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Multilocus microsatellite typing (MLMT) reveals genetic homogeneity of *Leishmania donovani* strains in the Indian subcontinent

Mohammad Zahangir Alam [a,b,*], Katrin Kuhls [a], Carola Schweynoch [a], Shyam Sundar [c], Suman Rijal [d], Abul Khair M. Shamsuzzaman [e], Balaraju Venkata Subba Raju [f], Poonam Salotra [f], Jean-Claude Dujardin [g], Gabriele Schönian [a]

[a] *Institut für Mikrobiologie und Hygiene, Charité Universitätsmedizin Berlin, Dorotheenstr. 96, D-10117 Berlin, Germany*
[b] *Department of Parasitology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh*
[c] *Kala-azar Medical Research Centre, Banaras Hindu University, Varanasi, India*
[d] *B.P. Koirala Institute of Health Sciences, Dharan, Nepal*
[e] *Department of Microbiology, Mymensingh Medical College, Mymensingh, Bangladesh*
[f] *Institute of Pathology, Indian Council of Medical Research, Safdarjang Hospital Campus, Post Box No. 4909, New Delhi 110029, India*
[g] *Department of Parasitology, Unit of Molecular Parasitology, Institute of Tropical Medicine, Antwerp B-2000, Belgium*

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ABSTRACT

In this population genetic study of *Leishmania donovani* parasites in the Indian subcontinent, 132 isolates obtained from patients in Bangladesh, India, Nepal and Sri Lanka suffering from Kala-azar (100), post-Kala-azar dermal leishmaniasis (PKDL) (25) and cutaneous leishmaniasis (CL) (2), and from 5 patients whose clinical patterns were not defined, were analysed by using 15 hyper-variable microsatellite loci. Multilocus microsatellite typing (MLMT) data were analysed by using a Bayesian model-based clustering algorithm and constructing phylogenic tree based on genetic distances. In total, 125 strains from Bangladesh, Bihar (India) and Nepal formed a very homogeneous population regardless of geographical origin, clinical manifestation, and whether they presented in vitro or in vivo susceptibility to antimonial drugs. Identical multilocus microsatellite profiles were found for 108 strains, other strains differed in only one marker. Considerably different microsatellite profiles were identified for three Indian strains most closely related to *L. donovani* from Kenya, and for four strains from Indian and Sri Lankan CL cases. The circulation of a single homogeneous population of *L. donovani* in Bihar (India), Bangladesh and Nepal is, most probably, related to the epidemic spread of visceral leishmaniasis in this area.

1. Introduction

Since the 1970s, South Asia has experienced a major resurgence of anthroponotic visceral leishmaniasis (VL) (Rahman and Islam, 1983), also known as Kala-azar ('Black fever' in Hindi). The disease seemed to be eliminated in the 1960s due to wide-spread DDT spraying during a national malaria control campaign. More than 100,000 cases of VL occur in India every year where the State of Bihar accounts for more than 90% of these cases. Bordering Bangladesh is contributing 40,000–45,000 cases yearly, with the total population at risk being around 20 million (Bern and Chowdhury, 2006). The Nepalese low land regions adjoining the border to Bihar report almost 1850 cases annually (Bern et al., 2000). India, Bangladesh and Nepal have long common borders in areas endemic for VL with frequent migrations of people. The aetiological agent, *Leishmania donovani*, clinical manifestation of the disease, and anthroponotic transmission cycle are the same in these countries. Treatment failure is well documented for Bihar, India, with more than 60% of unresponsiveness to antimonial treatment (Sundar et al., 2000). For Nepalese districts bordering Bihar, an unresponsiveness rate of up to 24% has been reported (Rijal et al., 2003). So far, no data were published about treatment failure from Bangladesh, however recent results showed that 12.8% of the studied strains showed a low response to antimonials (own unpublished data). In India and Nepal, antimony-resistant parasites have been reported (Lira et al., 1999; Rijal et al., 2007), however the reasons for the emergence of drug resistance and its geographic patterns in the Indian subcontinent are not at all understood.

* Corresponding author at: Institut für Mikrobiologie und Hygiene, Charité Universitätsmedizin Berlin, Dorotheenstr. 96, D-10117 Berlin, Germany.
Tel.: +49 30 450524058; fax: +49 30 450524902.
E-mail address: mzalam12@yahoo.com (M.Z. Alam).

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Foci of cutaneous leishmaniasis (CL) due to *L. donovani* have been recently emerged in Sri Lanka (Karunaweera et al., 2003), from which >400 cases were reported since 2001 and in the western Himalayas in northern India, in the state Himachal Pradesh, where cases were caused by both *L. donovani* and *Leishmania tropica* (Sharma et al., 2005). Post-Kala-azar dermal leishmaniasis (PKDL) is a dermal complication, following recovery from a VL infection (Zijlstra et al., 2003). It is not known whether the parasite in PKDL lesions is the residual parasite after VL infection or is introduced upon re-infection. The role of PKDL patients as putative reservoir hosts in the transmission of the disease remains unclear.

It is important to know whether specific genetic traits of the *L. donovani* strains are related to drug resistance or to different clinical manifestations of the disease, VL, PKDL or CL. Highly variable markers may significantly contribute to this exploration as they enable identification of different genetic populations of these parasites. Currently, multilocus enzyme electrophoresis (MLEE) is considered the 'gold standard' for identification and classification of species and strains in *Leishmania* (Rioux et al., 1990). Because this method requires cultured parasites, and is very laborious and time-consuming only very few strains from the study area have been typed so far. Of the genotyping methods that have been developed to overcome the disadvantages of MLEE, kDNA PCR-RFLP and multilocus microsatellite typing (MLMT) were most powerful for discriminating closely related strains of the *L. donovani* complex (Botilde et al., 2006).

