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Molecular arguments for splitting of *Schistosoma intercalatum*, into two distinct species

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Abstract The taxonomic status of the two known strains of *Schistosoma intercalatum*, the Lower Guinea strain (originating from Edea, Cameroon) and the Zaire strain (originating from Kinshasa, Democratic Republic of Congo, formerly Zaire) was examined using random amplified polymorphic DNA (RAPD) markers. Two additional species within the *S. haematobium* group, *S. haematobium* and *S. mattheei*, were included in the study. DNA was extracted from four male and four female worms of each species and strain under investigation. In all, 13 primers gave reproducible and informative marker patterns; the monomorphic bands in all the males and females of each sample were scored, and 138 bands were included in the final analysis. Overall, 14 RAPD fragments were shared by all the schistosomes studied, and 19 RAPD fragments were considered to be sex markers. Only 22% (20/91) of the RAPD fragments were shared between *S. intercalatum* Zaire and *S. intercalatum* Cameroon. The mean values recorded for the Nei and Li's genetic distances between *S. haematobium* and *S. mattheei* and between *S. intercalatum* Zaire and *S. intercalatum* Cameroon were 0.546 and 0.596,

respectively. A principal component analysis and one-way analysis of variance (ANOVA/MANOVA) showed a significant separation between *S. intercalatum* Zaire and *S. intercalatum* Cameroon. The data support the hypothesis that *S. intercalatum* Zaire and *S. intercalatum* Cameroon are distinct species. Additional molecular-biology studies are in progress that involve the use of nuclear and mitochondrial markers to confirm the extent of the genetic divergence prior to the establishment of final decision on the taxonomic status of the two strains of *S. intercalatum*.

Introduction

Schistosoma intercalatum, the agent of rectal schistosomiasis in Africa, continues to arouse interest because of its biological diversity and its puzzling distribution. Since the work of Wright et al. (1972) on "What is *Schistosoma intercalatum*?", the taxonomic status of the species has been debated by several parasitologists (Frandsen 1978; Bjerneboe and Frandsen 1979; Wright et al. 1979), but without definitive conclusion. If biological, clinical and, more recently, molecular-biology studies allow us to confirm the validity of *S. intercalatum* as a distinct species within the *S. haematobium* group of schistosomes, the inter-population polymorphism displayed by the species raises further taxonomic questions.

S. intercalatum, described by Fisher (1934) from material originating from the upper Zaire river (Kisangani area), appears to consist of two geographically separate entities, the Zaire strain and the Lower Guinea strain, which differ from each other in a number of characteristics. The Zaire strain, which corresponds to the taxon described by Fisher (1934), has been reported only from the Democratic Republic of Congo and develops naturally in *Bulinus globosus*, which belongs to the *B. africanus* group. The Lower Guinea strain includes the populations of the parasite

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present at the level of the Guinea area (essentially Gabon and Cameroon), developing exclusively in *B. forskalii*. Therefore, the two strains of the parasite develop in different intermediate host species without possible interchange. Beyond their respective intermediate host specificity, the two strains differ from each other in a number of other characteristics, including the duration of the pre-patent period in the intermediate host (Wright et al. 1972); egg biometry (Frandsen 1978); iso-enzyme patterns (Wright et al. 1979); and small differences in egg morphology, cercarial shedding pattern and biological characters in the definitive host (Wright et al. 1972; Bjerneboe and Frandsen 1979). No difference has been noted in the morphology of the adults, cercariae, or miracidia, in the behaviour of the cercariae, or in the results of Ziehl-Nielsen staining of the eggshells. A post-isolating reproductive barrier between the two strains has been observed in preliminary experimental work, characterized by F₂ hybrid nonviability (Frandsen 1978).

