Letter to the Editor

Phylogenies Inferred from Mitochondrial Gene Orders—A Cautionary Tale from the Parasitic Flatworms

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Mitochondrial genomes have been used in numerous studies to investigate phylogenetic relationships among eukaryotes at many levels (e.g., Smith et al. 1993; Boore et al. 1995; Boore, Lavrov, and Brown 1998). In recent years, the arrangement of genes in the mitochondrial genome has been regarded as a powerful record of historical relationships (Boore 1999). Changes in mitochondrial gene order are infrequent, even over considerable spans of time (Boore 1999), and are unlikely to exhibit homoplasy. Our research has focused on the relationships between two groups of human blood flukes within the genus *Schistosoma*. Our investigations on the mitochondrial genomes of these worms revealed striking divergences in mitochondrial gene order within the genus.

The schistosomes are among the most significant parasites of humans in the developing world. The disease they cause, schistosomiasis, is second only to malaria in public health importance, affecting some 200 million people in 75 countries and giving rise to severe morbidity or mortality in tens of millions. Recent molecular studies (Barker and Blair 1996) have demonstrated that the deepest split in the genus is between the Asian species utilizing prosobranch snail hosts and the African species utilizing pulmonate snails. The depth of this split has led some authors to propose an early Tertiary divergence (Després et al. 1992). Species closely allied with the African group also occur in the Middle East, India, and parts of Southern Asia. One African species, *Schistosoma mansoni,* was probably introduced into the Americas by the slave trade during the 18th and 19th centuries (Després, Imbert-Establet, and Monnerot 1993). The Asian group contains fewer recognized species, and these are found primarily in East Asia (the Philippines, China, Malaysia, Indonesia, Cambodia, and Laos). There is a growing realization that African and East Asian schistosomes differ in many biological attributes, including morphological characters, infectivity to snails, range of definitive hosts, growth rates, egg pro-

duction, prepatency periods, pathogenicity, and immunogenicity (McManus and Hope 1993). We expected our investigations of mitochondrial genomes in these two groups of species to provide more evidence of their phylogenetic distance. However, we were startled by the remarkable differences in mitochondrial gene order which came to light between the two groups and which we report here.

Partial sequences (totaling 8 kb) for *S. mansoni* were obtained from two genomic clones derived from a Brazilian strain (Deprés, Imbert-Establet, and Monnerot 1993; Blair et al. 1999). The sequences were used in the design of oligonucleotides that permitted amplification of the mitochondrial genome in a Puerto Rican strain using "long PCR." Overlapping fragments of up to 8.3 kb were generated, cloned, and sequenced by primer walking. Sequences from the Brazilian and Puerto Rican strains were almost identical. The sequences of *Schistosoma japonicum* (Anhui strain, China), *Schistosoma mekongi* (Khong Island, Laos), *Schistosoma haematobium* (Mali, West Africa), and *Fasciola hepatica* (Geelong strain, Australia) were generated by long PCR (the last using published partial sequences [Garey and Wolstenholme 1989] as a guide to primer design). The sequence of *Paragonimus westermani* (triploid specimen from Korea) was generated partly from genomic clones and partly from the products of long PCR. Four genomic clones containing between them the entire mitochondrial genome of *Taenia crassiceps* (Zarlenga and George 1995) were sequenced by primer walking, followed by PCR amplification and sequencing of mtDNA regions that bridged connections between the clones. In most of the trematodes, long PCR was difficult across one segment of the genome. Preliminary data (not shown) indicate that such segments contain numerous repeats, and we assume that growing nucleotide chains can jump over this region during PCR. As a result, the long PCR fragments we have sequenced do not contain this region (variable noncoding region; VNR), which is known to be variable in length in *S. mansoni* (Deprés, Imbert-Establet, and Monnerot 1993). Southern blotting of digested genomic DNA and the use of appropriate probes determined the exact locations and lengths of such regions in some species.

We obtained complete or near-complete DNA sequences for the mitochondrial coding regions of *S. mansoni* (Africa/Americas), *S. japonicum,* and *S. mekongi* (East Asia), *P. westermani* (human lungfluke, family