Microsatellites are repeated motifs of 1–6 nucleotides found in all eukaryotic genomes that mutate much faster than the bulk of DNA. Length polymorphisms in these sequences result from the gain and loss of single repeat units and can be easily screened. The results are reproducible and exchangeable between laboratories. Microsatellite sequences are, however prone to homoplasy and it is, therefore recommended to use a panel of 10–20 unlinked microsatellite markers in all studies. Recently developed panels of highly polymorphic and co-dominant microsatellite markers have proven useful for strain typing and population genetics studies in the *L. donovani* complex (Kuhls et al., 2007; Ochsenreither et al., 2006). Previously, 21 strains of *L. donovani*, mostly from Bihar, India, were shown to have identical microsatellite profiles (Kuhls et al., 2007). The present study used a more comprehensive sampling to cover as much as possible VL foci on the Indian subcontinent. Furthermore, it aimed to investigate whether specific genetic traits are related to different clinical manifestations of *L. donovani* infections, such as VL, PKDL and CL, or to in vitro and in vivo responses to antimonial treatment. Finally, it was tested whether the MLMT approach can be applied directly to clinical materials without prior culturing of parasites. For this, 15 microsatellite markers, polymorphic for the *L. donovani* complex (Kuhls et al., 2007) have been used for analysing 132 isolates of *L. donovani* isolated from patients in different VL regions of Bangladesh, India, Nepal and Sri Lanka.

2. Materials and methods

2.1. Parasite culture and DNA extraction

Sources, clinical manifestation, geographic origin of 132 strains of *L. donovani* analysed in this study are listed in Table 1. Ninety-two of these strains originated from Bangladesh (21), India (49), Nepal (20), and Sri Lanka (2) were typed by using a panel of 15 microsatellite markers. Additional 40 strains of which 20 were isolated from VL and 20 from PKDL patients in areas of low, moderate and high VL endemicity in India were analysed using 11 microsatellite markers. These 40 strains were all tested in vitro for their susceptibility against antimony (Singh et al., 2006). The Nepalese strains were isolated from patients responding and non-responding to standard antimonial therapy.

Previously obtained MLMT profiles of 15 East African (Sudan, Ethiopia, Kenya) *L. donovani* strains (Kuhls et al., 2007) were included for comparison. Most of the strains were obtained as promastigote cultures using conditions as described previously (Schonian et al., 1996). We also used nine bone marrow samples from Bangladesh spotted on filter paper or glass slides. DNA was extracted from promastigotes and clinical materials on filter paper or glass slide by the phenol–chloroform method described previously (el Tai et al., 2000; Schonian et al., 1996). ITS1-PCR was performed in order to confirm that samples on filter paper or slides were *Leishmania* positive (Schonian et al., 2003).

2.2. PCR amplification of microsatellite markers

Fifteen microsatellite markers (Li22-35, Li23-41, Li41-56, Li45-24, Li46-67, Li71-5, Li71-7, Li71-33, Lm2TG, Lm4TA, TubCA, CS19, CS20, LIST7031, and LIST7039) were used in this study as previously described (Kuhls et al., 2007; Ochsenreither et al., 2006). The microsatellite-containing sequences were amplified in a volume of 25 μl, using 1.5 μl of DNA isolated from clinical material (slide or filter paper) or 15 ng of DNA obtained from parasite cultures. PCR has been performed applying conditions as described elsewhere (Kuhls et al., 2007).

2.3. Electrophoretic analysis of microsatellite variation

Screening of microsatellite variation was performed by either using MetaPhor agarose gels or capillary electrophoresis (CE) by the CEQ 8000 automated genetic analysis system of Beckman Coulter, USA as described previously (Kuhls et al., 2007;

Table 1
The strains of *Leishmania donovani* analysed in this study.

Lab. code	WHO code	Origin	Region/district	Source	Zymodeme	Clinical pattern[a]	Population assignment[b]	MLMT genotype
DON-1	MHOM/IN/1980/DD8	India	nd	LRC	MON-2, LON-41	VL	1	1b
DON-39	MHOM/IN/0000/DEVI	India	Bihar	LSHTM	MON-2	VL	1	1a
DON-40	MHOM/IN/1996/THAK35	India	Bihar	LSHTM	MON-2	nd	1	1a
DON-45	MHOM/IN/2001/BHU20140	India	Bihar	BHU	nd	VL	1	1g
DON-28	MHOM/IN/1993/B12302	India	nd	LRC	nd	nd	1	1a
DON-51	MHOM/IN/2002/BHU1	India	Bihar	BHU	nd	VL	1	1a
DON-52	MHOM/IN/2002/BHU2	India	Bihar	BHU	nd	VL	1	1a
DON-53	MHOM/IN/2002/BHU3	India	Bihar	BHU	nd	VL	1	1a/1c
DON-54	MHOM/IN/2002/BHU4	India	Bihar	BHU	nd	VL	1	1a
DON-55	MHOM/IN/2002/BHU5	India	Bihar	BHU	nd	VL	1	1a
DON-56	MHOM/IN/2002/BHU6	India	Bihar	BHU	nd	VL	1	1h
DON-57	MHOM/IN/2002/BHU7	India	Bihar	BHU	nd	VL	1	1a/1b
DON-58	MHOM/IN/2002/BHU8	India	Bihar	BHU	nd	VL	1	1a

Table 1 (*Continued*)

