FOLIA PARASITOLOGICA 46: 10-14, 1999

A phylogenetic analysis by multilocus enzyme electrophoresis and multiprimer random amplified polymorphic DNA fingerprinting of the *Leishmania* genome project Friedlin reference strain

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Key words: *Leishmania major*, MLEE, RAPD, genetic characterization, genetic diversity

Abstract. We have assessed the phylogenetic status of the *Leishmania* genome project Friedlin reference strain by MLEE and multiprimer RAPD including a set of 9 stocks representative of the main *Leishmania* species and of the whole genetic diversity of the *Leishmania* genus. To our knowledge, the detailed genetic characterization of the Friedlin strain has never been published before. As previously recorded (Tibayrenc et al. 1993), MLEE and RAPD data gave congruent phylogenetic results. The Friedlin reference strain was definitely attributed to *Leishmania* (*Leishmania*) *major* Yakimoff et Schokhor, 1914. Five specific RAPD patterns made it possible to distinguish between the Friedlin strain and the 2 other *L.* (*L.*) *major* stocks included in the study. Various specific MLEE and RAPD characters permitted to distinguish between the *Leishmania* species included in the study. All these characters are usable to detect accidental laboratory mix-ups involving the Friedlin reference strain. In confirmation with previous studies involving a more limited set of genetic markers, the general genetic diversity of the *Leishmania* genus proved to be considerable. It must be made clear that only one strain cannot be considered as representative of the whole genetic variability of the genus *Leishmania*. In the future, it is therefore advisable to complement the results obtained in the framework of the *Leishmania* genome project with data from other strains that should be selected on a criterion of important genetic differences with the Friedlin strain.

The genus *Leishmania* Ross, 1903 shows considerable genetic polymorphism, as amply demonstrated by the use of various genetic markers, including MLEE (Chance and Gardener 1975), RFLP analysis of kinetoplast DNA variability (Jackson et al. 1984), pulse field gel electrophoresis (Holmes Giannini et al. 1986) and RAPD (Tibayrenc et al. 1993).

The Friedlin reference strain has been selected to be circulated among all teams involved in the *Leishmania* genome project. Nevertheless, until now, the detailed genetic characterization of this reference strain has never been published. Considering both the extreme intraspecific phylogenetic diversity of the *Leishmania* genus and the high risk of experimental stock mix-up, it is important to have an accurate idea of the phylogenetic situation of this reference strain. With this intention, we performed an extensive analysis of the Friedlin strain by both MLEE (14 genetic loci) and RAPD (29 primers).

MATERIALS AND METHODS

Selection and culturing of the stocks. In order to reliably set the phylogenetic status of the Friedlin strain, 9 stocks representative of the whole genetic diversity and of the main species of the *Leishmania* genus have been included in this study. Table 1 gives the name of the stocks, as well as their origin, date of isolation and taxonomic identification. The stocks were bulk-cultured in disposable plastic flasks in RPMI 1640 culture medium containing 10% fetal calf serum v/v and 50 µg/ml gentamycin.

Preparation of the samples. Stocks were harvested by centrifugation (2,800 *g* for 20 min at 4°C) and washed twice in phosphate-buffered saline (PBS, pH 7.3: Na_2HPO_4 (0.01 M), NaH_2PO_4 (0.01 M), NaCl (0.15 M)). Cells were lysed in an equal volume in a hypotonic enzyme stabilizer (EDTA, Dithiotreitol, ε−Aminocaproic acid, aa 2 mM) on an ice bed for 20 min. The lysates were again centrifuged in eppendforf 1.5 ml tubes (13,000 *g* for 10 min at 4°C). The water soluble fraction was stored at -70°C until use for MLEE analysis, whereas the pellet of lysed cells was used for DNA extraction, according to the following protocol. Pellets were resuspended in 400 µl Tris HCl pH 8.0, NaCl 400 mM, EDTA.Na_2 10 mM. SDS was then added to a final concentration of 1%, and the tubes were incubated for 2 h at 37°C in the presence of 100 mg/ml Rnase A and then overnight at 55°C with 200 µg/ml proteinase K. After two phenol extractions, two chloroform: isoamylic alcohol (24 : 1) extractions and ethanol precipitation, the DNA was resuspended in sterile bidistilled water. DNA concentration was estimated by spectrophotometry at 260 nm.