Another character seems to separate the two strains: the difference in the ability to spread outside endemic areas. Over the last 15 years the Lower Guinea strain has apparently spread geographically either within endemic countries or into new countries, including Equatorial Guinea (Simarro et al. 1989), São Tomé (Southgate et al. 1994), Nigeria (Arene et al. 1989) and Mali (Corachan et al. 1992; Visser et al. 1992). In contrast, the Zaire strain seems to be less capable of spreading, as only one new focus, in Kinshasa city, has been reported outside the Upper Zaire river (De Clercq 1987; Tchuem Tchuente et al. 1997).

Overall, all the different characteristics noted between the two strains indicate that *S. intercalatum* is not a homogeneous taxonomic entity. Are the differences observed indicative of the existence of usual genetic divergence between two allopatric populations or of the existence of two cryptic species?

The aim of this work was to determine the value of the genetic divergence between the two strains of *S. intercalatum* using a molecular approach and to determine whether the magnitude of the genetic divergence would support the hypothesis of the existence of two distinct species. For comparison, two other species belonging to the *S. haematobium* group, i.e., *S. haematobium* and *S. mattheei*, were included in the study.

Materials and methods

Schistosome material

The geographic origins and isolation dates of parasite strains and species are given in Table 1. Parasites had been maintained in Swiss mice, apart from *Schistosoma haematobium*, which had been maintained in jirds (*Meriones unguiculatus*). Four male and four female adult schistosomes of each species and geographic strain were used for the molecular-biology study.

Template DNA isolation and random amplified polymorphic DNA reaction

Template DNA for each schistosome adult was extracted according to the protocol described by Barral et al. (1993). DNA extracted from four males and four females of *S. haematobium*, *S. mattheei*, *S. intercalatum* Lower Guinea and *S. intercalatum* Zaire was amplified. A 20- μ l polymerase chain reaction (PCR) mixture containing approximately 20 ng of DNA in 2 μ l of the DNA extract, 2 μ l of buffer [10 mM TRIS-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100], 3 mM MgCl₂, 0.1 mM of each deoxynucleoside triphosphate (dNTP), 0.2 μ M random primer and 1.0 unit of *Taq* polymerase was incubated in an MJ-Research PTC100 thermal cycler. The random amplified polymorphic DNA (RAPD) program was initialized with 3 min 50 s at 92 °C followed by 40 cycles of 1 min at 92 °C, 2 min at 35 °C, and 2 min at 72 °C and was terminated with 5 min at 72 °C. The PCR products were separated by electrophoresis through a 1% agarose gel in TBE buffer, observed under UV light after staining with ethidium bromide, and photographed. Amplifications were performed twice and only reproducible bands were included in the data analysis. A control tube with no template DNA was run with each primer.

Identification of polymorphic primers

A preliminary assay using 18 10-mer primers from Kits A, B and G (Operon Technologies Inc., Alameda, Calif.) was carried out on all individuals. In all, 13 primers (Kit A: primers 4, 10, 15, 17, 18, and 20; Kit B: primers 1, 2, and 5; Kit G: primers 6, 10, 15, and 16) gave reproducible and informative marker patterns and were selected for the final study. These bands were considered independent phenotypic markers and were scored as being present or absent for each individual. The monomorphic bands in all males or females of each sample were scored, but not the polymorphic bands among males or females, which were in very low numbers (data not shown).

Data analysis

After the exclusion of markers that were monomorphic for the entire data set, presence/absence markers for each individual were used to compute Nei and Li's genetic distances matrix (Nei and Li

Table 1 Date, biological sources and geographical origin and experimental snail hosts of the four *Schistosoma* isolates

Species/strains	Biological source	Locality	Year of isolation	Snail used in the laboratory
<i>S. intercalatum</i>	Human	Edea, Cameroon	1990	<i>Bulinus forskalii</i> from Ivory Coast
<i>S. intercalatum</i>	Human	Kinshasa, Democratic Republic of Congo	1994	<i>B. globosus</i> from Zambia
<i>S. haematobium</i>	Human	Akakra, Ivory Coast	1991	<i>B. globosus</i> from Ivory Coast
<i>S. mattheei</i>	Naturally infected snails	Site near Lusaka, Zambia	1991	<i>B. globosus</i> from Zambia