Lab. code	WHO code	Origin	Region/district	Source	Zymodeme	Clinical pattern[a]	Population assignment[b]	MLMT genotype
DON-59	MHOM/IN/2002/BHU9	India	Bihar	BHU	nd	VL	1	1a/1b
DON-60	MHOM/IN/2002/BHU11	India	Bihar	BHU	nd	VL	1	1a
DON-61	MHOM/IN/2002/BHU12	India	Bihar	BHU	nd	VL	1	1a
DON-62	MHOM/IN/2002/BHU13	India	Bihar	BHU	nd	VL	1	1a
DON-63	MHOM/IN/2002/BHU15	India	Bihar	BHU	nd	VL	1	1a
DON-64	MHOM/IN/2002/BHU17	India	Bihar	BHU	nd	VL	1	1a
DON-65	MHOM/IN/2002/BHU20	India	Bihar	BHU	nd	VL	1	1a/1b
DON-69	MHOM/IN/2003/BHU50	India	Bihar	BHU	nd	VL	1	1a
DON-70	MHOM/IN/2003/BHU53	India	Bihar	BHU	nd	VL	1	1a
DON-71	MHOM/IN/2003/BHU55	India	Bihar	BHU	nd	VL	1	1a
DON-72	MHOM/IN/2003/BHU37	India	Bihar	BHU	nd	VL	1	1a
DON-73	MHOM/IN/2003/BHU41	India	Bihar	BHU	nd	VL	1	1a
DON-74	MHOM/IN/2003/BHU32	India	Bihar	BHU	nd	VL	1	1a
DON-75	MHOM/IN/2003/BHU52	India	Bihar	BHU	nd	VL	1	1a
DON-76	MHOM/IN/2003/BHU54	India	Bihar	BHU	nd	VL	1	1a
DON-77	MHOM/IN/2003/BHU33	India	Bihar	BHU	nd	VL	1	1a
DON-105	MHOM/IN/0000/NARESH-RAI	India	Bihar	LEMPP	nd	nd	1	1a
DON-106	MHOM/IN/1977/MUNNI	India	Bihar	LSHTM	nd	VL	1	1a
DON-136	MHOM/IN/1982/NANDI1	India	Kowgachi	LSHTM	LON-41	VL	1	1a
DON-137	MHOM/IN/1982/NANDI2	India	Kowgachi	LSHTM	LON-41	VL	1	1a
DON-138	MHOM/IN/1982/NANDI3	India	Bihar	LSHTM	LON-41	PKDL	1	1a
DON-143	MHOM/IN/1979/STL1-79	India	nd	LSHTM	LON-41	VL	1	1i
DON-144	MHOM/IN/1980/STL39-80	India	Calcutta	LSHTM	LON-41	PKDL	1	1a
DON-146	MHOM/IN/1977/Chowdhury-III	India	nd	LSHTM	LON-41	VL	1	1a/1e
DON-147	MHOM/IN/1977/Chowdhury-IV	India	nd	LSHTM	nd	VL	1	1a
DON-148	MHOM/IN/1977/Chowdhury-X	India	nd	LSHTM	nd	VL	1	1a
DON-150	MHOM/IN/1978/STL2-78	India	nd	LSHTM	nd	VL	1	1a
DON-152	MHOM/IN/1979/STL2-79	India	nd	LSHTM	LON-41	VL	1	1a
DON-153	MHOM/IN/1977/Chowdhury-V	India	nd	LSHTM	nd	VL	1	1a
DON-154	MHOM/IN/1979/DD5	India	Bihar	LSHTM	LON-41	VL	1	1a
DON-155	MHOM/IN/1981/STL106-81	India	Bihar	LSHTM	LON-41	PKDL	1	1a
K 1	MHOM/IN/1999/K59[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 2	MHOM/IN/1999/K75	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 3	MHOM/IN/1999/K80	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 4	MHOM/IN/2000/K111	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 5	MHOM/IN/2000/K131[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 6	MHOM/IN/2000/K132	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 7	MHOM/IN/2000/K133	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 8	MHOM/IN/2000/K135	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 9	MHOM/IN/2001/K149[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 10	MHOM/IN/2001/K155[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 11	MHOM/IN/2002/K172[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 12	MHOM/IN/2002/K188	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 13	MHOM/IN/2002/K192[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 14	MHOM/IN/2003/K216	India	West Bengal	SHC	nd	VL	1[h]	1a[h]
K 15	MHOM/IN/2003/K217[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 16	MHOM/IN/2003/K251[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 17	MHOM/IN/2005/K338[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 18	MHOM/IN/2005/K339[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 19	MHOM/IN/2005/K341[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
K 20	MHOM/IN/2006/K383[c]	India	Bihar	SHC	nd	VL	1[h]	1a[h]
P 1	MHOM/IN/1998/P21	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 2	MHOM/IN/1998/P44	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 3	MHOM/IN/1998/P48	India	Bihar	SHC	nd	PKDL	1[h]	1b[h]
P 4	MHOM/IN/1999/P49	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 5	MHOM/IN/1999/P56	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 6	MHOM/IN/2000/P68	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 7	MHOM/IN/2000/P69	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 8	MHOM/IN/2000/P75	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 9	MHOM/IN/2000/P82	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 10	MHOM/IN/2000/P83	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 11	MHOM/IN/2000/P84	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 12	MHOM/IN/2000/P85	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 13	MHOM/IN/2000/P86	India	West Bengal	SHC	nd	PKDL	1[h]	1a[h]
P 14	MHOM/IN/2001/P93	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 15	MHOM/IN/2001/P94	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 16	MHOM/IN/2002/P100	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 17	MHOM/IN/2002/P101	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 18	MHOM/IN/2005/P136	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 19	MHOM/IN/2005/P137	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
P 20	MHOM/IN/2006/P157	India	Bihar	SHC	nd	PKDL	1[h]	1a[h]
DON-128	MHOM/BD/2006/BD9	Bangladesh	Muktagacha	MMC	nd	VL	1	1a
BD-10	MHOM/BD/2006/BD10[e]	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
BD-11	MHOM/BD/2006/BD11[e]	Bangladesh	Fulbaria	MMC	nd	PKDL	1	1a
DON-129	MHOM/BD/2006/BD12	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
DON-130	MHOM/BD/2006/BD14	Bangladesh	Fulbaria	MMC	nd	VL	1	1a

