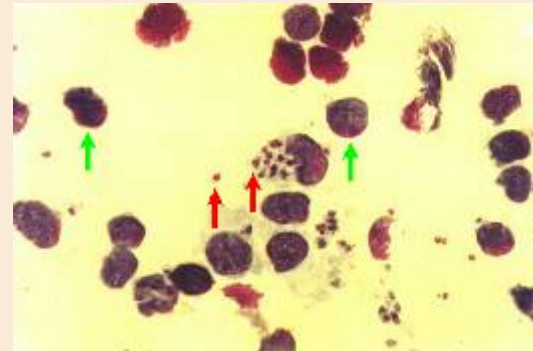


Variation of the parasite causing visceral leishmaniasis in East Africa



Prof. Asrat Hailu
Addis Ababa University, Ethiopia

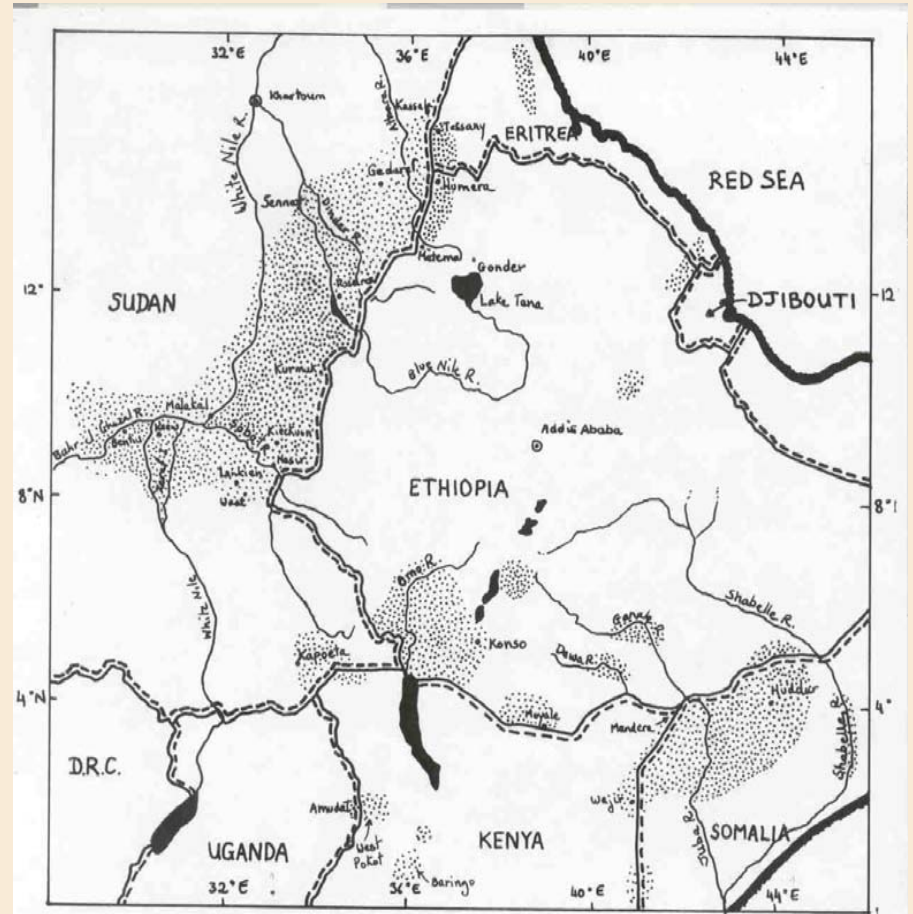
Ecotypes of VL

1. *P. orientalis*, Acacia-Balanites woodland, black-cotton soil

[Eastern Sudan, Northern, and North Eastern Ethiopia]

2. *P. martini/celiae*, eroded termite hills

[South & SE Ethiopia, Kenya, Uganda, SE Sudan]



Map: Courtesy of HealthNet Intl, 2003

What we know, and don't know

1. Disease phenotype
2. Response to PM treatment
3. Genetic diversity
4. *In vitro* drug susceptibility
5. Relationship between 3 and 4

VL in East Africa:

Clinical presentation and diagnosis

	Sudan	N-Eth	S-Eth	India
Lymphadenopathy	72-86%	$\leq 20\%$	$< 15\%$	Low
PKDL	$> 50\%$	$< 5\%$	$< 5\%$	$< 1\%$

Response to paromomycin treatment (East Africa & India)

Response to PM is variable between trial sites in Africa:

+ efficacy ranges of low – 96%

Efficacy is much lower in Sudan cf. India:

+ low cf. 93%

Hypothesis

East African visceral leishmaniasis is caused by geographically (and genetically) isolated populations of *Leishmania donovani*

Objectives

- ✓ To describe the genomic polymorphisms
- ✓ To determine if genotypic variations segregate by geographical location
- ✓ *To describe if genotypic variation correlates with drug sensitivity / resistance (planned)*

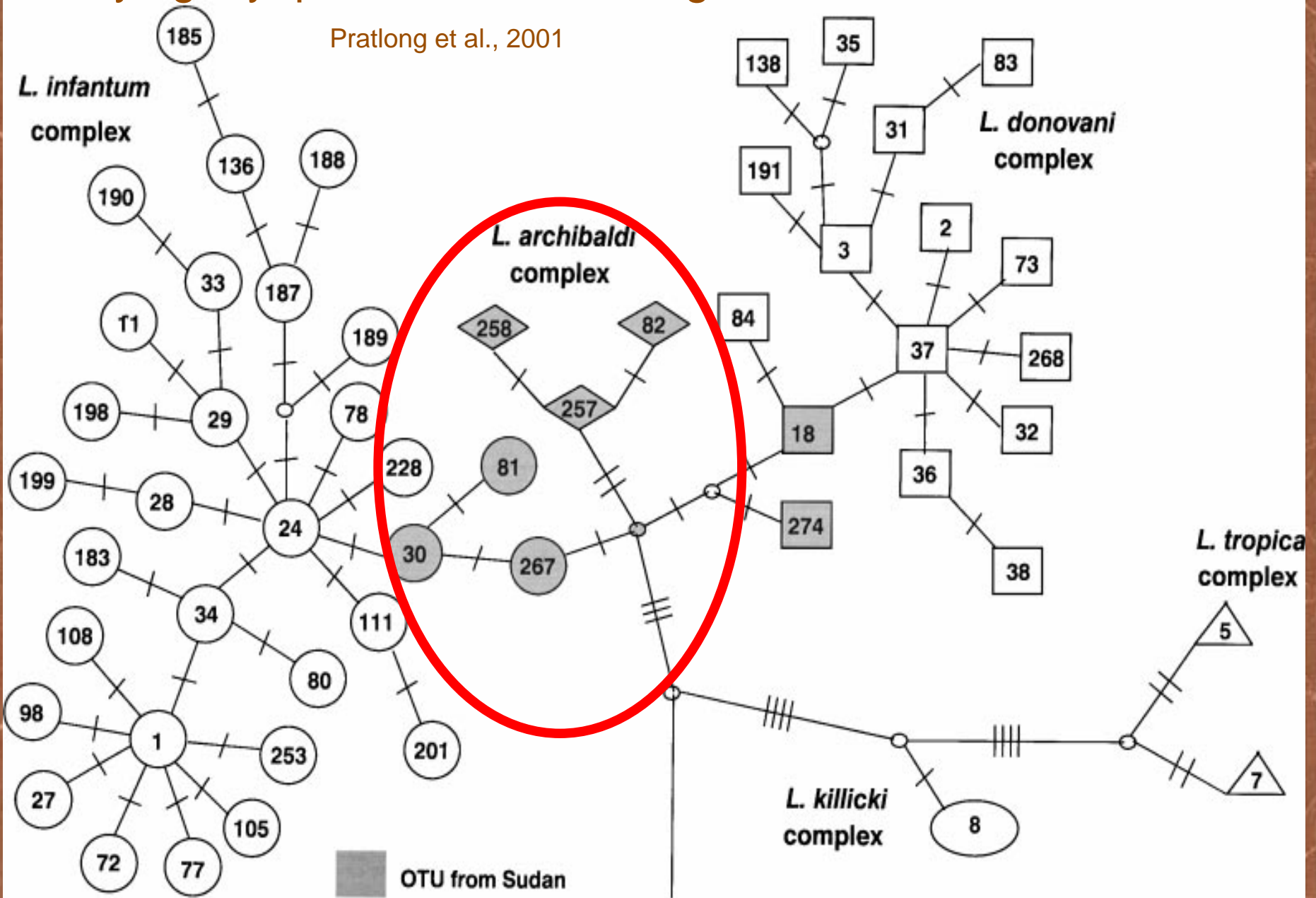
Species and zymodemes within *Leishmania donovani* complex