1979) by the RAPDistance program (Armstrong et al. 2000). In addition, a principal component analysis (PCA) and one-way ANOVA/MANOVA were used to assess the potential for statistical differentiation between males and females within populations and between geographic isolates. These analyses were undertaken with the STATISTICA version 5.0 program (StatSoft Inc., Tulsa, Okla., USA). The input data for the PCA were represented by a matrix of the presence/absence of 30 weighted variables (redundant RAPD phenotypes) for the 32 schistosomes studied. The one-way ANOVA/MANOVA was applied on the scores of observations of the first three factors of the PCA.

Results

Polymorphic markers

The 13 primers selected yielded a total of 138 scoreable RAPD fragments (apart from the rare polymorphic bands among males or females of each strain). The 138 bands included in the final analysis ranged between 190 and 1,800 bp in length by comparison with a 100-bp ladder (Life Technology). Of the 138 bands, only 14 (10.1%) were shared by all the schistosomes included in the study (the RAPDistance program does not take these fragments into account in the analysis), and 124 bands were polymorphic among the 4 samples. Overall, 19 fragments considered to be sex markers of schistosome adults were detected (Table 2).

Species-specific RAPD patterns

Only 22% (20/91) of the total number of bands observed for *Schistosoma intercalatum* Zaire and *S. intercalatum* Cameroon were shared. In all, 8, 13, 11 and 10 specific fragments were detected in *S. intercalatum* Lower Guinea, *S. intercalatum* Zaire, *S. haematobium* and *S. mattheei*, respectively. These RAPD fragments are diagnostic between the 4 strains. Figure 1 is an example of the patterns obtained using the primers A17 and B5.

Genetic distances

The values computed for Nei and Li's genetic distances between all pairs of males and females of the four schistosome isolates are given in Table 2. The genetic

distances are almost 10-fold lower within strains (range 0.018–0.063) than between strains (range 0.525–0.679). The value recorded for the genetic distance between the *S. intercalatum* strains is of the same magnitude as that found between *S. haematobium* and *S. mattheei*.

PCA and ANOVA/MANOVA

The first three factors of the PCA taken together account for 80% of the overall inertia. Figure 2 shows the projection on the plane defined by the two major axes (factor 1 and factor 2), which represent 55.4% of the overall variability of the isolates. The first axis (29.5%) shows a great opposition between *S. haematobium* and *S. mattheei* and the second axis (25.9%) reveals a well-defined separation of the *S. intercalatum* Zaire and *S. intercalatum* Cameroon. The values of inertia recorded for these two principal axes are similar, which means a high level of differentiation between the four samples studied. The one-way ANOVA/MANOVA (Table 3) attests the absence of significant differentiation among males and females within strains or species ($P < 0.999$) but a highly significant differentiation between all pairs of schistosome isolates under investigation ($P < 0.001$).

Discussion

Using genetic characters to evaluate the taxonomic status of *Schistosoma intercalatum*, Wright et al. (1979) identified differences between the Zaire and Lower Guinea strains at three of seven enzyme systems examined. The authors considered the differences insufficient for a conclusion to be reached on the relative status of the two strains. They studied an insufficient number of enzyme loci, thereby preventing a genetic analysis using Nei's distance values or percentages of fixed genetic differences. Thus, it was not possible for them to evaluate exact genetic divergence between the strains. Therefore, the present study is the first to use genetic data to define further the systematic status of the Zaire and Lower Guinea strains of *S. intercalatum*.