Table 1 (*Continued*)

Lab. code	WHO code	Origin	Region/district	Source	Zymodeme	Clinical pattern[a]	Population assignment[b]	MLMT genotype
DON-131	MHOM/BD/2006/BD15	Bangladesh	Guforgaon	MMC	nd	VL	1	1a
BD-16	MHOM/BD/2006/BD16[e]	Bangladesh	Trisal	MMC	nd	VL	1	1a
DON-132	MHOM/BD/2006/BD17	Bangladesh	Fulbaria	MMC	nd	PKDL	1	1a
BD-18	MHOM/BD/2006/BD18[e]	Bangladesh	Fulbaria	MMC	nd	VL	1	1f
DON-133	MHOM/BD/0000/IEDCR1	Bangladesh	nd	IEDCR	nd	VL	1	1a
DON-156	MHOM/BD/2006/BD21	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
DON-157	MHOM/BD/2006/BD22	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
DON-158	MHOM/BD/2006/BD24	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
DON-159	MHOM/BD/2006/BD25	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
DON-160	MHOM/BD/2006/BD27	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
DON-140	MHOM/BL/1981/BL1	Bangladesh	nd	LSHTM	nd	VL	1	1a
BD19	MHOM/BD/2006/BD19[e]	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
BD-20	MHOM/BD/2006/BD20[e]	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
BD-23	MHOM/BD/2006/BD23[e]	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
BD-26	MHOM/BD/2006/BD26[e]	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
BD-28	MHOM/BD/2006/BD28[e]	Bangladesh	Fulbaria	MMC	nd	VL	1	1a
DON-85	MHOM/NE/2002/BPK025/0	Nepal	Sunsari	ITM	nd	VL	1	1a
DON-86	MHOM/NE/2002/BPK035/0	Nepal	Saptari	ITM	nd	VL	1	1b
DON-87	MHOM/NE/2002/BPK043/0	Nepal	Sunsari	ITM	nd	VL	1	1b
DON-88	MHOM/NE/2002/BPK077/0[d]	Nepal	Saptari	ITM	nd	VL	1	1a
DON-89	MHOM/NE/2002/BPK080/0	Nepal	Sunsari	ITM	nd	VL	1	1a
DON-90	MHOM/NE/2002/BPK085/0[d]	Nepal	Saptari	ITM	nd	VL	1	1a
DON-91	MHOM/NE/2002/BPK090/0	Nepal	Sunsari	ITM	nd	VL	1	1a
DON-92	MHOM/NE/2002/BPK091/0	Nepal	Sunsari	ITM	nd	VL	1	1a
DON-93	MHOM/NE/2002/BPK164/1[d]	Nepal	Dhanusa	ITM	nd	VL	1	1a
DON-94	MHOM/NE/2002/BPK177/0[d]	Nepal	Dhanusa	ITM	nd	VL	1	1a
DON-95	MHOM/NE/2002/BPK178/0[d]	Nepal	Sunsari	ITM	nd	VL	1	1a
DON-96	MHOM/NE/2003/BPK190/0[d]	Nepal	Morang	ITM	nd	VL	1	1a
DON-97	MHOM/NE/2003/BPK191/0[d]	Nepal	Morang	ITM	nd	VL	1	1j
DON-98	MHOM/NE/2003/BPK206/0	Nepal	Sunsari	ITM	nd	VL	1	1a
DON-99	MHOM/NE/2003/BPK208/0	Nepal	Sunsari	ITM	nd	VL	1	1a
DON-100	MHOM/NE/2003/BPK275/0[d]	Nepal	Morang	ITM	nd	VL	1	1a
DON-101	MHOM/NE/2003/BPK276/0	Nepal	Sunsari	ITM	nd	VL	1	1a/1b
DON-102	MHOM/NE/2003/BPK279/0[d]	Nepal	Morang	ITM	nd	VL	1	1a
DON-103	MHOM/NE/2003/BPK282/0	Nepal	Sunsari	ITM	nd	VL	1	1a
DON-104	MHOM/NE/2003/BPK294/0	Nepal	Siraha	ITM	nd	VL	1	1d
DON-161	MHOM/LK/2002/L60c	Sri Lanka	nd	LSHTM	nd	CL	4	4b
DON-162	MHOM/LK/2002/L60b	Sri Lanka	nd	LSHTM	nd	CL	4	4b
DON-10	MHOM/IN/1971/LRC-L51a[f]	India	Calcutta	KIT	nd	VL	2	2a
DON-48	MHOM/IN/1954/SC23[g]	India	nd	LEMPP	MON-38	VL	2	2a
DON-9	MHOM/IN/1954/LRC-L51p[f]	India	Calcutta	KIT	nd	VL	2	2a
DON-2	MHOM/KE/1983/NLB 189	Kenya	nd	KIT	MON-37	PKDL	2	2a
DON-3	MHOM/KE/1984/NLB 218	Kenya	nd	KIT	nd	PKDL	2	2b
DON-4	MHOM/KE/1985/NLB 323	Kenya	nd	KIT	MON-37	VL	2	2c
DON-66	MHOM/KE/1955/LRC-L53	Kenya	nd	LRC	MON-36	nd	2	2d
DON-67	MHOM/KE/0000/LRC-L445	Kenya	nd	LRC	nd	nd	2	2e
DON-78	MHOM/KE/1973/MRC74	Kenya	nd	LSHTM	MON-2	nd	2	2f
DON-79	MHOM/KE/1962/LRC-L57	Kenya	nd	LSHTM	MON-37	nd	2	2g
DON-139	MHOM/IN/1961/L13	India	nd	LSHTM	nd	PKDL	3	3a
DON-151	MHOM/IN/1983/CHANDIGARH	India	Himachal Pradesh	LSHTM	nd	VL	4	4a
DON-13	MHOM/SD/1993/GE	Sudan	Gedaref	KIT	MON-82	VL	3	3b
DON-46	MHOM/SD/1997/LEM3429	Sudan	Gedaref	LEMPP	MON-257	nd	3	3c
DON-47	MHOM/SD/1997/LEM3463	Sudan	Gedaref	LEMPP	MON-258	nd	3	3d
DON-38	MHOM/ET/0000/HUSSEN	Ethiopia	nd	LSHTM	LON-42	nd	3	3e
DON-24	MHOM/ET/1967/HU3	Ethiopia	nd	LRC	MON-18	VL	5	5a
DON-41	MHOM/SD/1982/GILANI	Sudan	nd	LSHTM	MON-30	VL	5	5b
DON-21	MHOM/SD/1993/35-band	Sudan	Gedaref	KIT	MON-82	VL	5	5c
DON-17	MHOM/SD/1993/9S	Sudan	Gedaref	KIT	MON-18	VL	5	5d