Species	Zymodemes
<i>L. infantum</i> *	MON1, etc.. MON-30, MON-81 MON-267, MON-278
<i>L. archibaldi</i> *	MON-82, MON-257, MON-258
<i>L. donovani</i>	MON-18, MON2 MON-32, MON-36, MON-37, MON-38, MON-274, MON-276, MON-277

*Both spp., not found in South Ethiopia and Kenya

Phylogeny, parsimonious cladogram, based on MLEE

Pratlong et al., 2001



Species of *L. donovani* complex

Recommendations, based on molecular tools:

- Only two species: “*L. archibaldi* is non-existent”
 - *L. infantum*
 - *L. donovani*

- ***Leishmania donovani*** is the **only** cause of VL in East Africa;

“previous descriptions of *L. infantum* and *L. archibaldi* from this region are a consequence of convergent evolution in the isoenzyme data” [[Jamjoom et al. Parasitology. 2004; 129, 399 – 409](#)]

Targetted molecular markers

- **ITS-1** (Internal Transcribed Spacer) sequences
- **MER** (Mini Exon Repeats)
- **LEG** (T_2B_4)
- **CpbEF** (Cysteine proteinase b)

Molecular markers analyzed

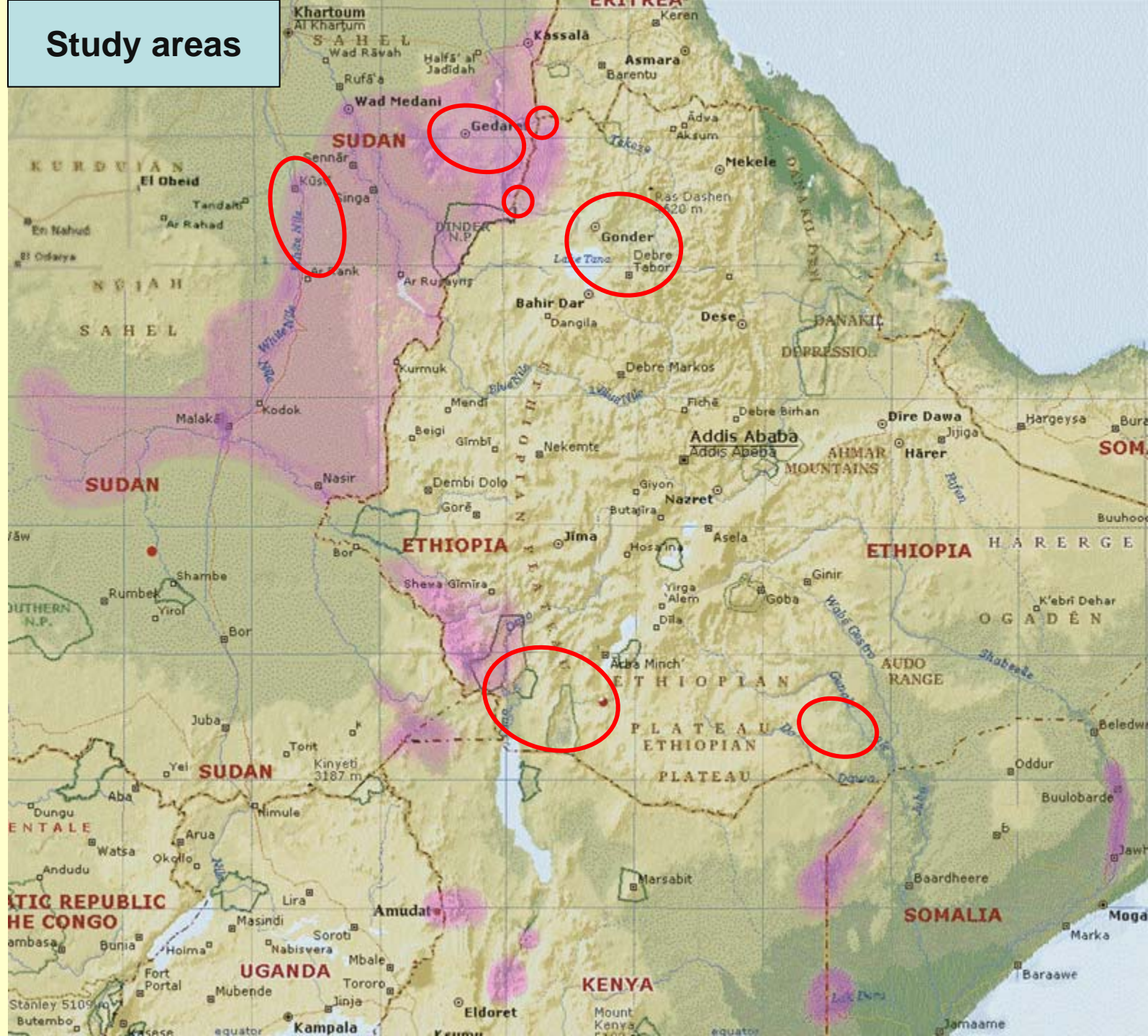
Methods	S	Markers
PCR	1	CpbEF
PCR-RFLP	2	MER (Eae I)
	3	MER (Nco I)
	4	MER (Hae III)
	5	LEG (Hae III)
	6	ITS-1(Hae III)
Analysis of sequences (PCR products)	7	MER
	8	LEG
	9	ITS-1
	10	CpbEF

PCR amplifications (N=111)

- Majority of samples collected during LEAP0104 study
- n = 53 Sudan; n = 58 Ethiopia

Markers	Sudan	N. Ethiopia	S. Ethiopia
MER	53/53	30/30	24/26
ITS-1	41/53	32/32	24/26
LEG	49/53	32/32	24/26
CpbEF	45/53	32/32	26/26

