First isolation of a new species of *Leishmania* responsible for human cutaneous leishmaniasis in Ghana and classification in the *Leishmania enriettii* complex

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Abstract

An active case detection approach with PCR diagnosis was used in the Ho District of the Volta Region that identified individuals with active cutaneous leishmaniasis. Three isolates were successfully cultured and DNA sequences from these were analysed (ribosomal RNA ITS1; RPL23a intergenic; RNA polymerase II large subunit), showing them to be *Leishmania*, identical to each other but different to all other known *Leishmania* species. Phylogenetic analysis showed the parasites to be new members of the *Leishmania enriettii* complex, which is emerging as a possible new subgenus of *Leishmania* parasites containing human pathogens.

Keywords: Ghana, *Leishmania*, cutaneous leishmaniasis, phylogeny

Cutaneous leishmaniasis (CL) is a significant emerging disease in the Volta Region of Ghana (Kweku et al., 2011) and has become sufficiently common to acquire a local name, “agbamekanu”, with estimates of high prevalence in some communities. Reported infections have occurred mainly in the Ho District, a moist semi-deciduous forest zone with villages dotted around the district capital and an estimated population of 235,000 (Ghana Statistical Service, 2002 Population and Housing Census). From clinical signs, 8,533 cases were recorded in Ho District in 2002 and 2003, which represents ~3-4% of the population, and in surveys of schoolchildren prevalence of leishmanial-like lesions ranged from 12.2 to 32.3 % (Kweku et al., 2011). However, the number of cases with parasitologically confirmed diagnoses is very small (Fryauff et al., 2006; Villinski et al., 2007), and prior to the current study parasites causing CL in Ghana had never been isolated into culture. The identity of the species responsible remain uncertain, one PCR-confirmed case from a biopsy was identified as *L. major* by ribosomal RNA ITS1 sequencing (Fryauff et al., 2006), but in a second study conducted in the same area further biopsies found no match to any known *Leishmania* species (Villinkski et al., 2007). Regarding transmission, leishmaniasis is a vector-borne disease usually transmitted by sand flies but the majority caught to date in Ghana have been various *Sergentomyia* species, which are not generally regarded as likely vectors of human leishmaniasis (Ready, 2013). However, low numbers of the possible vectors *Phlebotomus rodhaini* and *P. duboscqi* were found (Fryauff et al., 2006). It has been recently reported that *L. tropica* DNA was found in *Sergentomyia hamoni* and *S. ingrami*, and *L. major* DNA in *S. ingrami*, in flies collected from the endemic region (Nzelu et al., 2014). However, the finding of parasite DNA by itself does not prove vector status, as blood meal infections can persist in non-vectors for some days but will not result in established transmissible infections (Ready, 2013). The current study was undertaken to isolate and characterize parasites causing CL in Ghana. Cultures were established for the first time, and here we present

evidence that these represent a new species of *Leishmania*, which is related to several other species grouped within the *Leishmania enriettii* complex. These parasites are the first new human-infective *Leishmania* species to be isolated in Africa for over 40 years.

Fifteen villages in Ho District with previous records of suspected or reported cases of CL were initially visited, and of these five with recent cases were followed up: Matse-Lotus, Sokode-Gbogame, Dodome-Doglome, Dodome-Awiausu, and Lume-Atsyame. The study was assessed by and approved by the University of Ghana Medical School Ethical and Protocol Review Board, Protocol Identification Number MS-Et/M.6.1 – P.3/2006-07 and the Noguchi Memorial Institute for Medical Research Institutional Review Board, CPN 062/11-12. The purpose of the study and the procedures to be followed were explained and written informed consent was obtained from all participants or their legal guardians prior to any intervention. A total of 68 people with suspected CL were seen, of these 44 were recruited into the study and 41 subsequently confirmed as infected with *Leishmania*, 38 by PCR from dermal scrapings and 3 cases by successful culture and DNA sequence analysis (further details of sampling and other methods in Supplementary Data S1). Typical households included 10 to 14 inhabitants, and usually 1-2 people per household had healed lesions on the body suggestive of past CL. Two sets of PCR primers were used for diagnosis: Mincr2 and Mincr3 are derived from the conserved region of *Leishmania* species minicircle DNA of the parasite kinetoplast, generating a product of 120 bp (Degrave et al., 1994; da Silva et al., 2004); R221 and R332 are *Leishmania*-specific and amplify a region of the 18S rRNA gene, generating a product of 603 bp (van Eys et al., 1992; Meredith et al., 1993). The Mincr2/Mincr3 primer pair demonstrated better efficacy, amplifying 38 out of the 41 dermal scraping samples taken (93 %; Fig. 1A, Supplementary Fig. S1A), with the R221/R332 pair amplifying 27 out of the 41 samples (66 %; Fig. 1B, Supplementary Fig. S1B). All samples that were positive with R221/R332 were also positive with Mincr2/Mincr3, no additional positive cases were discovered with R221/R332.