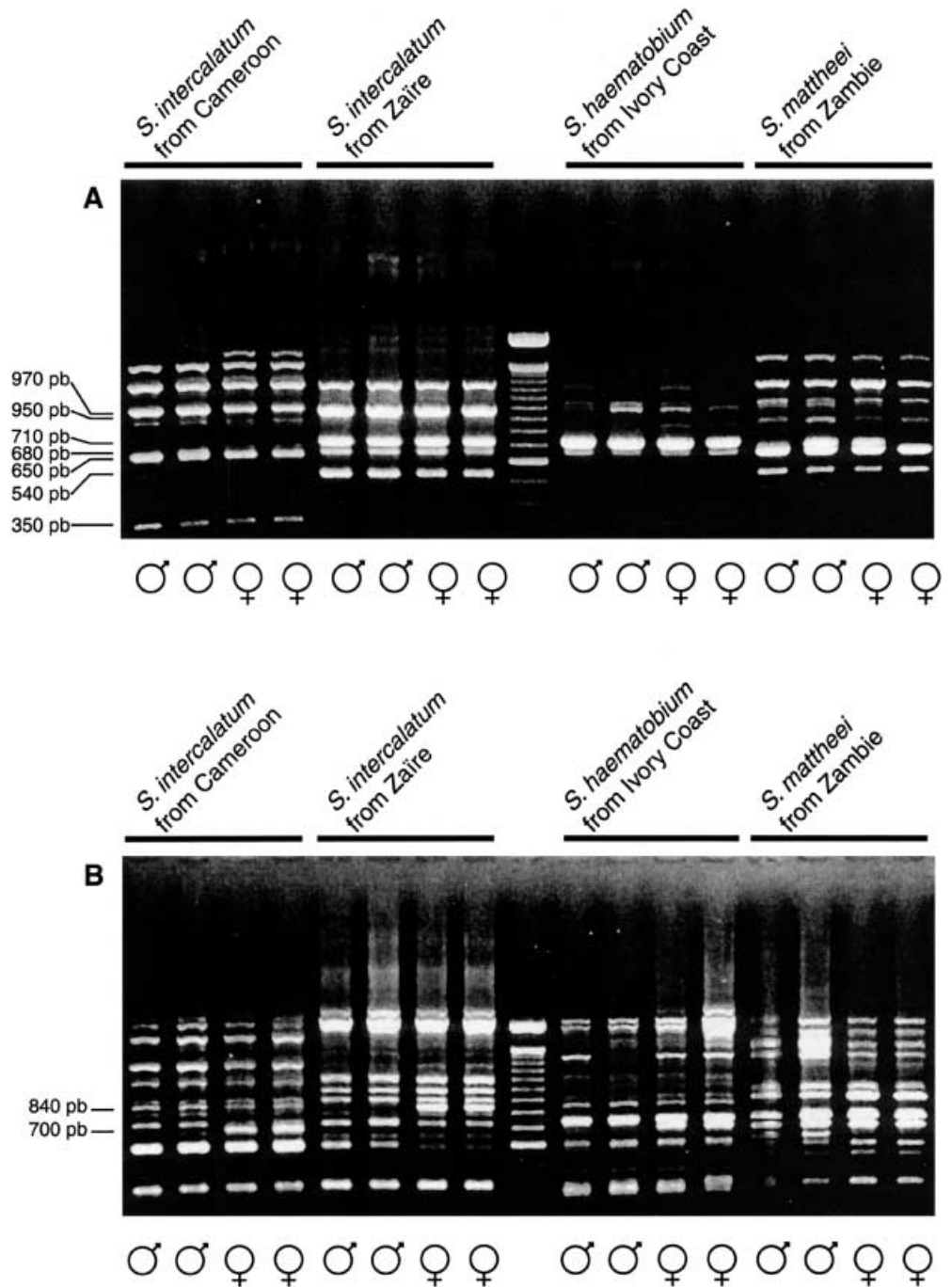
Results of RAPD profiling indicate that RAPD fingerprinting allows the distinguishment of the two species

Table 2 Number of shared RAPD markers (*below the diagonal*), sex markers (*given diagonally*) and Nei and Li's genetic distance matrix (*above the diagonal*) among *S. intercalatum* Cameroon males

