slightly less nodal support within each clade. The relationships between *S. spindale*, *S. haematobium* and *S. mansoni* are the least resolved, where several genes give an alternative to the most common solution (*S. mansoni* (*S. spindale* + *S. haematobium*)). Without a comparative analysis of mt genomes, it is unlikely that

Fig. 5. Average nucleotide diversity (π) in the *nad1* (a) and the *rrnL-rrnS* alignments (b) of *Schistosoma mekongi* + *Schistosoma malayensis*, *Schistosoma haematobium* + *Schistosoma spindale*, and *Schistosoma mansoni* (from Morgan et al., 2005) datasets; * indicates significant correlations of interspecific variation and intraspecific variation in *S. mansoni* (see Table 5).

genes such as *cox3* or *nad4* would be suggested as alternatives to the more commonly chosen *cox1*. Readily available primers for *cox1* and a wealth of comparative information for this gene allow for a potentially complacent approach to gene and primer selection. Moreover, poorly performing genes such as *cox2* and *nad6* could easily be chosen without recourse to preliminary phylogenetic analyses.

In a recent study assessing the phylogenetic performance of mt protein-coding genes from 11 neodermatan (parasitic platyhelminth) mt genomes (Hardman and Hardman,

2006), the authors concluded that any 4000 bp of alignable mt sequence data would yield accurate phylogenetic estimates. All genes performed as well as randomly selected regions except *nad2* and *nad5*, which performed significantly better and worse, respectively. This contrasts with the present study, which found *nad5* was both accurate in resolving the benchmark phylogeny amongst schistosomes and provided a high level of information content (Table 4). Together with *cox3*, in the present study *nad5* is considered to be a worthwhile gene for future sampling in phylogenetic

Table 6

Primers identified as potential population markers among SE Asian schistosomes, from alignments of *Schistosoma mekongi* and *Schistosoma malayensis* mitochondrial genomes, capturing high levels of sequence variability; see Figs. 2 and 7

Location	Primer sets (Fig. 2)	Forward (F) Reverse (R) primers (all 5' to 3')	Start ^a	End ^a	T _m (°C)	Product (bp)	% divergent sites in amplicon
Middle to end of <i>nad5</i>	p1	F – GKTTATCTGKTTTCCWTTTATGGG R – TGAGATCTCYACTTGGAAAGAAAG	1067 1759	1091 1735	53.3 50.0	~693	15
<i>cytb</i> – <i>nad4L</i>	p2	F – ATWAAGGATTGTTTGYTTTATG R – AYAAAGCCATCATCGAAATG	3244 3926	3267 3907	45.4 47.0	~683	18
End of <i>nad5</i> , through <i>trnG</i> to beginning of <i>cox3</i>	p3	F – TTGATTWTTTGRTTKATATGTATA R – TGAGATCTCYACTTGGAAAGAA	1469 1759	1492 1737	48.7 58.2	~236	15
<i>nad1</i>	p4	F – TTTATTTGAGCRCGTGGTAC R – CGGAGTAACCTGCTTAGCA	7852 8200	7871 8182	45.4 48.1	~349	10
End of <i>nad4</i> , over <i>trnQ</i> and <i>trnF</i> and ending in <i>trnM</i>	p5	F – TGGWTTTGATAAATTWTTGTYTTGTA R – KTTTMTTAACTTAATATCCACCTCT	5175 5488	5200 5463	49.1 48.6	~314	14

^a Numbers refer to mitochondrial genome sequence of *Schistosoma japonicum* (NC_002544).

and molecular ecological studies. The taxonomic diversity of the study by Hardman and Hardman (2006) encompassed cyclophyllidean cestodes as well as schistosomes and other digeneans. As with other phylogenetic studies, the results from comparative mitogenomic analyses need to be considered with care and with reference to the questions being addressed, before embarking on large-scale sampling. Whilst *nad5* may be appropriate for studies amongst schistosomes, it appears to have its limitations if more distantly related taxa are sampled. Denser sampling of mt genomes of Platyhelminthes will allow further studies of gene utility to be undertaken across different taxonomic ranges. If lengths of mt genomes are preferred over specific genes, then such studies will be necessary to assess the differences in mt gene order within and between flatworm groups; novel gene orders have now also been found amongst the Neodermata (Park et al., 2007). Gene order changes have a bearing on which genes or which gene regions can be amplified with universal primers to produce sufficiently long sequences (>4000 bp) for alignment and accurate phylogenetic analysis.

The distribution of divergence (differences between species) and polymorphism (differences within a species) over different scales are crucial for this study. The notion that “optimal” phylogenetic and population genetic markers can be constructed depends on two conditions: (i) an uneven distribution of informative characters, and (ii) a certain amount of conservatism in the distribution. Because of the functional constraints or absence of function for a particular region, we expect the distribution of variation to evolve in a predictable manner. Forces of positive and negative selection can affect evolutionary rates in entire proteins, regions of proteins or among individual base pairs and, in the case of mt genes, can affect the entire mt genome. If random mutations are sorted by selection in this way, one might expect to see a pattern of correlation between (a) nucleotide diversity in datasets of closely related taxa (b) inter- and intraspecific patterns of variation

and (c) intraspecific patterns of variation between closely related species. These principles, although not always explicitly stated, are used quite often; for example, when a researcher uses PCR primers for the amplification of molecular markers that have given good results from one species, on a different, but closely related species.