Thus the R221/R332 primers detected *Leishmania* in fewer samples, which is consistent with the lower copy number of their targets at about 160 copies per genome (van Eys et al., 1992), compared to ~10,000 copy number of the minicr1/minicr2 targets (Degrave et al., 1994). The 3 negative samples from dermal scrapings are either true negative non-leishmanial skin lesions that can arise from a number of other causes, or are *Leishmania* infections below the level of detection. There was no evidence of other differential diagnoses from Buruli ulcer, yaws or cutaneous fungal infections amongst the participants. Use of dermal scrapings for diagnosis of *Leishmania* is a minimally invasive sampling method for CL, and another useful aspect of this study was the ability of the primers to detect *Leishmania* from the lesion material on FTA cards, without the need for separate isolation of DNA from clinical samples.

Three lesion aspirate samples from separate individuals were used to establish promastigote cultures. Sloppy Evans semi-solid medium was prepared by mixing 350 ml of Locke's solution (9 g NaCl, 0.42 g KCl, 0.4 g CaCl, 0.2 g $NaHCO_3$, 1 g Glucose and dH_20 to 1 litre) with 1.3 g Agar No 1, 2 g bacteriological peptone, and 0.2 g Beef extract (Bovril), which was autoclaved, after which 50 ml defibrinated sterile rabbit blood was added, mixed and 2 ml aliquots dispensed into sterile bijou tubes. Lesion aspirates were transferred into 2ml volumes of Sloppy Evans, incubated at 26 ºC and checked every 48 hours for up to a month by phase contrast microscopy. Upon observing promastigotes, aliquots were transferred into liquid culture medium comprised of Medium 199 (Life Technologies 12350-039) supplemented with 20 % (v/v) fetal bovine serum, BME vitamins (Sigma B6891) and 25 µg/ml gentamicin sulphate. Cultures were expanded and sub-passaged as required and promastigotes cryopreserved in 7.5 % glycerol at -80 ºC and liquid nitrogen. The WHO codes for these isolates are MHOM/GH/2012/GH5;LV757, MHOM/GH/2012/GH10;LV758 and MHOM/GH/2012/GH11;LV759, hereafter referred to as GH5, GH10 and GH11, respectively. Amplification of the ITS1 sequence from each isolate was performed, and the

resulting sequences were found to be very similar or identical to each other and to the sequence previously reported by Villinski et al. (2007) derived from CL lesion biopsies, showing 99.2-99.6% identity (Supplementary Fig. S2). These sequences are more similar to each other than to any other known *Leishmania* sequence, and the degree of similarity is what would be expected for ITS1 sequences from different isolates of the same species.

Infection in confirmed cases was predominantly in younger age groups (~58% were up to 10 years old), although the age range included from infants to adults above fifty years (Supplementary Table S2). The majority of these were recent infections with open sores in which the observed lesions were either crusted or ulcerated (Fig. 2). No nodular or papular forms were observed. The lesions of the majority of participants were circular, except for one person who had both circular and oval lesions. Their appearance was relatively uniform, perhaps indicating the presence of a single species responsible for CL. On the other hand, there is often wide variation in the appearance of lesions even with a single species (Reithinger et al., 2007). More work is required to see if any pattern or significant variation of lesion form emerges in Ghana. The number of lesions per person ranged from 1 to 3, and no multiple diffused lesions were observed on any of the participants. In all, a total of 51 lesions were found on confirmed cases, of these 33 individuals had single lesions, 6 had two lesions and 2 had three lesions. The average size in diameter and reported age of the lesions (according to the participants) were 11.1 mm and 3.9 weeks, respectively. The sites of the lesions were classified into five regions and approximately half (53%), were located on the head (Supplementary Table S3). This could simply mean that the vector preferred uncovered parts of the body, consistent with the fact that the vector if active at night will bite the exposed head parts, since people will typically cover the body but not the head when asleep, or be due to some behavioral property of the vector. There were no reports of severe clinical symptoms accompanying the lesions, there were a few reports of low grade fever, and slight pain and discomfort associated with

the ulcers. The recruitment of participants was not evenly distributed through time, with the number of cases detected increasing from July, peaking in September and declining to November (Supplementary Fig. S3). This peak in recruitment is approximately three months after the peak in rainfall in June, the rainy season running from May to November, which could have contributed to increased transmission by elevating vector numbers. This is similar to findings in Afghanistan, where a rise in numbers of CL in various age groups from August to November and then a decline in December were reported (Faulde et al., 2007). This pattern is also consistent with the typical evolution of CL, where a lesion will develop over a period of 2-3 months before beginning to heal and form scar tissue. There were old scars in some individuals, which were dappled, somewhat depressed and de-pigmented, suggestive of earlier CL in the communities (Okwori et al., 2001; Mendonca et al., 2004).