	SiCm	SiCf	SiZm	SiZf	Shm	Shf	Smm	Smf
SiCm	(0)	0.018	0.600	0.604	0.558	0.571	0.621	0.583
SiCf	56	(2)	0.589	0.593	0.565	0.561	0.593	0.556
SiZm	22	23	(3)	0.063	0.664	0.679	0.655	0.652
SiZf	15	23	51	(4)	0.632	0.646	0.675	0.672
Shm	24	25	18	20	(2)	0.026	0.546	0.559
Shf	24	25	17	19	55	(1)	0.525	0.556
Smm	30	25	19	18	26	27	(4)	0.058
Smf	25	27	19	18	25	25	57	(3)

(*SiCm*) and females (*SiCf*), *S. intercalatum* Zaire males (*SiZm*) and females (*SiZf*), *S. haematobium* males (*Shm*) and females (*Shf*) and *S. mattheei* males (*Smm*) and females (*Smf*)

Fig. 1A, B RAPD profiles visualized by the agarose/ethidium bromide method for the 4 isolates of *Schistosoma* using the primers **A** OPA17 and **B** OPB05. Polymorphic PCR bands distinguishing each strain of *S. intercalatum* (**A**) and sex-specific bands among each strain of *S. intercalatum* (**B**) are indicated



and two strains. Each of the 13 primers gave a specific RAPD profile for each taxon as characterized by the presence or absence of a variable number of bands according to the primer. When the RAPD pattern obtained with primer A17 is considered (Fig. 1A), the 540-, 680-, 710- and 950-bp fragments are present in *S. intercalatum* Zaire and absent from the Cameroon strain. Conversely, the 350-, 650- and 970-bp fragments are specific to the Cameroon strain. The only reproducible differences between individuals from the same strain are associated with sexual polymorphism (with the use of primer B5, for example, the 840-bp fragment is

specific to Zaire *S. intercalatum* females and the 700-bp fragment is specific to the Cameroon *S. intercalatum* females; Fig. 1B).

If Nei and Li's distance values are taken into account, it can be seen that the mean value (0.596) recorded for the distance between the two strains of *S. intercalatum* (the mean of the four values corresponding to the four male and female combinations) is of the same magnitude as the mean value noted between *S. haematobium* and *S. mattheei*. The mean values recorded for Nei and Li's distance between *S. haematobium* and *S. mattheei* and between *S. haematobium* and *S. intercalatum* Cameroon

Fig. 2 PCA plot (first versus second) on RAPD multimarkers using individual profiles involving 32 observations \times 30 weighted variables (*SiCm* *S. intercalatum* Cameroon males, *SiCf* *S. intercalatum* Cameroon females, *SiZm* *S. intercalatum* Zaire males, *SiZf* *S. intercalatum* Zaire females, *Shm* *S. haematobium* males, *Shf* *S. haematobium* females, *Smm* *S. mattheei* males, *Smf* *S. haematobium* females)