Study areas



ITS-1

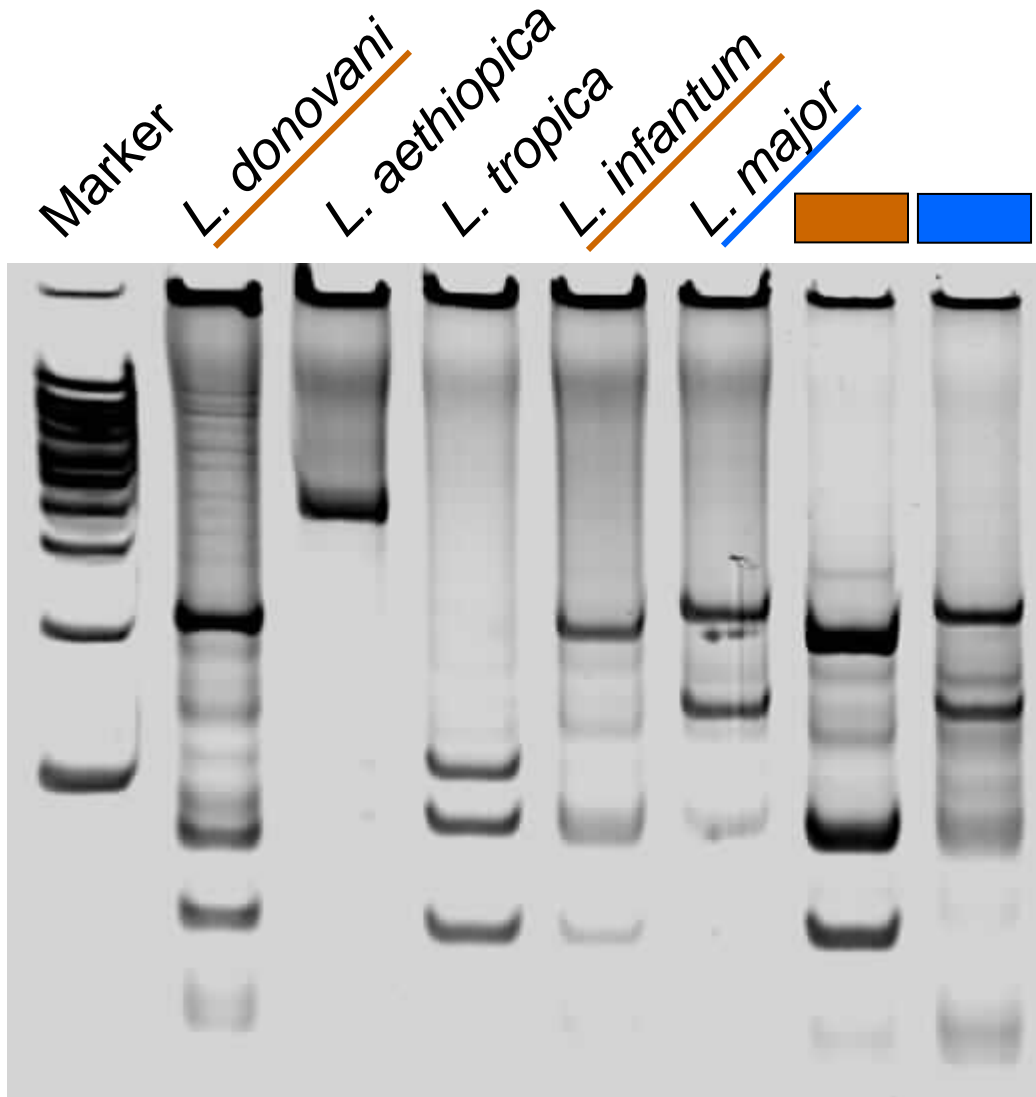
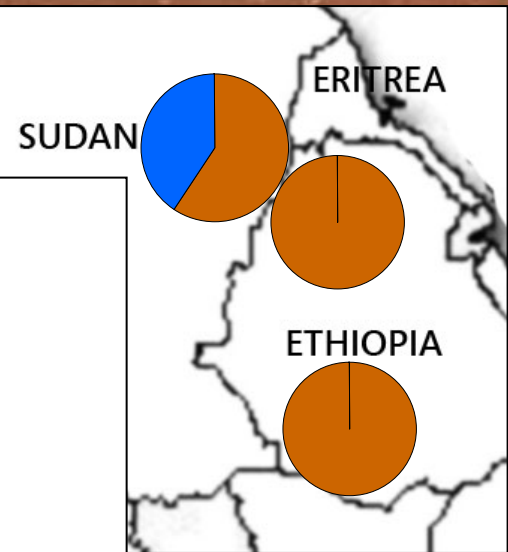
PCR, PCR-RFLP:

PCR: (320 bp); 1.0% agarose gel electrophoresis

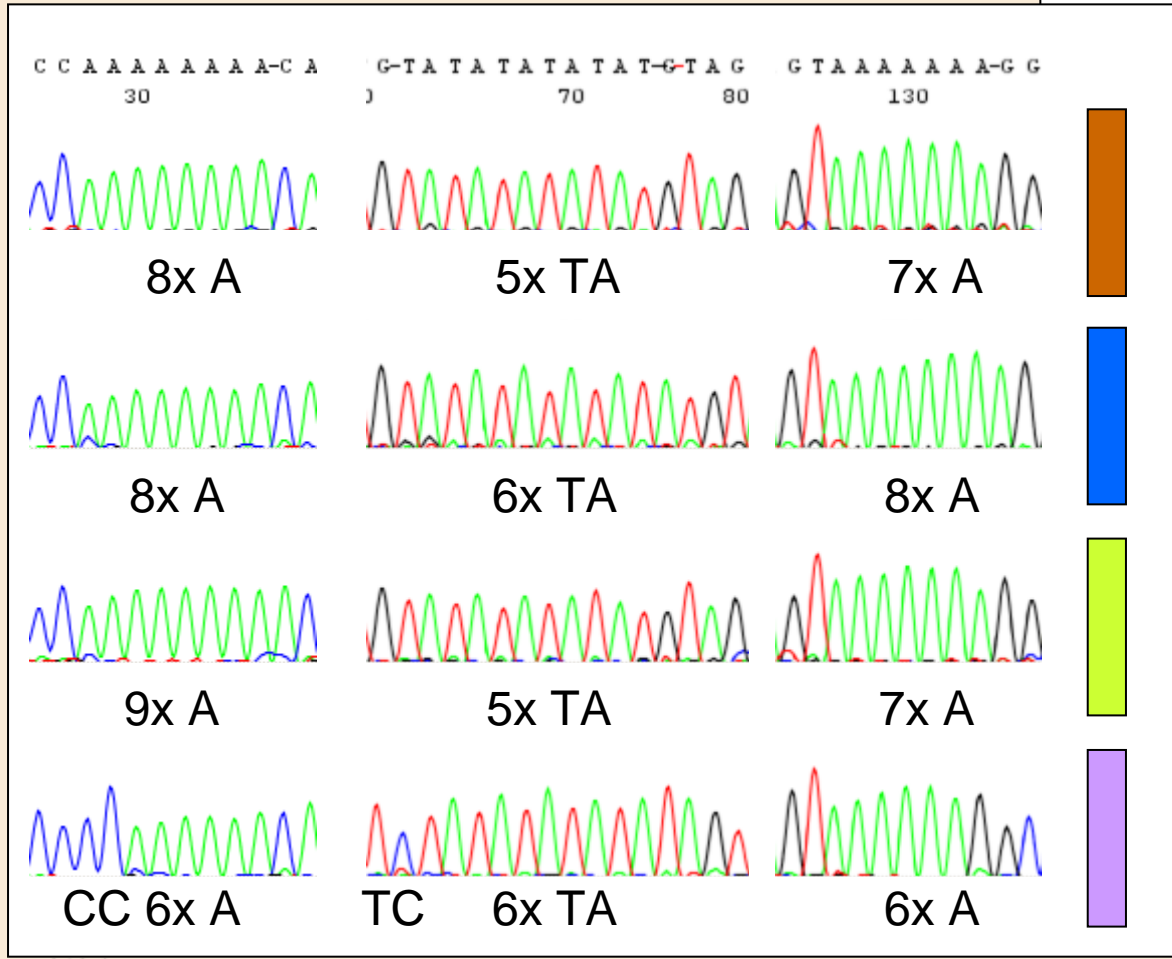
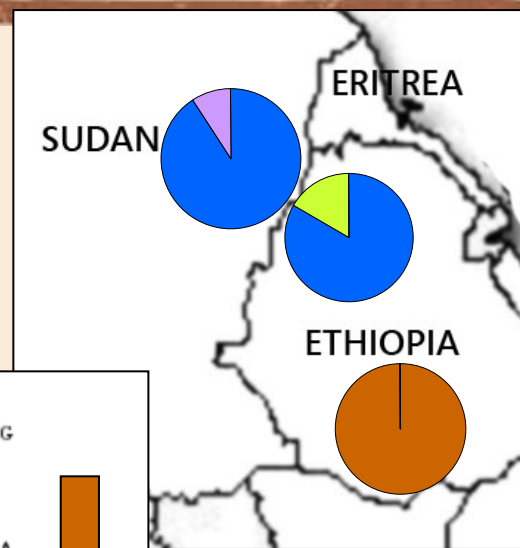
Primers: L5.8S = 5'-TGATACCACTTATCGCACTT-3'
LITSR = 5'-CTGGATCATTTCGATG-3'