DNA was isolated from the three isolates established in culture. Initial identification of these was performed using a PCR-RFLP test we have developed that amplifies across a single copy intergenic region of approximately 1.5 kb between the RPLS7A and RPS7B genes (*L. major* chromosome 1) using primers AM1/AM2. This target has been found to give species-specific banding patterns in all of a wide range of *Leishmania* species examined to date. A single product was generated from each of the three isolates, GH5, GH10 and GH11, which when digested with *Msp*I generated identical bands in each case that were completely different to those found with reference strains (Fig. 1C). In addition to the data shown, the banding pattern of the Ghanaian isolates was different to that seen in a wide range of additional reference strains examined, including *L*. *infantum*, *L*. *donovani*, *L*. *amazonensis*, *L*. *mexicana*, *L*. *braziliensis* and *L*. *guyanensis*. These results are consistent with the Ghanaian parasites representing a new species. To investigate this further, PCR was performed on GH5, GH10 and GH11 DNA using the BN1/BN2 primers that amplify a single copy intergenic region of 450-500bp between two RPL23a genes (*L.*

major chromosome 6), followed by sequencing, as used previously for phylogenetic analysis (Dougall et al., 2011). Each isolate generated a 468bp product of identical sequence. Analysis of these sequences and comparison with the homologous sequences from a range of other *Leishmania* species in a maximum likelihood (ML) tree is shown in Figure 3A. The three established subgenera, *Leishmania* (*Leishmania*), *Leishmania* (*Sauroleishmania*) and *Leishmania* (*Viannia*) were all supported with high bootstrap values, as was the *L. enriettii* complex and the paraleishmania. The Ghanaian parasites clustered within the *L. enriettii* complex, which includes *L. enriettii* itself (Lainson, 1997), *L. martiniquensis* (Desbois et al., 2014), a new as yet un-named species from Australia (AM-2004) (Rose et al., 2004) and "*L. siamensis*" (Bualert et al., 2012). It should be noted that "*L. siamensis*" has not been formally described, despite appearing in the literature several times, and is therefore currently a *nomen nudum*, hence the name is used in quotation marks. Further, although the majority of "*L. siamensis*" appear to be *L. martiniquensis*, the isolate analysed here is the PCM2 Trang strain, which is not *L. martiniquensis* (Pothirat et al., 2014). Within the *L. enriettii* complex the most closely related species to the Ghanaian parasites is "*L. siamensis* PCM2 Trang" (98.29% identity, 460/468 nucleotides) followed by *L. enriettii* (90.26% identity) according to the RPL23a tree. Finally we also generated sequences for the RNA polymerase II large subunit gene (RNA PolII; *L. major* chromosome 31), a conserved single copy housekeeping gene that has also been previously used to construct *Leishmania* phylogenies (Croan et al., 1997; Noyes et al., 2002; Yurchenko et al., 2006; Dougall et al., 2011; Pothirat et al., 2014). The sequences from the three isolates were again identical to each other and the resulting ML tree is shown in Figure 3B. This tree is topologically very similar to Figure 3A, the main groups were well supported, and again the Ghanaian isolates clustered within the *L. enriettii* complex. In this tree the most closely related species to the Ghanaian parasites appears different and to be *L. enriettii* (98.42% identity, 1181/1200 nucleotides) followed by "*L.*

siamensis" (98.08% identity), however, there is relatively low bootstrap support (48%) on the branch including *L. enriettii*. Thus the sequences derived for both the RPL23a intergenic and RNA Pol II sequences were identical between the three isolates, but different from all the *Leishmania* species examined, which includes all the major human pathogens.