MLEE protocol. MLEE analysis was performed on cellulose acetate plates (Helena Laboratories) according to Ben Abderrazak et al. (1993), with slight modifications. The 13 following enzyme systems were used: Aconitase (ACON, E.C.4.2.1.3.), Alanine aminotransferase (ALAT, E.C.2.6.1.2.),

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Glucose-6-phosphate dehydrogenase (G6PD, E.C.1.1.1.49.), Glucose-phosphate isomerase (GPI, E.C.5.3.1.9.), Glutamate oxaloacetate transaminase (GOT, E.C.2.6.1.1.), Isocitrate dehydrogenase (IDH, E.C.1.1.1.42.), Malate dehydrogenase NAD^+ (MDH, E.C.1.1.1.37.), Malate dehydrogenase ($NADP^+$) or Malic enzyme (ME, E.C.1.1.1.40.), Mannose-phosphate isomerase (MPI, E.C.5.3.1.8.), Nucleoside hydrolase (deoxyinosine) (NHD, E.C.2.4.2.*), Nucleoside hydrolase (inosine) (NHI, E.C.2.4.2.*), 6-Phosphogluconate dehydrogenase (6PGD, E.C.1.1.1.44.), and Phosphoglucomutase (PGM, E.C.2.7.5.1.). These 13 enzyme systems permit the analysis of 14 different genetic loci, since NHI has 2 loci (*Nhi* 1 and *Nhi* 2, *Nhi* 1 being the fastest migrating locus on gels).

RAPD protocol. 120 different decameric primers were screened, corresponding to the A, B, F, N, R and U kits from Operon Technologies (Alameda, California). Amplification was performed according to Williams et al. (1990) with slight modifications. Briefly, the amplification reactions were performed in a final volume of 60 µl containing 0.9 units *Taq* Polymerase (Boehringer, Mannheim, Germany), 100 µM each dNTP, 200 nM primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 20 ng template DNA. Fortyfive cycles (denaturation: 1 min at 94°C; annealing: 1 min at 36°C; elongation: 2 min at 72°C) were followed by a final elongation step of 7 min at 72°C. Amplification was performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, Massa-chusetts). RAPD products were analyzed on 1.6% agarose gels in TAE buffer (Tris-acetate 0.04 M; EDTA 0.001 M) stained with ethidium bromide and visualized by UV. Electrophoresis is carried out with a voltage of 3.3 V/cm for 4 hours.

Data analysis. MLEE and RAPD genetic diversity was computed with the *genetics* software, designed in our laboratory and operated on SUN stations (S. Noël). For both MLEE and RAPD data, the Jaccard's distance (Jaccard 1908) was used to estimate the genetic differences among the stocks. Each MLEE and RAPD gel band was coded with a number, starting with 1 for the fastest band in the case of MLEE, for the slowest band in the case of RAPD. The distance was estimated after the following formula:

D = 1 - (a / (a+ b + c))

a = number of bands that are common to the two compared genotypes

b = number of bands present in the 1st genotype and absent in the 2nd

c = number of bands absent in the 1st genotype and present in the 2nd

The UPGMA method (Unweighted Pair-Group Method with Arithmetic Averages) (Sneath and Sokal 1973) was used to cluster the genotypes together according to their Jaccard's distances.

Agreement between MLEE and RAPD genetic distances was tested by a correlation analysis based on a nonparametric Mantel test (Mantel 1967). Briefly, this test relies on a Monte Carlo simulation with 10^4 iterations, which randomly permutes the different cells of one of the distance matrices. Differently from the classical correlation test, this randomization procedure does not need any assumptions about the number of degrees of freedom.