RFLP: Hae III restriction; PAGE, 10% acrylamide

ITS RFLP



ITS sequence types



MER

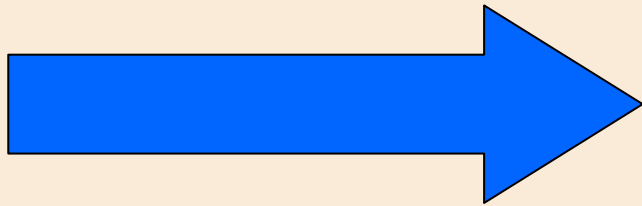
PCR, PCR-RFLP:

PCR: (378-435 bp); 1.0% agarose gel electrophoresis

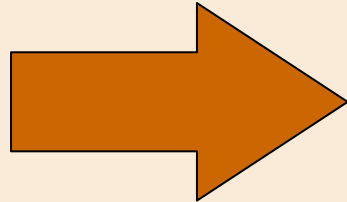
Primers: Fme2 = 5'-ACTTATTGGTATGCGAAACTTCCGG-3'
Rme2 = 5'-ACAGAAACTGATACTTATATAGCGTTAG-3'

RFLP: Eae I, Nco I, Hae III (*L. tropica*, *L. major*)
PAGE, 10% acrylamide

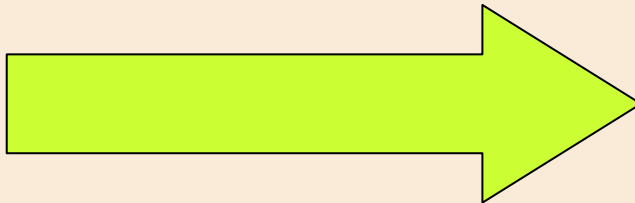
MER sequence types



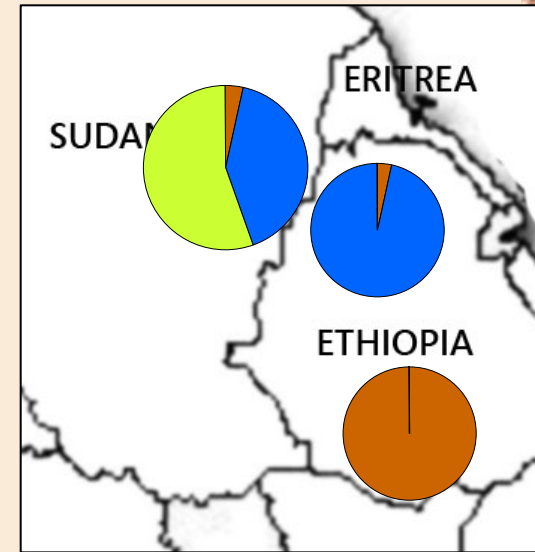
L. donovani



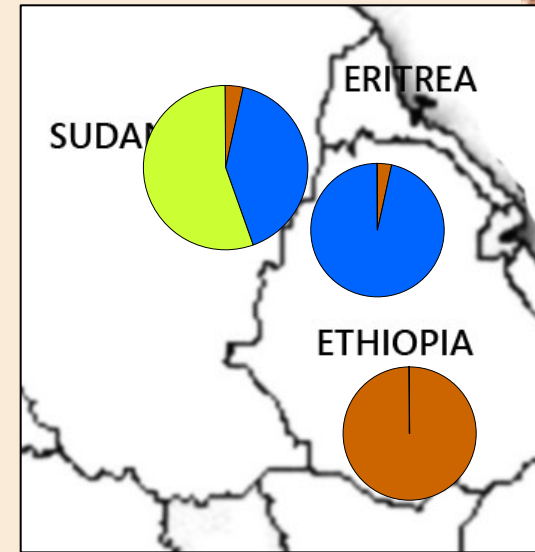
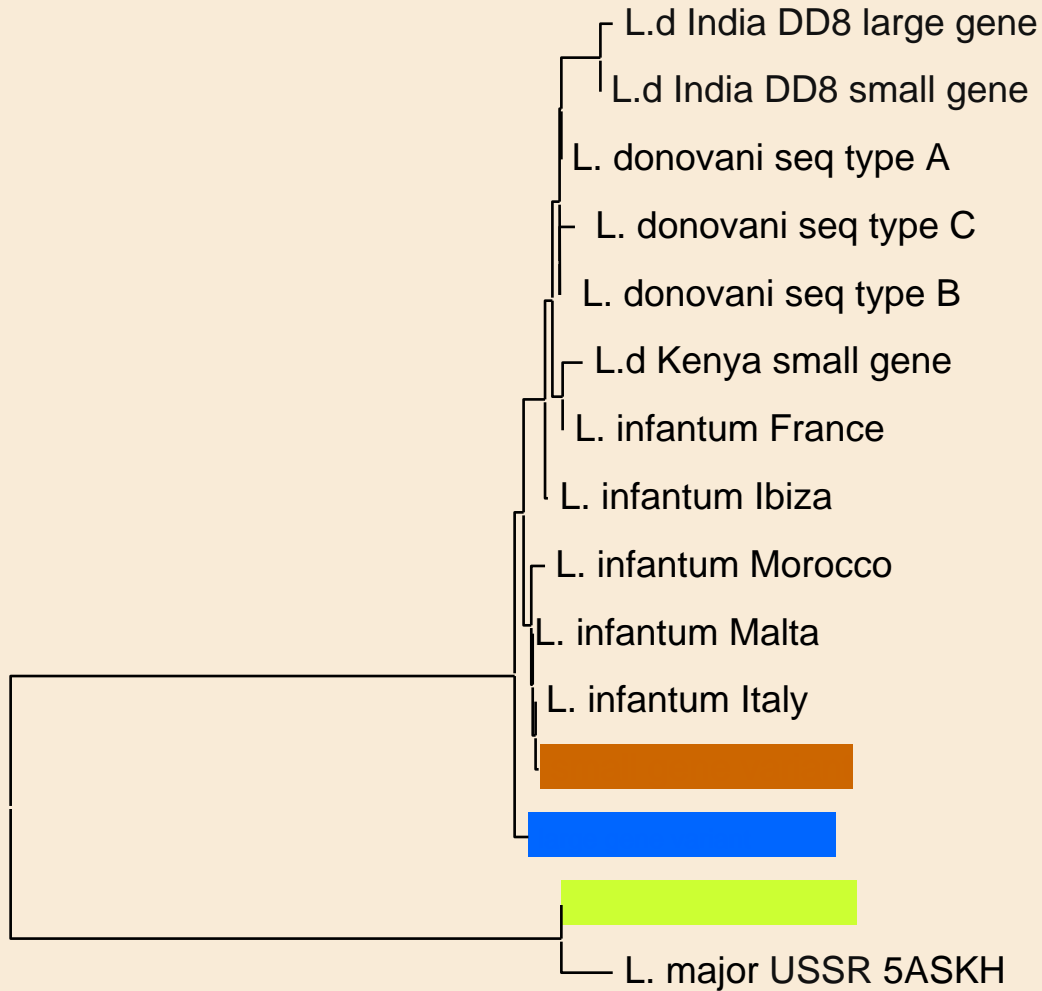
L. donovani small gene variant



L. major seq.



MER sequence types



LEG (T₂B₄)

PCR, PCR-RFLP:

PCR: (250 bp); 1.0% agarose gel electrophoresis

Primer T2: 5'-CGGCTTCGCACCATGCGGGTG-3'

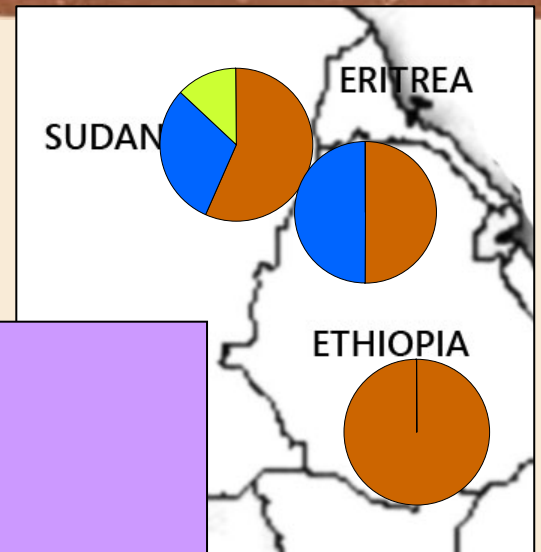
Primer B4: 5'-ACATCCCTGCCACATACGC-3'