The phylogenetic analysis showed the Ghanaian parasites to be members of the *L. enriettii* complex, a possible new subgenus of *Leishmania* parasites. To date, the evidence for a new subgenus is by inference from phylogenetic analyses such as those presented here and previously (Dougall et al., 2011; Pothirat et al., 2014). Members of the *L. enriettii* complex are clearly not placed within any of the existing subgenera, *L*. (*Leishmania*), *L*. (*Viannia*) or *L*. (*Sauroleishmania*), and the root of the branch leading to the *L. enriettii* complex is deep and of similar separation to the other subgenera. However, many important details of the biology of these parasites that would be required to make a precise definition of the possible subgenus are still unknown, so until there is progress in that regard this proposition remains to be decided. For example, there is little to no current information on natural reservoir hosts for any species in the *L. enriettii* complex. In both ML trees the location of the Ghanaian isolates within the *L. enriettii* complex was supported by high bootstrap values. The most closely related species are *L. enriettii* and "*L. siamensis*", but the extent of the sequence variation clearly discriminates these species from each other and the Ghanaian parasites. Taking the RNA PolII gene as an example, the extent of sequence identity between the Ghanaian parasites and "*L. siamensis*" is 98.08%, which is less than between *L. major* and *L. tropica* (98.75%), or *L. braziliensis* and *L. panamensis* (98.92%), which most authorities would regard as valid species. Although more work needs to be done and genetic divergence is not the only consideration, the data currently available supports the notion that the Ghanaian parasites are a distinct species of *Leishmania*. *L. enriettii* is not a human pathogen, having only been isolated from domestic

guinea pigs in southern Brazil (Lainson, 1997), whereas "*L. siamensis*" is a human pathogen (Bualert et al., 2012). Of the other two known members of the *L. enriettii* complex, the un-named species from Australia is also not a human pathogen, having only been found in kangaroos and other macropods (Rose et al., 2004; Dougall et al., 2009), whereas *L. martiniquensis* causes human disease manifesting as both CL and visceral leishmaniasis (Desbois et al., 2014; Pothirat et al., 2014; Liautaud et al., 2014). Allied to this, the *L. enriettii* complex consistently appears as the most basal clade within the *Leishmania*, excluding paraleishmania, an informal grouping that requires re-classification but not regarded as *Leishmania* species sensu stricto (Cupolillo et al., 2000). Thus what emerges is an early diverging group of parasites that has become geographically widely dispersed, distributed among a wide diversity of hosts, but including some species with the potential to cause human disease. One of the most intriguing aspects of the *L. enriettii* complex is the identity of the vectors responsible for transmission, which have not been established with certainty for any species. However, recent evidence indicates that day-biting midges are responsible for transmitting leishmaniasis to kangaroos in Australia (Dougall et al., 2011). Therefore, it is possible that midges may be involved in transmission of the Ghanaian parasites, despite the presence of candidate sand flies.

Cumulatively these data demonstrate that the GH5, GH10 and GH11 isolates are representative of a new human-infective *Leishmania* species in Ghana. Given the previous report of one case of *L. major* infection (Fryauff et al., 2006) we cannot exclude the possibility that other *Leishmania* species may be present in this endemic focus, and identification of further human isolates is required to assess this possibility. However, the evidence for the presence of other species is currently not strong, and typically only one species is found in a particular landscape and ecological niche (Ready, 2013), although multiple species may be geographically sympatric, even if not typically present in exactly the same ecological niche. However, it would not be surprising if this proposed new

species is responsible for the majority or even all of the CL cases in the Ho region of Ghana. The local name agbamekanu is also of interest, meaning "gift from somebody who has returned from a journey", and refers to the local belief that the disease has been brought in from neighbouring Togo, travel across the border between the Volta region and Togo being quite frequent. Whether this implied importation of CL is true remains uncertain, but other than young children, when the disease is found in older children and adults it is frequently the case that they are newcomers to the area. Thus the current pattern of infection more likely reflects an exposure of naïve individuals to what has become an established endemic focus in Ghana. Many aspects of this new focus require investigation, including the nature of the vector and presumed animal reservoir hosts, and a proper understanding of epidemiology, but these are important so that appropriate control measures can be considered to help those afflicted by agbamekanu.

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Figure Legends

Figure 1. PCR diagnosis and RFLP. A. An example of diagnosis using kDNA minicircle primers. Lesions were cleaned with 70 % alcohol and scrapings stored on FTA cards (Whatman BioScience). Three 2 mm discs from each FTA sample were processed for PCR and the products examined by agarose gel electrophoresis. Numbers 59-65 above lanes refer to participant sample numbers; M1 is a 100 base pair ladder marker; N is a negative control; 357, 546, and FV1 are positive controls for *L. tropica*, *L. aethiopica* and *L. major*, respectively. B. An example of diagnosis using 18S rRNA primers. Numbers 11-22 refer to participant sample numbers; M1 is a 100 base pair ladder marker; N is a negative control; 357, 546, JPC, LV9 and FV1 are positive controls for *L. tropica*, *L. aethiopica*, *L. infantum*, *L. donovani* and *L. major*, respectively. C. Analysis of isolates by PCR-RFLP. DNA was purified from cultures of isolates GH5, GH10, GH11 and positive controls, amplified using primers AM1/AM2, and products digested with restriction enzyme *Msp*I. M contains size markers; LV546, LV357 and FV1 are controls for *L*. *aethiopica*, *L*. *tropica* and *L*. *major*, respectively; N is a negative control. Further technical details are given in Supplementary Data S1, and further results in Supplementary Figure S1.