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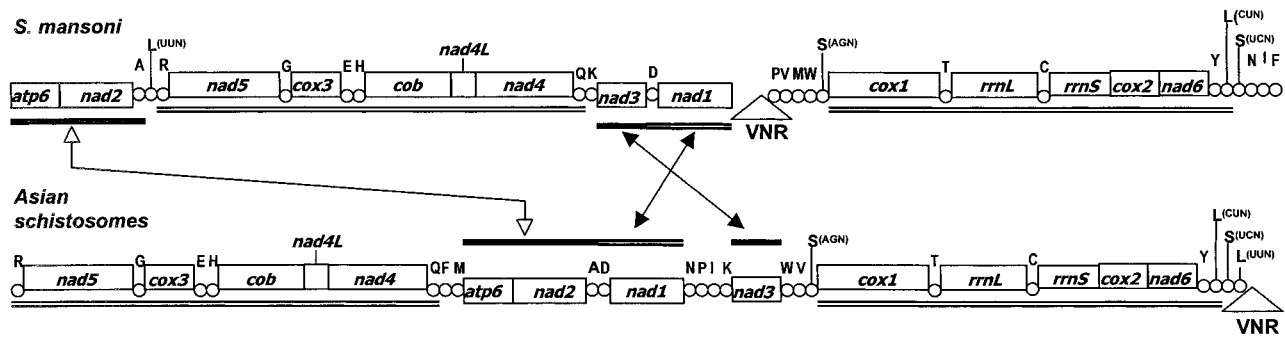
FIG. 1.—Linear representation of mitochondrial genome organization for African and Asian blood flukes. The total length of the coding regions is approximately 14 kb. Arrows indicate the rearrangements that have taken place. Individual genes are boxed, and transfer RNAs are shown as circles. Triangles indicate the locations of the long variable noncoding regions (VNRs, 2–9 kb in each case) that have not yet been sequenced. The Asian schistosome species analyzed were *Schistosoma japonicum* and *Schistosoma mekongi*. The abbreviations used for individual genes are those recommended in www.biology.lsa.umich.edu/~jboore.

Paragonimidae), and *T. crassiceps* (tapeworm, family Taeniidae). In addition, partial sequences were obtained for *S. haematobium* (Africa) and *F. hepatica* (liverfluke, family Fasciolidae). Nucleotide sequence data referred to in this paper are available in the GenBank database under accession numbers AF216698, AF216699, AF215860, AF216697, AF217449, AF219379, and AJ271051. Successful PCR amplification experiments with a further six African species (*Schistosoma bovis, Schistosoma curassoni, Schistosoma intercalatum, Schistosoma mattheei, Schistosoma rodhaini,* and *Schistosoma margrebowiei*) suggested that these have the same gene order as the sequenced portion of *S. haematobium*. Figure 1 presents the results for the schistosomes, and differences between these and other parasitic flatworms are given below.

All genes are transcribed from the same strand. The gene complement is typical of metazoans (Boore 1999), but the gene order is unique. Twenty-two tRNA genes, 2 rRNA genes and 12 protein-coding genes were identified. The gene for *atp8* was not found in any species. Gene order differs considerably between *S. mansoni* and *S. japonicum* and in each case is unlike that seen in any other organism. The differences are as follows (fig. 1): (1) the block of sequence containing *atp6* and *nad2* occurs in different places; (2) the genes for *nad3* and *nad1* have exchanged positions; (3) the large VNRs are in different places; and (4) the tRNAs for F, P, M, N, I, F, $S^{(AGN)}$, W, and V are not flanked on either side by any of the same genes (tRNA, rRNA, or protein-coding) in the two species (although in the case of the last three, this is due to local exchanges of position among them). The sequenced portion from *S. haematobium* (*rrnL* > *nad2*) exhibits the same gene order as *S. mansoni*. *Paragonimus westermani, F. hepatica, T. crassiceps,* and *Echinococcus multilocularis* (another cestode; GenBank accession number AB018440) have the same mitochondrial genome structure as the Asian schistosomes (fig. 1), with the following differences: (1) the tRNAs for $S^{(AGN)}$, W, and V have been rearranged; (2) the VNR (~6 kb) in *P. westermani* lies between *nad5* and *cox3*; (3) *T. crassiceps* lacks a long noncoding region and therefore has the shortest genome (14 kb) of any of the

species investigated here; and (4) the tRNA for *E. multilocularis* lies between *cox2* and *nad6*.

Published fragments of mitochondrial genomes from other trematodes (families Campulidae and Nasitrematidae) (Fernández et al. 1998) and cestodes (order Pseudophyllidea; Kokaze et al. 1997) indicate that the order of tRNAs around the *nad3* gene in these species is identical to that in *P. westermani, F. hepatica,* and *T. crassiceps*. A tRNA $S^{(AGN)}$ is present at the 3′ end of *nad3* in the cestodes, contrary to an earlier report (Kokaze et al. 1997).

The magnitude of the differences in mitochondrial gene order between African and Asian schistosomes is unprecedented among metazoans belonging to the same genus (reviewed in Boore 1999). This result is nevertheless consistent with the deep phylogenetic divide between these groups of schistosomes inferred previously from nucleotide sequences (and reinforced by trees constructed from our newly available sequences; results not shown). The possibility of major changes occurring even within a single genus cautions us that studies using mitochondrial gene order for phylogenetic inference should include as diverse a sampling of species within each major taxon as possible.

Acknowledgments

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