A confounding issue when investigating correlation is that the co-variation can be measured on many different levels; in genes, predicted protein structures or codons, in regions of single to thousands of nucleotides. Which scale is investigated can dramatically affect the level of co-variation. Therefore, we used several different window and step sizes in the sliding window analyses (1/1, 9/3, 24/3, 300/10; e.g., see Fig. 6); we would encourage this approach in future similar studies. The interspecific variation in the SE Asian schistosome clade was always correlated with the divergence in the African-Indian clade, except for *nad1*. When using a window and step size of 1 bp, the correlation in the latter region was also significant.

Using the mitochondrial dataset from Morgan et al. (2005) we took intraspecific gene sequences for *S. mansoni* ($n = 99$) and compared them with the corresponding alignment of *S. spindale* + *S. haematobium* and *S. malayensis* + *S. mekongi* (Fig. 5a and b). Fig. 6 indicates the gene/genome regions used in Morgan et al. (2005). For each region (*cox1*, *rrnL*–*rrnS*, *cytb*–*nad4L*–*nad4* and *nad1*), the average nucleotide diversity ($\times 10$) for the marker in the *S. mansoni* data is also shown. Separate sliding window analyses showed significant levels of correlation between the divergent and the polymorphic sites compared with the *S. spindale* and *S. haematobium* dataset. For *S. malayensis* and *S. mekongi*, a positive correlation was found in the *cytb*–*nad4L*–*nad4* and the *rrnL*–*rrnS* dataset; in *nad1* and *cox1*, significant values were only obtained with window size and step size 1. *Schistosoma mansoni*, *S. haematobium* and *S. spindale* all belong to the African-Indian clade, characterised by a different gene order

Fig. 6. Sliding window analysis (window size 300 bp, step size 50) of the alignment of the African-Indian species *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma spindale*. On the y-axis is the nucleotide diversity (π) and on the x-axis the relative position on the mitochondrial genome (bp). The alignment starts at *trnM* and is annotated showing positions of protein-coding and ribosomal genes. Marker regions defined by Morgan et al. (2005) from their study on *S. mansoni* are shown, with black bars indicating the average nucleotide diversity for each fragment.

compared with the SE Asian clade (*S. mekongi* and *S. malayensis*) and other neodermatan parasites. Thus, the correlation between polymorphism in the *S. mansoni* dataset and the African-Indian alignment is stronger than the correlation between the *S. mansoni* dataset and the SE Asian alignment. This finding may relate to the effect of phylogenetic constraint, or it might be conflated by the effect of a different gene order. As demonstrated, the level of divergence in the beginning of *cox1* was higher in the African-Indian species. Due to gene rearrangement, *cox1* is located adjacent to the non-coding region in the African-Indian species, but not in the SE Asian species. The gene flanking the non-coding region on the other side is *nad1*, which was the most variable gene in the study by Morgan et al. (2005). The rate of evolution of these genes might be linked with the non-coding region, which evolves at a different rate due to different selective pressures. Another explanation might be linked with the gene arrangement itself. According to Szczepanik et al. (2001), sequences in which gene orders have changed their positions recently are the most susceptible to mutation accumulation. The position of a gene with respect to the direction of replication fork movement is important; this position defines the duration that the heavy strand spends in the mutagenic single-strand state during replication (Szczepanik et al., 2001). Reyes et al. (1998) found a posi-

tive correlation between the number of variable sites for a gene and its single-stranded state duration.

Thus, the selection on a gene, and therefore the evolutionary rate of nucleotide substitution, will depend on its position in the mt genome and on changes in mt gene order. Whether or not these issues have played a role in the present study, we can only speculate. However, if the species under study have the same genomic arrangement, the distribution of polymorphic and divergent characters is more likely to be correlated, such that an informed primer design is effective. Certainly, the correlation between polymorphism within a species (in this study *S. mansoni*) and divergence between species is more conserved over short phylogenetic distances.

Generally, population genetic markers need to be more variable than sequences used for phylogenetic studies. They need to amplify readily and give easily alignable and unambiguous sequence data. The mt genome has several desirable features, since it is rich in variable regions and it exists in multiple copies within cells, making it easy to amplify. We have developed and tested five sets of primers from the alignment of *S. mekongi* and *S. malayensis* (see Table 6). The primers are identical for both species, and attempt to capture as many of the variable sites between the species as possible. The primers were tested in silico against the mt genome sequence of *S. japonicum* using

MacVector (Accelrys), and indeed worked extremely well against this template when tested experimentally (see Fig. 7). MacVector takes several features into account including self-duplexing, hairpins, specificity and mismatches. The primers are designed to amplify fragments of *nad1*, *nad4*, *nad5*, *cox3* and some tRNA genes, capturing 20–27% of the variation present in the mt genome with less than 2300 bp, ~16% of any SE Asian *Schistosoma* genome. These genes were predicted to be the most useful for phylogenetic reconstruction within the Schistosomatidae.