Figure 2. Appearance of lesions from confirmed cases of cutaneous leishmaniasis. Examples of typical active lesions are shown on several participants: A, on the arm; B, C, D, on the head; and E, on the back. In F is an example of the scar developing in a healing lesion.

Figure 3. Phylogenetic analysis of Ghanaian *Leishmania*. A. Maximum likelihood tree based on RPL23a intergenic sequences, with 22 species of *Leishmania* and *Endotrypanum monterogeii*, and using *Crithidia fasciculata* as an outgroup, based on alignment of 405-547 homologous nucleotide sequences. AM-2004 is an un-named

394 species of *Leishmania* from Australia. The accession numbers for the sequences used are
395 given in Table S1 and the various subgenera and other groups are as indicated. Bootstrap
396 values from 1000 replicates are given at the nodes. B. Maximum likelihood tree based on
397 RNA Polymerase II large subunit gene sequences, with 22 species of *Leishmania* and
398 *Endotrypanum monterogeii*, using *Crithidia fasciculata* as an outgroup, based on alignment
399 of 1191-1200 homologous nucleotide sequences. Accession numbers are given in Table
400 S1. Further technical details are given in Supplementary Data S1.
401
402

Supplementary Information Legends

Figure S1. Further examples of PCR diagnosis. A. Four panels showing PCR results from diagnosis using kDNA minicircle primers. Numbers above lanes refer to participant sample numbers; M1 is a 100 base pair ladder marker; N is a negative control; 357, 546, and FV1 are positive controls for *L. tropica*, *L. aethiopica* and *L. major*, respectively. B. Two panels showing PCR results from diagnosis using 18S rRNA primers. Numbers above lanes refer to participant sample numbers; M1 is a 100 base pair ladder marker; N is a negative control; 357, 546, JPC, LV9 and FV1 are positive controls for *L. tropica*, *L. aethiopica*, *L. infantum*, *L. donovani* and *L. major*, respectively.

Figure S2. Multiple sequence alignment of ITS1 sequences. The sequences derived from three independent CL cultures (GH5, GH10 and GH11; accession numbers KP006688-KP006690) are aligned with the sequence from Villinski et al. [17] (TAVE; EF524071), previously attributed to a new species of *Leishmania*. Identical residues are indicated by asterisks and the two variable residues are shaded.

Figure S3. Participant recruitment. Villages were visited through the year, **t**he number of participants recruited into the study during different months is shown.

Table S1. Accession numbers of sequences used for phylogenetic analysis.

Species	WHO Code	Accession number for RPL23a IGS	WHO Code	Accession number for RNAPolII
GH5	MHOM/GH/2012/GH5;LV757	KP006691 [a]	MHOM/GH/2012/GH5;LV757	KP054394[a]
GH10	MHOM/GH/2012/GH10;LV758	KP006692 [a]	MHOM/GH/2012/GH10;LV758	KP054395[a]
GH11	MHOM/GH/2012/GH11;LV759	KP006693 [a]	MHOM/GH/2012/GH11;LV759	KP054396[a]
L. adleri	RLIZ/KE/XXXX/LV30	KP025941[a]	RLIZ/KE/XXXX/LV30	AF009153
L. amazonensis	MHOM/BR/1997/M2269	FR693777	MHOM/BR/1973/LV78	AF009154
Leishmania new species from Australia	MMAC/AU/2004/AM-2004;Roo1	FR693774	MMAC/AU/2004/AM-2004;Roo1	HM775497
L. braziliensis	MHOM/BR/83/LTB300	FR693776	MHOM/VE/XXXX/LBV	AF009155
L. colombiensis	IHAR/CO/1996/CL500;LEM2334	KP025942[a]	IHAR/CO/1996/CL500;LEM2334	KM820662
L. donovani	MHOM/ET/67/HU3;L82;LV9	FR693771	MHOM/IN/1980/DD8	AF009157
L. enriettii	MCAV/BR/1945/LV90	FR693773	MCAV/BR/1945/LV90	AF151727
L. equatorensis	MCHO/EC/1982/Lsp1;L888	KP025943[a]	MCHO/EC/1982/Lsp1;L888	DQ383655
L. gerbilli	MRHO/CN/1960/Gerbilli;LON-25	FR693785	MRHO/CN/1960/Gerbilli;LON-25	AJ304947
L. guyanensis	MHOM/BR/75/M4147	FR693784	MHOM/SR/1987/TRUUSI	AJ304949
L. gymnodactyli	RGEC/SU/XXXX/LV247	FR693781	RGEC/SU/XXXX/LV247	AF009159
L. hertigi	MCOE/PA/1965/C-8;LV42	FR693775	MCOE/PA/1965/C-8;LV42	AF009161
L. hoogstraali	RLIZ/SD/XXXX/LV31	FR693782	RLIZ/SD/XXXX/LV31	AF009162
L. infantum	MCAN/ES/1998/LEM-935;JPCM5	FR796438	MCAN/ES/1998/LEM-935;JPCM5	XM_001467548
L. major	MHOM/IL/1980/Friedlin;FV1	FR796402	MHOM/IL/1980/Friedlin;FV1	XM_001685196
L. martiniquensis	MHOM/MQ/1992/MAR1; LEM2494	KP025945[a]	MHOM/MQ/1992/MAR1; LEM2494	KM820663
L. mexicana	MNYC/BZ/1962/M379	FR693769	MNYC/BZ/1962/M379	AF009164
L. panamensis	MHOM/PA/XX/53A;LV145	FR693783	MHOM/PA/XXXX/CIDEP004	AF009165
"L. siamensis"	MHOM/TH/2010/PCM2;Trang	KP025944[a]	MHOM/TH/2010/PCM2;Trang	KM820664
L. tarentolae	RTAR/SE/67/G10/LV108	FR693780	RTAR/DZ/1939/LV414	AF009166
L. tropica	MHOM/IR/60/LV357	FR693778	MHOM/SU/1958/Strain-OD	AF009167
L. turanica	MRHO/SU/83/MARZ-051	FR693786	IRAI/TR/1991/M87	AJ304946
Endotrypanum monterogeii	MCHO/CR/1962/LV88;A9	FR693788	MCHO/CR/1962/LV88;A9	AF009158
Crithidia fasciculata	Cf-C1	CfaC1_23 571,910-572,533[b]	Cf-C1	Cfa-C1_34 1,685,828-1,687,024 (minus strand)[b]