RFLP: *Hae* III restriction; PAGE, 10% acrylamide

L. dn = 2 bands: 180, 70 bp

L. in = 1 band: 250 bp// = 3 bands: 250, 180, 70bp

LEG RFLP



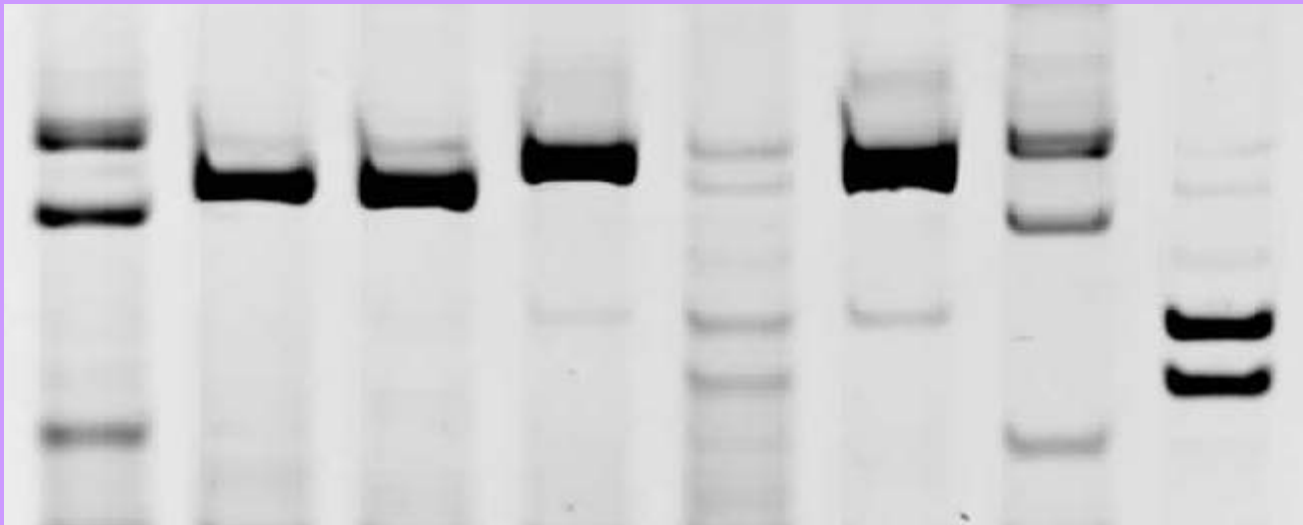
L. donovani

L. aethiopica

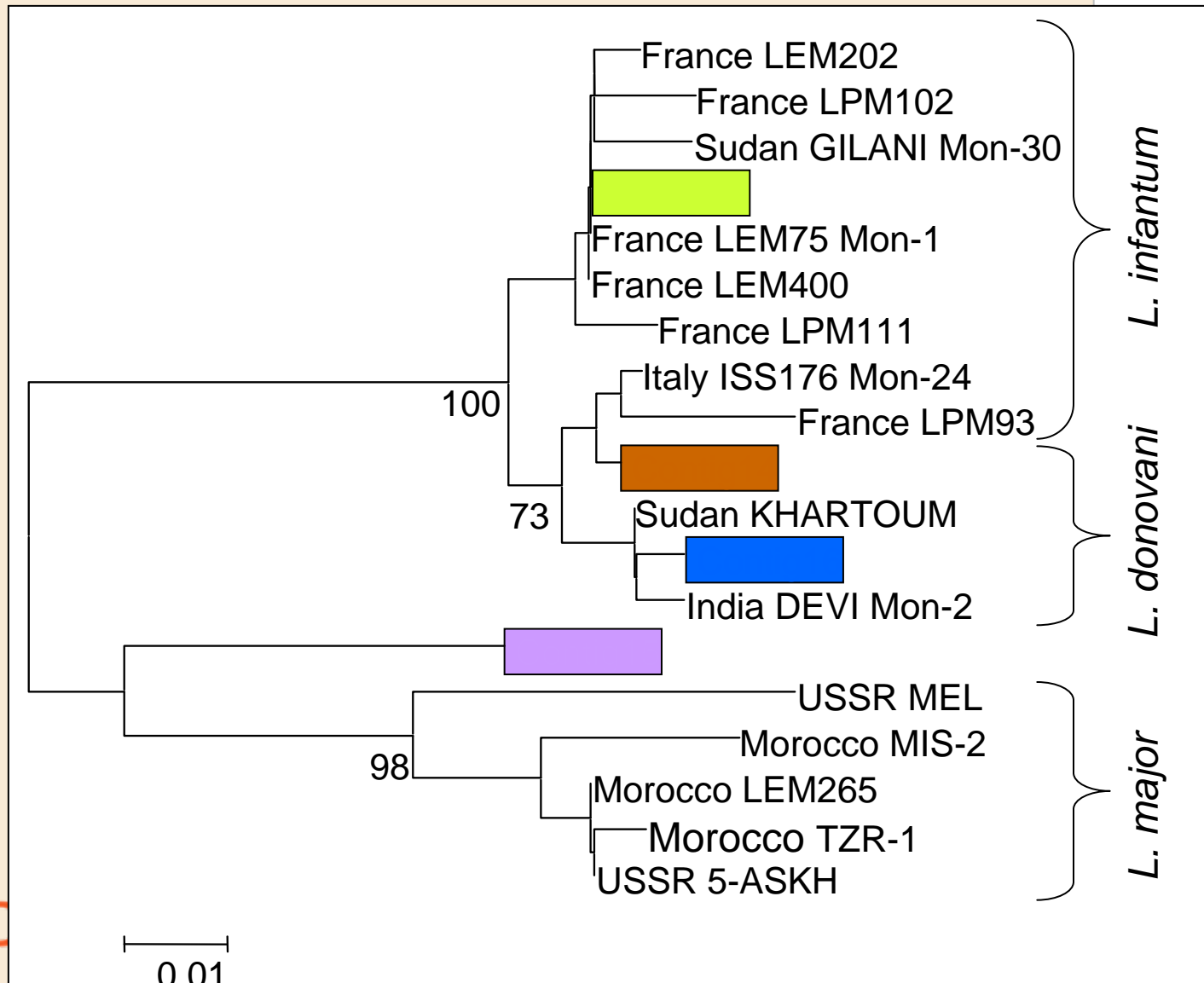
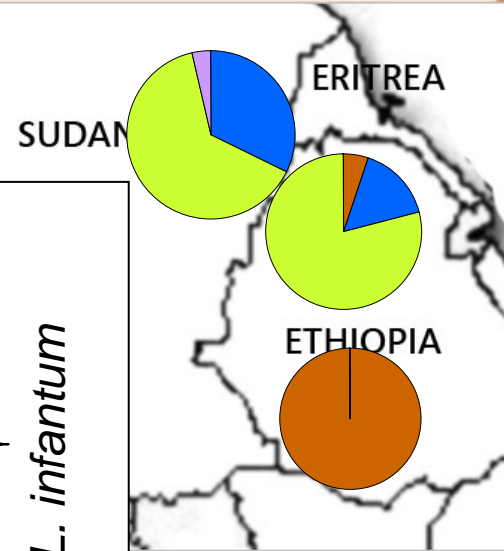
L. tropica

L. infantum

L. major



LEG sequence types



CpbEF

PCR:

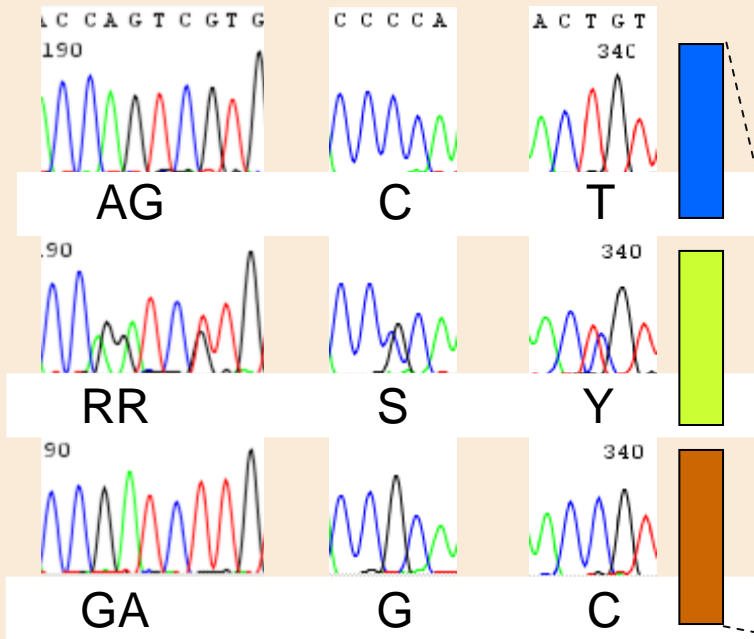
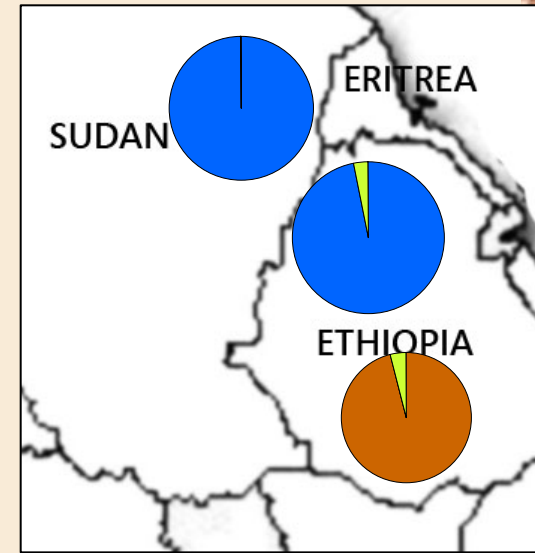
PCR: CpbE (702); CpbF (741)
1.0 - 2.0% agarose gel electrophoresis

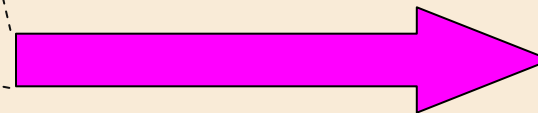
Primers: Forward= 5'-CGTGACGCCGGTGAAGAAT-3'
Reverse= 5'-CGTGCACTCGGCCGTCTT-3'


CpbE = *L. infantum*

CpbF = *L. donovani/archibaldi*

CpbEF sequence types



 *L. donovani*

 *L. infantum*

39 bp
deletion

Summary, multi-locus analysis

Seq. types	1	2	3	4	5	6	7	8	9
E-Sudan	1	1	1	1	1	1	1	1	7
N-Eth									
S-Eth									
Seq. types	10	11	12	13	14	15	16	17	18
E-Sudan			1	2	1			1	1
N-Eth	1	1				8	9		
S-Eth									