Only recently have microsatellites been isolated and used in schistosome research (Rodrigues et al., 2002; Srivastava et al., 2005; Sorensen et al., 2006). However, although a valuable resource, microsatellite markers are useful when studying very recent phenomena such as migration and bottlenecks. Few mt genes or gene regions have been used as population markers for *Schistosoma*. Although recognised as potentially useful from laboratory isolates of *S. japonicum* (see Sørensen et al., 1999), *nad1* has only been used in *S. mansoni*. Others have highlighted the potential utility of the polymorphic repeat element in the mt genome of *S. mansoni* (see Curtis and Minchella, 2000), and it has been used successfully in differentiating strains in snail hosts (Spotila et al., 1991; Minchella et al., 1994). However, Curtis and Minchella (2000) noted that there was a lot of heteroplasmy (noise) in this region, possibly related to a high mutation rate. The only study in which a combination of mt markers has been used with schistosomes is on *S. mansoni* by Morgan et al. (2005); these authors used partial fragments of *cox1*, *rrnL-rrnS*, *cytb-nad4L-nad4* and *nad1*. However, as shown in Fig. 6, the 3' ends of *cox1* and the *rrnL-rrnS* region have a low number of variable characters. Amplifying the 5' end of *cox1* in combination with *nad5* would substantially increase the number of variable characters per unit length. Thus, an informed primer design will be more cost-efficient, involve less sequencing and generate proportionally more information.

Finally, a comment on *cox1* as a DNA barcode in the context of schistosome research is warranted. Mitochondria are generally maternally inherited and this appears

to be the case for flatworms (Johnston, 2006), although there may be evidence for paternal leakage according to Bieberich and Minchella (2001). As such, mt markers cannot be used to detect hybrids or hybridisation events except when used in conjunction with nuclear markers. Hybridisation within the genus *Schistosoma* is common (Webster et al., 2006) making species diagnosis with mt markers potentially problematic. Partial mt *cox1*, corresponding to the region chosen by the Consortium for the Barcoding of Life (CBOL; <http://barcoding.si.edu/>), has been characterised for almost all members of the genus *Schistosoma* (Webster et al., 2006). The only within-species study on mt gene utility in terms of resolving as many haplotypes as possible, is that of Morgan et al. (2005) for *S. mansoni*. Unfortunately, the *cox1* fragment they chose begins some 40 amino acids after the fragment promoted by the “Barcoding of Life” consortium. However, both from our study and that of Morgan et al. (2005), it is clear that *nad1* and the *cytb-nad4L* fragment provide greater variation and resolving power than *cox1*, presumably by capturing considerably more nucleotide diversity. The present study provides additional advantages and disadvantages in considering *cox1* for species identification. A simple unrooted distance tree (not shown) differentiates individual species but with very short internodal and terminal branch lengths between members of the African-Indian species (a clade within which hybridisation is particularly common), suggesting the need for a more variable marker. In the current study, the *cox1* barcode region of the mt genome by no means encompasses the greatest molecular diversity or potential resolving power; it has the lowest number of phylogenetically informative characters. Therefore, given the availability of full mt genome sequences, we suggest *cox3* and *nad4*. Full mt genome sequences allow the assessment of such preferred barcoding regions, prior to their wide-scale use.

In conclusion, using Lockyer et al. (2003b) as a “benchmark”, this study has predicted that a single mt gene is sufficient to give a well-resolved, accurate phylogeny of the schistosomes. For accurate identification (sufficient variability) and a consistent phylogenetic signal (sufficient number of phylogenetically informative characters per total length), *cox3* is the preferred gene, with high variability in characters, but not in length. Genes *nad1*, *nad4*, *nad5* and *cox1* also give coherent topologies but they are also the longest genes. Of these, *nad4* gives the highest support values, while *cox1* gives high support values and correct topology, but has a low level (31%) of phylogenetically informative characters. The genes predicted to be uninformative are *cox2*, *nad6* and the rRNA genes; they also have a small number of informative characters. The so-called “barcoding gene” *cox1* had the lowest proportion of phylogenetically informative positions. For population markers, *cox3* is predicted to be a good alternative, but *nad4L* also has a high proportion of variable sites, with low variance in length. Sliding window analysis provided a rapid means of identifying

Fig. 7. Amplicons produced using primer sets p1–p5 with gDNA from *Schistosoma japonicum*, resolved by agarose gel electrophoresis; primer details and genes amplified given in Table 6 and indicated in Fig. 2.

novel oligonucleotide primers for capturing sequence variability in short PCR amplifications.

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