[a]Those that have been generated as part of this study. [b]TriTrypDB identifier (http://tritrypdb.org/tritrypdb/), Stephen M. Beverley and The Genome Institute, Washington University School of Medicine.

Table S2. Age group and gender distribution in confirmed CL cases. Diagnosis was confirmed by PCR from dermal scrapings (38 participants) or by culture and DNA sequencing (3 participants).

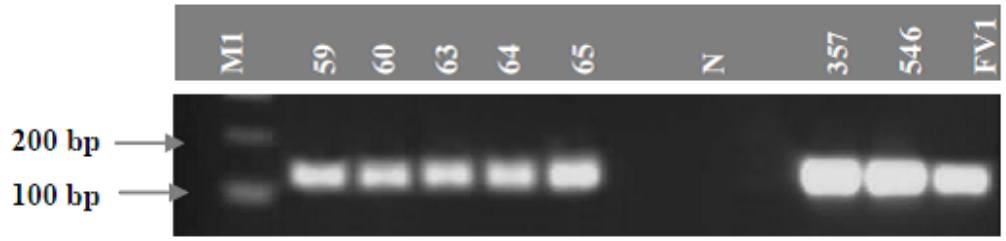
Age Group	Sex		Total
	Male	Female	
0-10	14	10	24
11-20	1	1	2
21-30	0	7	7
31-40	0	2	2
41-50	1	0	1
≥51	2	3	5
Total	**18**	**23**	**41**

Table S3. Numbers of lesions on different regions of the body in confirmed CL cases. The majority of cases presented with single lesions (33/41) but 8 individuals had multiple lesions.

Body Region	Number of Lesions
Head	27
Neck	1
Arm	11
Trunk	6
Leg	6
Total	**51**

Figure 1

A



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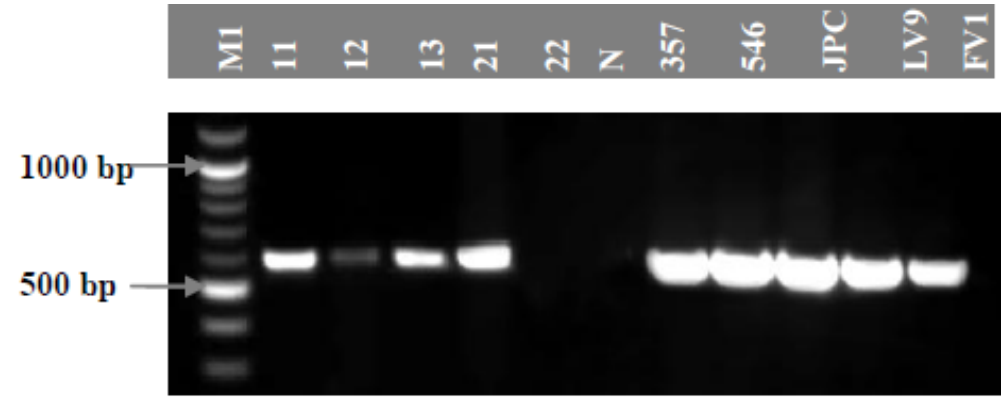