LRC: Leishmania Reference Centre, Hebrew University-Hadassah Medical School, Jerusalem (L.F. Schnur); LSHTM: London School of Hygiene and Tropical Medicine, UK (I. Mauricio); BHU: Banaras Hindu University, Varanasi, India (S. Sundar); MMC: Mymensingh Medical College, Bangladesh (A.K.M. Shamsuzzaman); IEDCR: Institute of Epidemiology, Disease Control and Research, Bangladesh (B.N. Ahmad); ITM: Institute of Tropical Medicine, Belgium (J.C. Dujardin); LEMPP: Centre National de reference des Leishmaniosis, Montpellier, France (J.P. Dedet; P. Bastien); KIT: Royal Tropical Institute Amsterdam, The Netherlands (W. Meide); SHC: Safdarjang Hospital Campus; nd: not defined.

[a] VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; PKDL, post-Kala-azar dermal leishmaniasis.

[b] Population assignment according to Structure results for $K = 5$.

[c] Antimonial-resistant strain.

[d] Strain from a unresponsive case.

[e] Clinical isolate (bone marrow aspirates).

[f] LRC-L51a is presumably the designation of a line subcultured from the strain LRC-L51, possibly as a re-isolation from a laboratory animal, at the Bernhard Noch Institute, Hamburg, in 1971, the original strain having been isolated in Calcutta in 1954 by P.C. Sen Gupta (L. Schnur, pers. comm.) and LRC-L51p was obtained from KIT and is another line of strain LRC-L51, and, with caution, LRC-L51a, LRC-L51p and LRC-L51 can be considered identical.

[g] SC23 is another designation of strain LRC-L52 synonym as is WR352 (Walter Reed Army Institute of Research (WRAIR)).

[h] Assignment of population genotype designations is based on the subset of 11 microsatellite markers, also for those strains, in population 1 showing polymorphism.

Ochsenreither et al., 2006). Both methods are able to detect fragments differing in one single dinucleotide repeat and the results obtained are reproducible and comparable (Ochsenreither et al., 2006). PCR products for CE have been amplified by using fluorescence-conjugated forward primers (Proligo, France).

2.4. Data analysis

The multilocus genotype data, consisting of the repeat numbers determined for 15 microsatellite markers, were processed by using the Bayesian model-based clustering algorithm implemented in STRUCTURE (Pritchard et al., 2000), which determines genetically distinct populations on the basis of allele frequencies, independent of a particular mutation model. For each strain the fraction of its genotype that belongs to each population is measured and then genetic clusters are constructed from a collection of individual genotypes. The following parameters were used: burn-in period of 10,000 iterations, 100,000 Markov chain Monte Carlo iterations, admixture model. A series of runs was performed, testing each value of K (estimated number of populations) between 1 and 10. The most appropriate number of populations was determined by comparing log-likelihood values for K between 1 and 10. The log-likelihood values were compared in a diagram. At the plateau of the derived Gaussian graph the value of K captures the major structure of the populations. Microsatellite-based genetic distances were calculated using the software MICROSAT (Minch et al., 1995). The distance measure Dps (D_{AS}) (proportion of shared alleles) was applied, which calculates multilocus pairwise distances as $1 - s/n$, where s is the total number of shared alleles at all loci and n is the number of loci compared (Bowcock et al., 1994). A neighbour-joining (NJ) tree was constructed with the programme Paup version 4.0b8 (Swofford, 2000). Confidence intervals were obtained by bootstrapping (100 replicates). Microsatellite data were analysed with respect to diversity of alleles (A), mean number of alleles (MNA), expected heterozygosity (H_e), and observed heterozygosity (H_o) by using the GDA software (http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php).

3. Results

3.1. Multilocus microsatellite typing – genotypes

In total, 15 different multilocus microsatellite genotypes (profiles) summarizing the repeat numbers obtained for the 15 microsatellite markers were assigned to 92 strains originating from different endemic regions in the Indian subcontinent (Tables 1 and 2). Sixty-nine of the strains from India (mainly from Bihar), Bangladesh and Nepal presented an identical microsatellite profile (1a). Most of the remaining strains from the Indian subcontinent had profiles (1b–j) that differed only in one locus (mainly in Li23-41, in single cases solely in Li22-35, CS20 or Lm4TA) from the predominating one, or presented a combination of two alleles in marker Li23-41 (six strains), where one of the alleles was always the predominating type 1a. All but one of the 40 additional strains from Indian VL and PKDL patients had the same fragment sizes for the 11 markers (Li22-35, Li23-41, Li41-56, Li46-67, Li71-7, Li71-33, Lm2TG, Lm4TA, TubCA, LIST7031, and LIST7039) tested, identical to that found for the predominating microsatellite profile 1a. One strain had in locus Li23-41 the allele characteristic for profile 1b (Table 1). Three strains from India, MHOM/IN/71/LRC-L51a, MHOM/IN/54/LRC-L51p and MHOM/IN/54/SC23, shared an identical genotype (2a) which was, however clearly different from those described before. The two strains from Sri Lanka showed identical MLMT profiles (4b), significantly different from all the previous ones. Two Indian strains (MHOM/IN/83/CHANDIGARH and MHOM/IN/61/L13) had unique microsatellite profiles (4a and 3a, respectively).