RESULTS

MLEE analysis. The 14 enzyme loci yielded a total of 68 scoreable isoenzyme characters. As expected, this set of stocks including the main species of the genus *Leishmania* exhibited considerable MLEE variability. As a matter of fact, as seen on the UPGMA dendrogram depicting the phylogenetic relationships among the stocks (Fig. 1), the upper clustering reached the value of maximum genetic distance, that is to say 1 in the case of the Jaccard's distance. This means that in the sample under study, no isoenzyme locus was monomorphic, and some stocks had zero bands in common. The present results are therefore in agreement with the well-established notion of considerable isoenzyme diversity in the genus *Leishmania* (see, for example, in Lanotte et al. 1986).

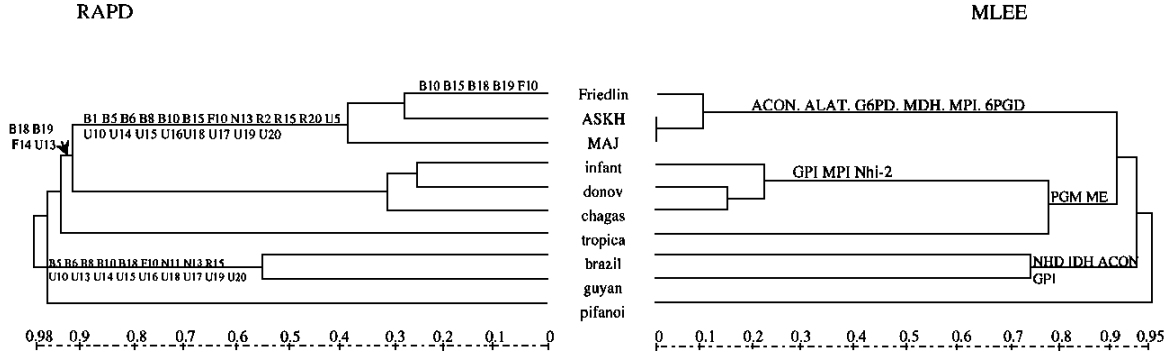

Fig. 1. Two UPGMA dendrograms (8) obtained from MLEE (right) and RAPD (left) data. The scales indicate the genetic distances that separate the genotypes studied. On the MLEE dendrogram, the loci mentioned along some branches correspond to isoenzyme genotypes that are synapomorphic of the corresponding clades. On the RAPD dendrogram, the primers mentioned yield either overall RAPD profiles or individual RAPD fragments that are synapomorphic of the corresponding clade or individual stock (in the case of the Friedlin strain).

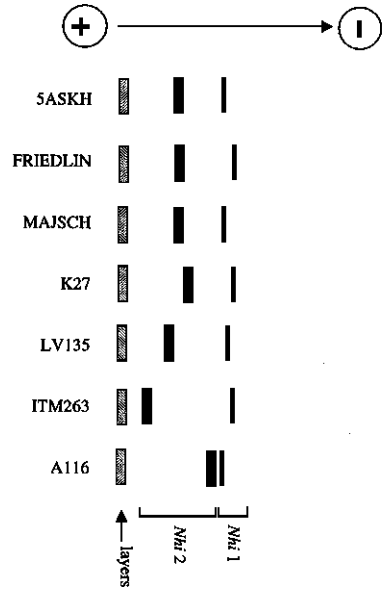

Fig. 2. Diagrammatic representation of the variability recorded for NHI enzyme system. The Friedlin strain exhibits a *Nhi* 1 pattern different from the two other *Leishmania (Leishmania) major* strains (5ASKH and MAJSCH).