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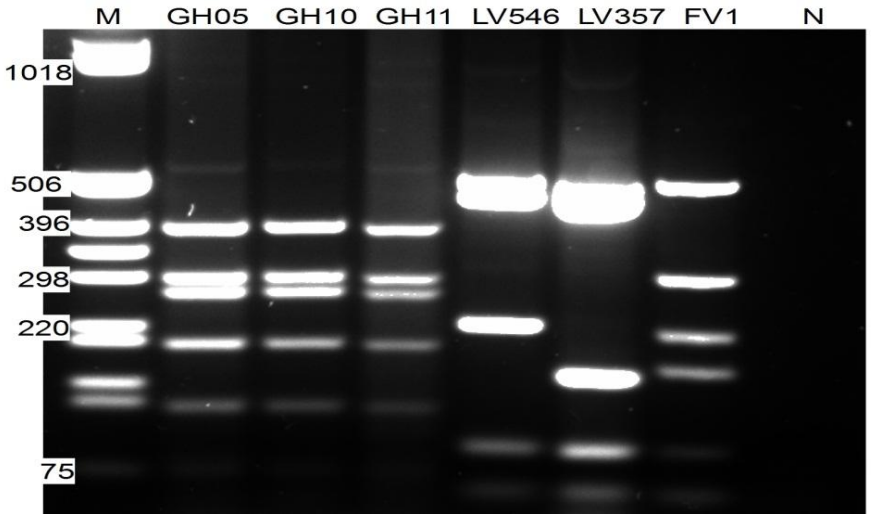