In addition to the above-mentioned microsatellite markers we have tested 24 markers developed from a genomic microsatellite enriched library of the strain MHOM/IN/2001/BHU20140 and 10 markers (LIST 7011, LIST 7021, LIST 7023, LIST 7027, LIST 7028, LIST 7030, LIST 7033, LIST 7035, LIST 7037, and LIST 7040) published by Jamjoom et al. (2002) for polymorphisms in 19 strains from the Indian subcontinent. None of the 34 additional markers was polymorphic for these strains, all of them were, however remarkably polymorphic for the East African strains included in the tests (data not shown).

3.2. Population structure of L. donovani from the Indian subcontinent

Two different methods were applied in order to infer the population structure of *L. donovani* in the Indian subcontinent, a Bayesian model-based clustering approach and calculation of genetic distances. In STRUCTURE, increasing values of K (2–10) were used to find out ancestral source populations as indicated by the bars next to the tree in Fig. 1. At $K = 2$, the method separated two main groups of *L. donovani* representing strains from Bangladesh/India (mainly Bihar)/Nepal, and East Africa/India (the single strain

Table 2
MLMT genotypes of strains from the Indian subcontinent based on the number of repeats of the microsatellite markers.

Genotype[a]	No. strains	Country	Lm2TG	TubCA	Lm4TA	Li41-56	Li46-67	Li22-35	Li23-41	Li45-24	Li71-33	Li71-5/2	Li71-7	CS19	CS20	LIST 7031	LIST 7039
1a	69 (+39)[b]	IN, BD, NE	9	10	6	11	6	14	31	10	10	8	8	13	22	8	11
1b	3 (+1)[b]	IN, NE	9	10	6	11	6	14	32	10	10	8	8	13	22	8	11
1a/1b	4	IN, NE	9	10	6	11	6	14	31/32	10	10	8	8	13	22	8	11
1a/1c	1	IN	9	10	6	11	6	14	29/31	10	10	8	8	13	22	8	11
1a/1e	1	IN	9	10	6	11	6	14	31/33	10	10	8	8	13	22	8	11
1d	1	NE	9	10	6	11	6	14	34	10	10	8	8	13	22	8	11
1f	1	BD	9	10	4/6	11	6	14	31	10	10	8	8	13	22	8	11
1g	1	IN	9	10	6	11	6	12	31	10	10	8	8	13	22	8	11
1h	1	IN	9	10	6	11	6	17	31	10	10	8	8	13	22	8	11
1i	1	IN	9	10	6	11	6	14	31	10	10	8	8	13	21	8	11
1j	1	NE	9	10	6	11	6	14	12	10	10	8	8	13	22	8	11
2a	3	IN	12	16	9	16	6	16	13	8	11	8	10	15	30	8	10
3a	1	IN	9	11	15/16	8	7	15	5	17/18	16/18	8	nd	15/16	20	7	18
4a	1	IN[c]	10	9	8	22	6	14	18	13	23	8	8	14	19	8	11
4b	2	LK	9	9	10	18	6	14	24/25	9	11	8	8	13	22	8	15

IN, India; BD, Bangladesh; NE, Nepal; LK, Sri Lanka.
[a] First number indicates the population number according to STRUCTURE analysis $K = 5$.
[b] Number in brackets refer to the strains analysed only with 11 microsatellite markers (Li45-24, Li71-5/2, CS19 and CS20 have not been tested).
[c] India – Himachal Pradesh focus.