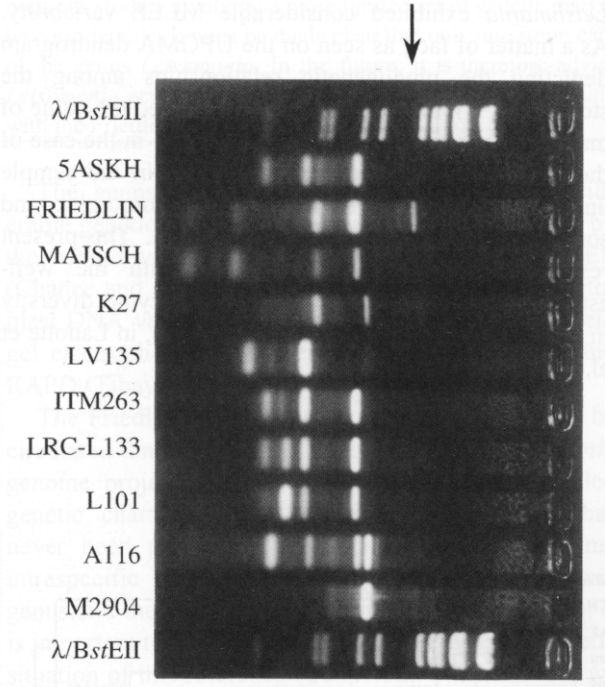

Fig. 3. RAPD patterns obtained with the primer OPB-18 (see Table 2). The Friedlin strain exhibits a specific fragment (arrow) that permits to distinguish it from the two other *Leishmania (Leishmania) major* stocks (5ASKH and MAJSCH).

Only two stocks had exactly the same isoenzyme profile ("zymodeme") in the present sample**.** They correspond to the two *L.* (*L.*) *major* reference stocks. The Friedlin stock unambiguously clustered with these two *L.* (*L.*) *major* reference stocks (Fig. 1).

For identification purposes, Friedlin can be distinguished from the two other *L.* (*L.*) *major* stocks by one isoenzyme locus, *Nhi* 1 (Fig. 2). Moreover, the cluster *L.* (*L.*) *major* can be distinguished from any other *Leishmania* stock by the 5 following enzyme systems (Fig. 1): ACON, ALAT, G6PD, MDH, MPI and 6PGD.

The phylogenetic picture obtained with MLEE (Fig. 1) is in agreement with the known taxonomy of the genus *Leishmania*. Nevertheless, the two subgenera *Leishmania* and *Viannia* (the latter represented by the two species *L.* (*Viannia*) *braziliensis* and *L.* (*V.*) *guyanensis* only) are not clearly separated on the dendrogram (see Discussion).

RAPD analysis. Out of 120 primers screened, the 29 primers that gave the more readable profiles were selected. This yielded a total of 328 scoreable RAPD fragments. All stocks proved to have a different RAPD genotype. Moreover, all primers exhibited polymorphism. It was impossible to record any RAPD fragment common to all stocks under survey. This means that, like for MLEE, the most distantly related stocks shared no RAPD fragment (genetic distance = 1). The dendrogram generated from RAPD data was roughly in agreement with the MLEE dendrogram; also some clustering discrepancies at the upper levels of phylogenetic divergence are apparent (Fig. 1). The overall agreement between MLEE and RAPD dendrograms was statistically verified by the Mantel test ($r = 0.95$, $p < 10^{-4}$).

For identification purposes, Friedlin can be distinguished from the two other *L.* (*L.*) *major* by 5 different primers (Figs. 1, 3; Table 2). Moreover, the cluster corresponding to Friedlin and the two *L.* (*L.*) *major* reference stocks can be distinguished from any other stock by various RAPD characters (Fig. 1).

DISCUSSION

Parity between MLEE and RAPD results. As recorded in previous studies (Tibayrenc et al. 1993, Bañuls et al. 1997), phylogenies built from MLEE and RAPD show a strong parity in the *Leishmania* stocks surveyed here, as confirmed by the Mantel test of correlation. Nevertheless, at upper levels of clustering, there are some discrepancies between MLEE and RAPD dendrograms (Fig. 1). This is probably explainable by the fact that hierarchical organization of the cluster becomes less reliable at these levels of phylogenetic divergence, close to, or equal to the maximum theoretically possible. At the lower levels of phylogenetic clusterings, corresponding to either species or com-plexes of species, parity between MLEE and RAPD dendrograms is a clear indication that the phylogenies obtained by the two methods are robust. The present analysis did not permit to separate clearly the two subgenera *Leishmania* and *Viannia*. This is due to

Table 1. Code, origin and taxonomic identification of the *Leishmania* stocks under study.