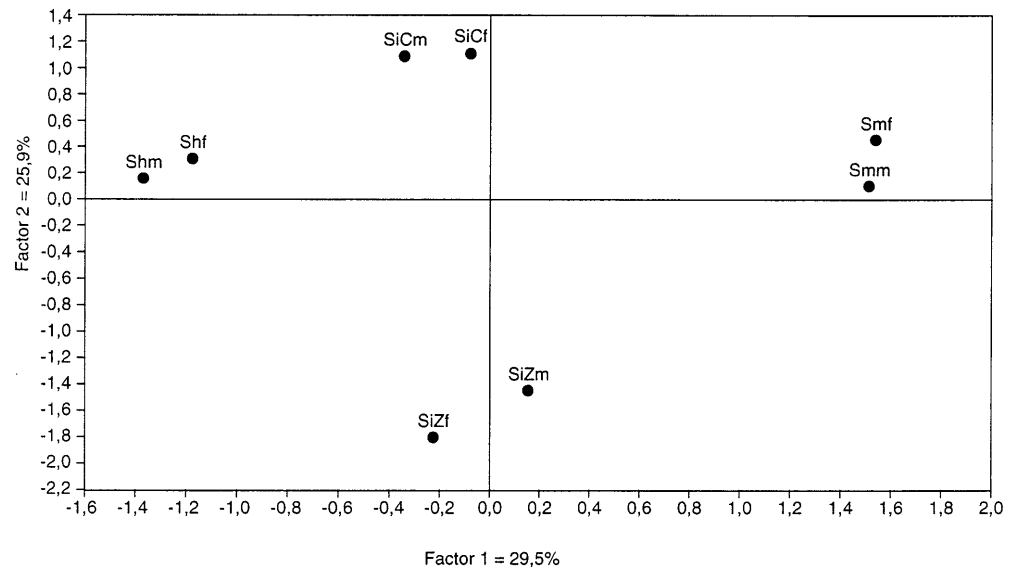


Table 3 Summary of all-effects one-way ANOVA/MANOVA (sex versus scores of observations and strains versus scores of observations)

Source of variation	Wilks' lambda	df 1	df 2	P-level
Sex/scores of observations on factors from 1 to 3	0.999	3	28	<0.998
Strains/scores of observations on factors from 1 to 3	<0.001	9	63	<0.001

are 0.546 and 0.563, respectively. The greatest mean genetic distance (0.663) was observed between *S. mattheei* and the *S. intercalatum* Zaire. The degree of divergence between the two populations of *S. intercalatum* appears to be similar to that observed between two “good species”, that is, *S. haematobium* and *S. mattheei*, suggesting that the four taxa separated at about the same time. Therefore, it would be relevant to question whether the value (0.596) noted for the genetic distance corresponds to a “usual” genetic distance between two allopatric geographic populations or is indicative of the existence of two species. Analysing the data on intra-specific variation, Barral et al. (1993) reported inter-population distances of *S. mansoni* between Brazil, Guadeloupe and Venezuela ranging from 0.05 to 0.06. Kaukas et al. (1994) used an RAPD approach and found a distance value of 0.046 between two populations of *S. mansoni* from Brazil and Liberia and values ranging from 0.155 to 0.447 between populations of *S. haematobium* from Nigeria, Zambia and Guinea Bissau. In all cases the intra-specific variation is lower than the inter-specific variation.

PCA analysis also clearly opposes the two strains of *S. intercalatum*, the differentiation noted between the two strains being as high as that seen between two “good species”. It is apparent from ANOVA/MANOVA analysis that the intra-population sex polymorphism does not carry weight with statistical differentiation.

From the three approaches – RAPD profiling, Nei and Li’ s distance values and PCA analysis – it appears that RAPD markers differentiate the two strains of *S. intercalatum* unambiguously. The similarity in Nei and Li’ s distance values observed between the two strains of *S. intercalatum* and between “good species” within the *S. haematobium* group suggests that the divergence of the two strains is ancient and certainly occurred during the diversification of the schistosomes with terminal-spined eggs (Després et al. 1992). Molecular-biology data associated with other characters (snail host specificity, pre-patent period in the snail and impaired viability of hybrid offspring) support the hypothesis of the existence of two distinct species. It must be emphasized that in schistosomes, undeniable difficulties are involved in the use of the biological species concept (inter-breeding concept) as a criterion for precise identification of the species because there is a considerable body of literature on natural and experimental hybridization between so-called “good species” of *Schistosoma* (for a review, see Jourdane and Southgate 1992). It seems that the best definition of species in schistosomes is the “genotypic cluster definition”, which considers the species as distinguishable groups of individuals (cluster) that can hybridize when in contact but remain distinct due not just to reproductive traits but also to stabilizing ecological adaptations that select disruptively against intermediates or hybrids, keeping the clusters apart (Mallet 1995).

Additional molecular-biology studies involving nuclear and mitochondrial informative markers are in progress to confirm the extent of the genetic divergence before a conclusion can finally be reached on the true taxonomic status of *S. intercalatum* Zaire and *S. intercalatum* Lower Guinea.

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