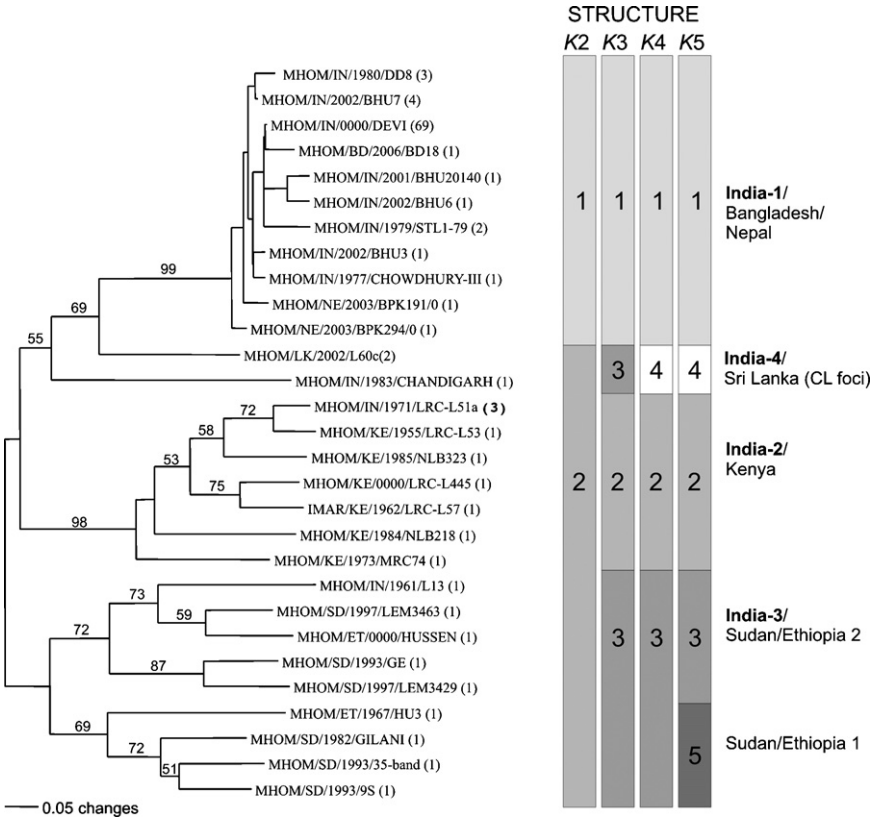

Fig. 1. Neighbour-joining tree (midpoint rooted) inferred from the Dps distances calculated for the data of 15 microsatellite markers and 29 *L. donovani* genotypes with MICROSAT and PAUP softwares. Numbers of strains sharing the same microsatellite profile are given in brackets. The values above the branches indicate the percentage with which a given branch is supported in 100 bootstrap replications. Bootstrap values above 50% are shown. The grey bars at the right side show the populations identified by STRUCTURE with increasing numbers of estimated populations (*K*). Only the 92 strains that were analysed by all 15 microsatellite markers were included in these analyses.

from Himachal Pradesh and a single strain (L13) of unknown Indian origin)/Sri Lanka, respectively. The successive splits in populations could be followed by increasing the number of *K*. All clusters were well defined and stable at $K = 5$. The five populations were: (1) Bangladesh (all strains)/India-1 (all strains from Bihar and Kowgachi, several of undefined Indian origin)/Nepal (all strains), (2) India-2 (three strains)/Kenya, (3) Sudan/Ethiopia 2 + India-3 (L13 – unknown Indian origin), (4) India-4 (single strain from Himachal Pradesh)/Sri Lanka (both strains), and (5) Sudan/Ethiopia 1 that consisted of 85, 10, 5, 3 and 4 strains of *L. donovani*, respectively (Table 1). Populations 3 and 5 have been described in an earlier study, in which more strains per population had been included (Kuhls et al., 2007). Here we used only a subset of strains as representatives for these two populations from Sudan/Ethiopia.

For the 29 microsatellite genotypes obtained for the strains of *L. donovani* from the Indian subcontinent and East Africa (Table 1) a NJ phylogram was constructed based on the Dps distance matrix obtained (Fig. 1). It displays exactly the same population pattern as STRUCTURE, albeit with varying bootstrap support. The phylogram falls into three main clusters: (1) India-1/Bangladesh/Nepal, (2) Sudan/Ethiopia with two sub-clusters, and (3) Kenya/India-2, all identical with the corresponding STRUCTURE populations. The cluster including 85 *L. donovani* strains from Bangladesh, India and Nepal, has 99% bootstrap support. The grouping of three Indian and six Kenyan strains is also confirmed by high bootstrap values. Minor differences concern the single Indian strain (MHOM/IN/83/CHANDIGARH) from the Himachal Pradesh focus and the two strains (MHOM/LK/2002/L60b and MHOM/LK/2002/L60c) from Sri Lanka, which were assigned to the same population by STRUCTURE but did not form a cluster in the tree. According to their position in the NJ tree these strains are most closely related to the Bangladesh/India-1/Nepal group. One single strain from India, MHOM/IN/61/L13, grouped in the Sudan/Ethiopia 2 cluster, as also found by STRUCTURE.

The genetic diversity within the two main populations (1) India-1/Bangladesh/Nepal and (2) India-2/Kenya was estimated by calculating the mean number of alleles per locus, the proportion of polymorphic loci (*P*), observed (H_o) and expected (H_e) heterozygosity (Table 3) and compared with the values for the Sudan/Ethiopia 1 and Sudan/Ethiopia 2 populations obtained previously (Kuhls et al., 2007), which were based on a higher number of strains analysed per population than included here. MNA, which is considered to be an indicator of genetic variation within a population, varied between 1.6 for India-1/Bangladesh/Nepal and 2.67 for Sudan/Ethiopia 1. *P* was also lowest for India-1/Bangladesh/Nepal (0.20) and highest for Sudan/Ethiopia 2 (0.8). The values obtained for H_e differed between 0.018 for India-1/Bangladesh/Nepal and 0.439 for Sudan/Ethiopia 2. In the population India-1/Bangladesh/Nepal homozygous allele combinations were predominating whereas Sudan/Ethiopia 1 represented the highest degree of heterozygosity. All these findings confirmed that the population India-1/Bangladesh/Nepal was least diverse and the two East African populations, Sudan/Ethiopia 1 and Sudan/Ethiopia 2, most heterogenous.

4. Discussion

Microsatellites belong to markers with the highest resolution potential, hence they are frequently used for population genetic studies and strain typing. By the use of MLMT we could show that

Table 3
Characterization of the populations found for the strains of *L. donovani* from different endemic regions.

Group	Population	*N*	*P*	MNA	H_o	H_e
1	India-1/Bangladesh/Nepal	85	0.200	1.600	0.005	0.018
2	India-2/Kenya	10	0.533	2.200	0.042	0.270
4[a]	Sudan/Ethiopia 2[a]	6[a]	0.800[a]	2.267[a]	0.267[a]	0.439[a]
5[a]	Sudan/Ethiopia 1[a]	18[a]	0.733[a]	2.667[a]	0.304[a]	0.376[a]

N, number of strains; *P*, proportion of polymorphic loci; MNA, mean number of allele per locus; H_o, observed heterozygosity; H_e, expected heterozygosity. Only the 92 strains that were analysed by all 15 microsatellite markers were included in these analyses.

[a] These values have been obtained for the same marker set including a higher number of strains for the populations Sudan/Ethiopia 1 and Sudan/Ethiopia 2 published in previous study (Kuhls et al., 2007). In the present study only some representatives of these two populations have been included.

the strains from different endemic areas in the Indian subcontinent are remarkably homogeneous, when compared to the *L. donovani* strains from East Africa (Kuhls et al., 2007) and to *Leishmania infantum* from the Mediterranean Basin (Kuhls et al., 2008). Four distinct populations were identified for the strains of *L. donovani* from the Indian subcontinent by Structure and all but one was confirmed by genetic distance analysis. Most strains from the Indian subcontinent presented a very homogeneous population that included all strains previously typed as MON-2.