Summary, multi-locus analysis

Seq. types	19	20	21	22	23	24	25	26	27
E-Sudan	2			1	1			2	
N-Eth	4	1	1				1		1
S-Eth									
Seq. types	28	29	30	31	32	33	34	35	36
E-Sudan							1		1
N-Eth								1	
S-Eth	8	2	8	1	1	2			

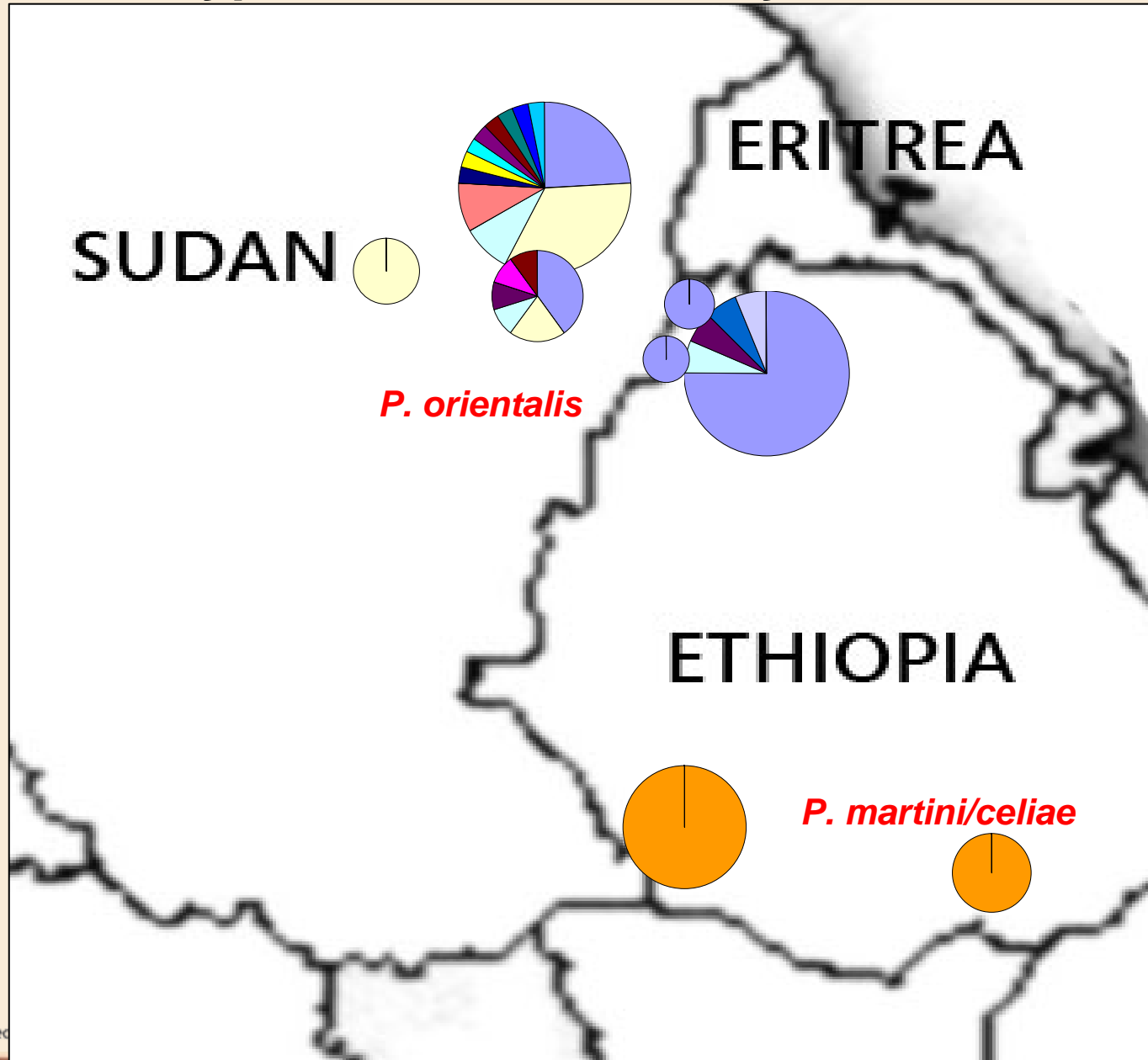
Summary, multi-locus analysis

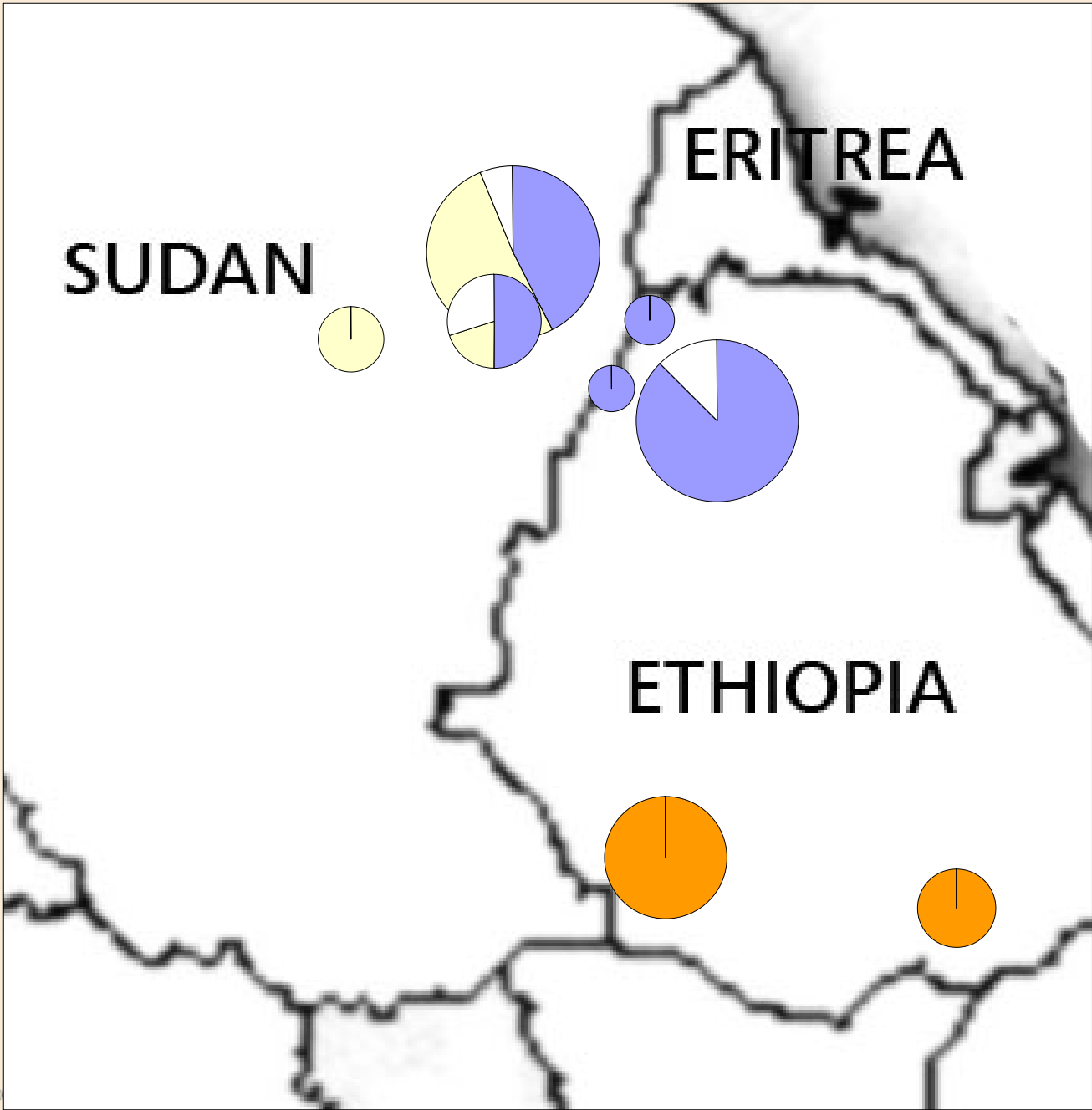
Seq. types	37	38	39	40	41	42	43	44	45
E-Sudan			1		1	1	1	1	2
N-Eth	2	1							
S-Eth				1					
Seq. types	46	47	48	49	50	51	52	53	54
E-Sudan		1	1			1	1	1	1
N-Eth		1			1				
S-Eth	1			1					