WHO code	Origin	Species	Complex	Subgenus
MHOM/SU/73/5ASKH	USSR	*L. major*	*L. major*	*Leishmania*
MHOM/IL/81/FRIEDLIN	Israel	*L. major*	*L. major*	*Leishmania*
MHOM/SA/--/MAJ SCH	Saudi Arabia	*L. major*	*L. major*	*Leishmania*
MHOM/SU/74/K-27	USSR	*L. tropica*	*L. tropica*	*Leishmania*
MHOM/VE/57/LV135	Venezuela	*L. pifanoi*	*L. mexicana*	*Leishmania*
MHOM/MA(BE)/67/ITM263	Morocco	*L. infantum*	*L. donovani*	*Leishmania*
MHOM/ET/67/LRC-L133	Ethiopia	*L. donovani*	*L. donovani*	*Leishmania*
MHOM/BR/72/L101	Brazil	*L. chagasi*	*L. donovani*	*Leishmania*
MCHO/FG/83/CAY A116	French Guyana	*L. guyanensis*	*L. guyanensis*	*Viannia*
MHOM/BR/75/M2904	Brazil	*L. braziliensis*	*L. braziliensis*	*Viannia*

Table 2. Sequences of primers allowing to distinguish *Leishmania (Leishmania) majo*r Friedlin strain from the two other *L. (L.) major* strains (5ASKH and MAJSCH).

Primers names	Sequences
OPB-10	CTGCTGGGAC
OPB-15	GGAGGGTGTT
OPB-18	CCACAGCAGT
OPB-19	ACCCCCGAAG
OPF-10	GGAAGCTTGG

the fact that both MLEE and RAPD have a too fast molecular clock to reliably explore this higher level of phylogenetic divergence which is beyond their level of resolution.

Taxonomic attribution and phylogenetic position of the Friedlin strain. From a phylogenetic point of view, our study permits to firmly confirm the attribution of the Friedlin strain to the species *Leishmania* (*L.*) *major*. Its clustering to the two *L.* (*L.*) *major* reference stocks is very tight, both for MLEE and RAPD dendrograms (Fig. 1). Moreover, there are clear-cut differences between this *L.* (*L.*) *major* group on one hand, and any other stock on the other hand (Fig. 1). When phylogenetic distances are considered, the evolutionary gap between the *L.* (*L.*) *major* group, including the Friedlin strain, is indeed considerable. The average Jaccard's distance between the *L.* (*L.*) *major* group on one hand, and the other *Leishmania* species from the Old World (*L.* (*L.*) *tropica* and the complex *L. donovani*/ *infantum*/ *chagasi*) is on the other hand, 0.927.

Practical identification of the Friedlin strain. Due to the clear phylogenetic separation between *L.* (*L.*) *major* on one hand and the other *Leishmania* species on the other hand, many MLEE and RAPD characters are available to verify the species attribution of the Friedlin strain in the course of experiments. In the present set of stocks, the Friedlin strain was distinguishable from the other *L.* (*L.*) *major* stocks by one isoenzyme character and 5 RAPD primers (Figs. 1, 3; Table 2). Nevertheless, it will be necessary to check the specificity of these markers on a broader set of *L.* (*L.*) *major* stocks.

Practical implication of phylogenetic diversity in the *Leishmania* genome project. This study, relying on a broad range of diversified genetic markers, has amply confirmed that the phylogenetic diversity of the genus *Leishmania* is considerable, since several stocks of the present study share no identical character, either MLEE or RAPD. This is true also at a supposedly lower level of phylogenetic divergence, within the subgenus *Leishmania*, which includes the Friedlin strain. One has therefore to keep in mind that the Friedlin strain is by no means representative of the whole phylogenetic diversity of the genus *Leishmania*. It will be therefore advisable to complete the studies presently involved in the *Leishmania* genome project with additional work dealing with one or more other *Leishmania* species.

Acknowledgements. The authors thank Dr. Al Ivens, Department of Biochemistry, Imperial College, Exhibition Road, London SW7 2AZ, UK, for providing the Friedlin strain. This work was performed thanks to a concerted action ACCSV7 from the French Ministry of research, a EEC Avicenne grant No. AVI*CT92-0013°, by a "groupement de recherche" CNRS/French Army grant, and by FGWO (National Loterij, grants 346/1990 and 9.0024.90).

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Received 16 March 1998

Accepted 30 July 1998