The identification of a single cluster of genetically almost identical strains of *L. donovani* in the regions of Bangladesh, Bihar and Nepal highly endemic for VL suggests that this population emerged only recently and underwent a very short evolutionary process since that. The most plausible explanation for the great genetic homogeneity of strains of *L. donovani* from Bangladesh, Bihar and Nepal is a bottleneck event that exterminated the original *L. donovani* population(s) leaving only a small pocket of survivors. In the 1960s, Kala-azar had virtually disappeared from the Indian subcontinent as a collateral effect of insecticide spraying under the Malaria Control Program. The completion of this campaign resulted, however in a dramatic resurgence of the disease in Bihar in the late 1970s (Sen Gupta, 1975) which then spread downstream to Bangladesh and to bordering regions in Nepal. It can, however not been excluded that Indian *L. donovani*, being parasites of humans only, may have undergone a long process of adaptation to human physiology during which they have lost their intraspecies diversity as previously suggested (Pandey et al., 2007).

Unfortunately, we succeeded only in receiving three strains isolated in the study area before the start of insecticide spraying. Two of them were isolated in 1954, and one in 1961. All three strains differed significantly from the strains in the main cluster, which included strains isolated between 1977 and 2007. Interestingly, these three old strains were grouping with East African strains. The two strains from 1954 were closely related to strains from Kenya isolated between 1955 and 1985 whereas strain MHOM/IN/61/L13 grouped with strains from Sudan/Ethiopia-2. We can only speculate whether this might be due to human migration or other reasons because there is no epidemiological information about these strains available. An East African origin as suggested by our study has been previously proposed for Indian *L. donovani* (Ashford, 1984). There are speculations about a recent introduction, in late 19th century, of the parasites to India since historical evidence for their presence in the region before about 1880 is missing. This would also have given rise to the population structure observed. The number of strains collected before insecticide spraying is, however very small and we cannot exclude cross-contamination as reason for their clustering with East African strains.

The great MLMT homogeneity of the strains isolated from patients in these areas is nevertheless surprising especially having in mind the different clinical manifestations that were caused by the strains investigated and the varying refractoriness to standard antimonial treatment in different countries of the Indian subcontinent (Laurent et al., 2007; Sundar et al., 2000). Thus, strains from KA and PKDL patients as well as antimony-resistant and antimony-sensitive strains from Nepal (Laurent et al., 2007) and India showed the same MLMT profile. Obviously, the homogeneity observed with a given genetic marker does not preclude heterogeneity with others. Indeed, polymorphism was evidenced among the same Nepalese strains by other markers: (i) kDNA mini-circles (albeit without correlation with antimony-susceptibility pattern (Laurent et al., 2007) and (ii) expression patterns of genes encoding oxidative stress protective proteins (Decuypere et al., 2005). This could be explained by a more rapid genetic drift (kDNA mini-circles) and by drug pressure (oxidative stress protective proteins), respectively. The mechanisms underlying treatment failure, so far only explored for antimonial drugs, are far from being fully understood. In Bihar, parasite resistance in vitro was consistently found to be correlated with SSG treatment failure (Sundar, 2001) but this was not the case in Nepal (Rijal et al., 2007). The first studies on naturally resistant strains of *L. donovani* point to two possible mechanisms leading to SSG unresponsiveness, up-regulated expression of genes encoding oxidative stress protective proteins (Decuypere et al., 2005) and specific alteration of macrophage transport functions (Roy, pers. comm.). Since in our study probably less than only about 5–50% of the genome has been surveyed, genetic changes leading to resistance to antimonials could easily have missed.

The *L. donovani* strains isolated from CL cases in Sri Lanka and Himachal Pradesh were different. They were related to each other and assigned to a distinct population by Structure. The Sri Lankan strains seem to be closely related to the India-1/Bangladesh/Nepal population, according to distance analysis (Siriwardana et al., 2007) and sequencing of ribosomal internal transcribed spacer 1 (ITS1) (data not shown). The isolate from Himachal Pradesh is, however likely to represent a distinct population, since it branched separately and early in the phylogram and represented a unique ITS sequence (data not shown), different from all other isolates. More strains of this focus have to be analysed to prove this indication. The topography, climatic conditions and epidemiology of CL in Himachal Pradesh differ from those of the Indian VL endemic zones. Most probably another vector, *Phlebotomus longiductus*, and probably a zoonotic reservoir are involved in the transmission of the parasite (Kuhls et al., 2007).

An important outcome of this study is that bone marrow aspirates spotted on filter papers or glass slides is a suitable material for microsatellite typing. Parasite culture is not easy to perform, especially under field conditions, and often not successful. Therefore, assays that can be carried out directly on clinical materials are of great advantage for surveys including high numbers of isolates. Old Giemsa-stained slides can be also used (own unpublished) thus permitting to type previously collected isolates that could not be cryo-preserved for different reasons.

In conclusion, our results demonstrate a remarkably homogeneous single clone of *L. donovani*, population India-1/Bangla-

desh/Nepal, related to the epidemic spread of VL in the Indian subcontinent. The existence of a single MLMT genotype for the majority of strains from a certain endemic region is so far unique for the strains from the Indian subcontinent and was not found for other endemic regions of visceral leishmaniasis. Questions about the reasons for increasing unresponsiveness to antimonial treatment, especially in Bihar, and for different clinical presentations, KA and PKDL, remain unanswered. Host factors or different gene expression profiles of parasites might be responsible for these phenomena and should be further explored.

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