Summary, multi-locus analysis

Seq. types	55	56	57	58
E-Sudan	2	1	4	1
N-Eth				
S-Eth				

Genotypes: Multi locus analysis





SUDAN

ERITREA

ETHIOPIA

P. orientalis

P. martini

P. celiae

Summary: main genotypes

	G-I	G-II	G-III
Sudan	19		25
North Ethiopia	30		
South Ethiopia		25	

2 major genotypes in E/Sudan
1 major genotype in N/Ethiopia
1 genotype in S/Ethiopia

Summary - Phase I study

Multi-locus analysis

- Genetic heterogeneity in Eastern Sudan & North Ethiopia
- Greater heterogeneity among isolates of Eastern Sudan
- *L. major* sequences within Sudanese *L. donovani* isolates
- Genetic homogeneity in southern Ethiopia

Evidences for distinct genotypes in Africa

Evidences from MLEE

African (Sub-saharan) *L. infantum* zymodemes

- = Not found in Mediterranean VL
- = Not found in S-Ethiopia, Kenya

Mediterranean *L. infantum* zymodemes

- = Not found in Sub-sahara Africa

African (Sub-saharan) *L. archibaldi* zymodemes

- = Not found in S-Ethiopia, Kenya

Evidences for distinct genotypes in Africa

Evidences from molecular tools

- | | |
|-------------------------|--|
| Oskam et al., 1998 | - Restriction analysis and sothern blotting

- Microsattelites |
| Zemanova E. et al. 2004 | - RAPD |
| Kuhls K. et al. 2007 | - Microsatellite markers |
| Lukes J et al., 2007 | - Multi-factorial (RAPD, RFLPs,
Microsatellites, DNA Sequences) |

Phylogenetic relationships, *L. donovani* complex

Sudan vs. Kenya vs. India

- gp63 intergenic region RFLP
Mauricio IL et al., 2001
- mitochondrial cytochrome oxidase II gene sequences
Ibrahim ME et al., 2001
- SCAR analysis
Lewin S et al., 2002

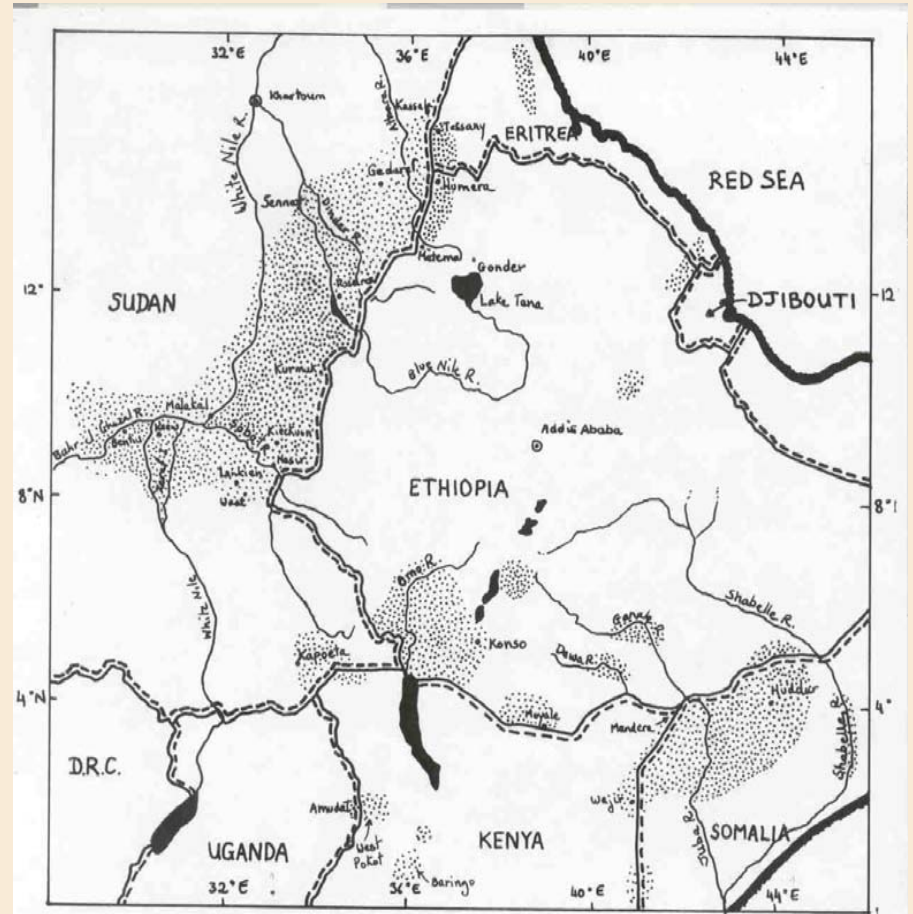
Ecotypes of VL

1. *P. orientalis*, Acacia-Balanites woodland, black-cotton soil

[Eastern Sudan, Northern, and North Eastern Ethiopia]

2. *P. martini/celiae*, eroded termite hills

[South & SE Ethiopia, Kenya, Uganda, SE Sudan]



Map: Courtesy of HealthNet Intl, 2003

Parasitology: next steps

Methods: - *In vitro* drug sensitivity testing (DST)
- Further genotypic characterization

✓ **230 isolates of *Leishmania donovani* complex from VL patients**

230 isolates:

- E/Sudan (n= 75)**
- N/Ethiopia (n= 40)**
- S/Ethiopia (n= 55)**
- Kenya (n= 30)**
- Uganda (n= 30)**

- ✓ **Infection of peritoneal macrophages, CD1 mice**
- ✓ **IC₅₀ values: PM, AmBisome®, miltefosine, SSG**
- ✓ **Microscopy, Alamar blue (optional)**

Acknowledgements

- Trial sites
 - Ethiopia: Arba Minch, Gondar
 - Sudan: Kassab
- University of Amsterdam
 - Dr. Aldert Bart
- Cooperating institutions
 - IEND, AAU
- Funding
 - DNDi

Thank you