Figure 1

Figure 2

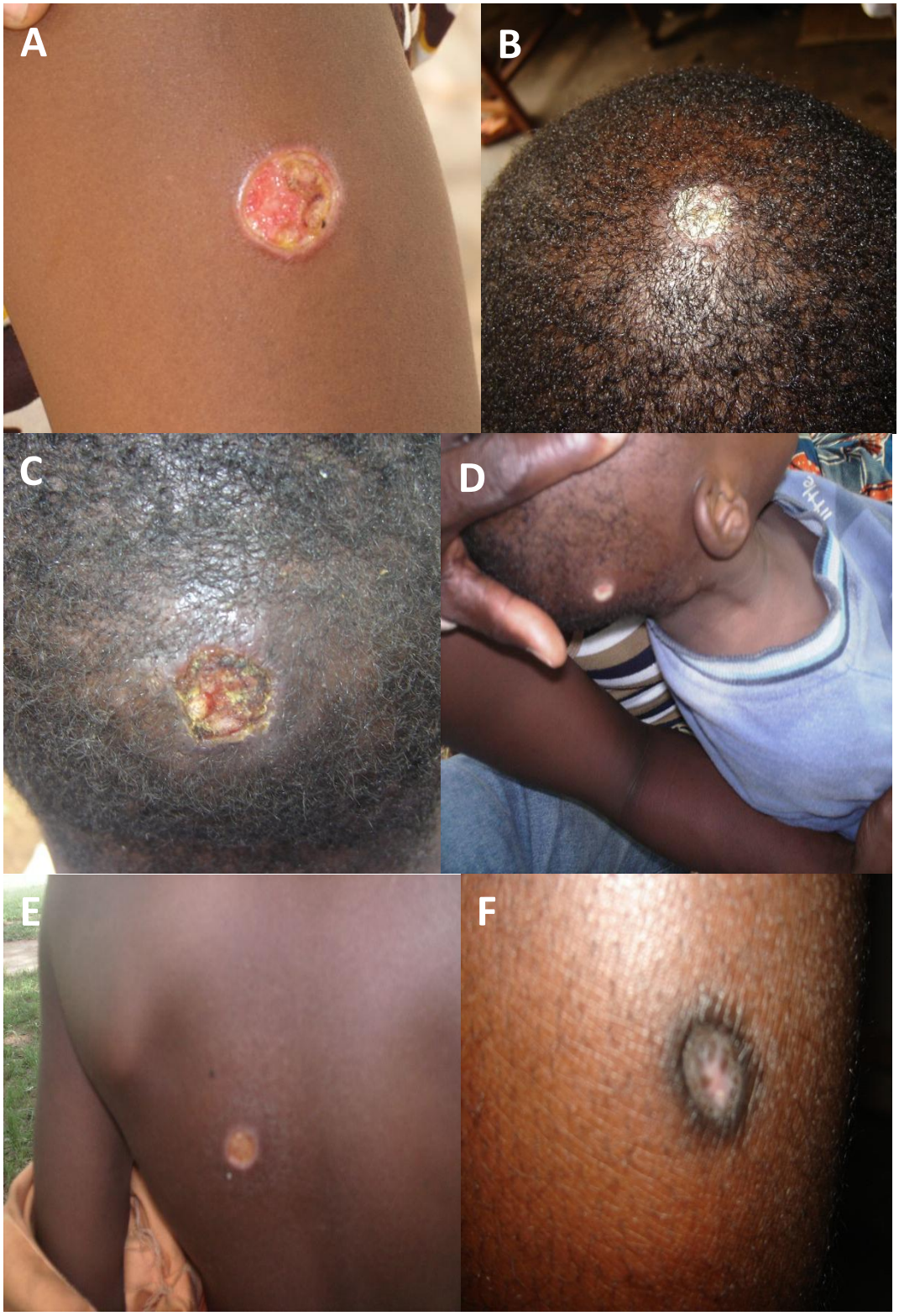
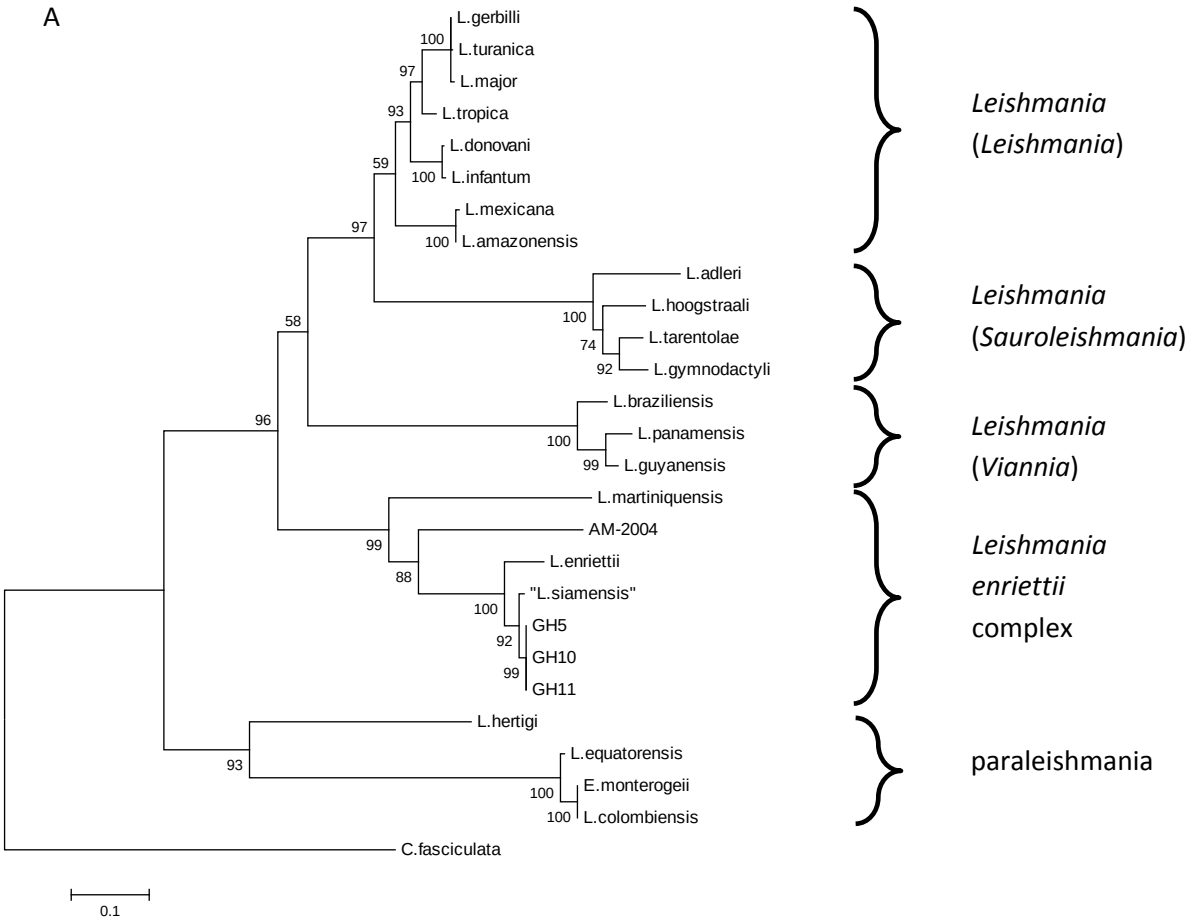


Figure 2

Figure 3



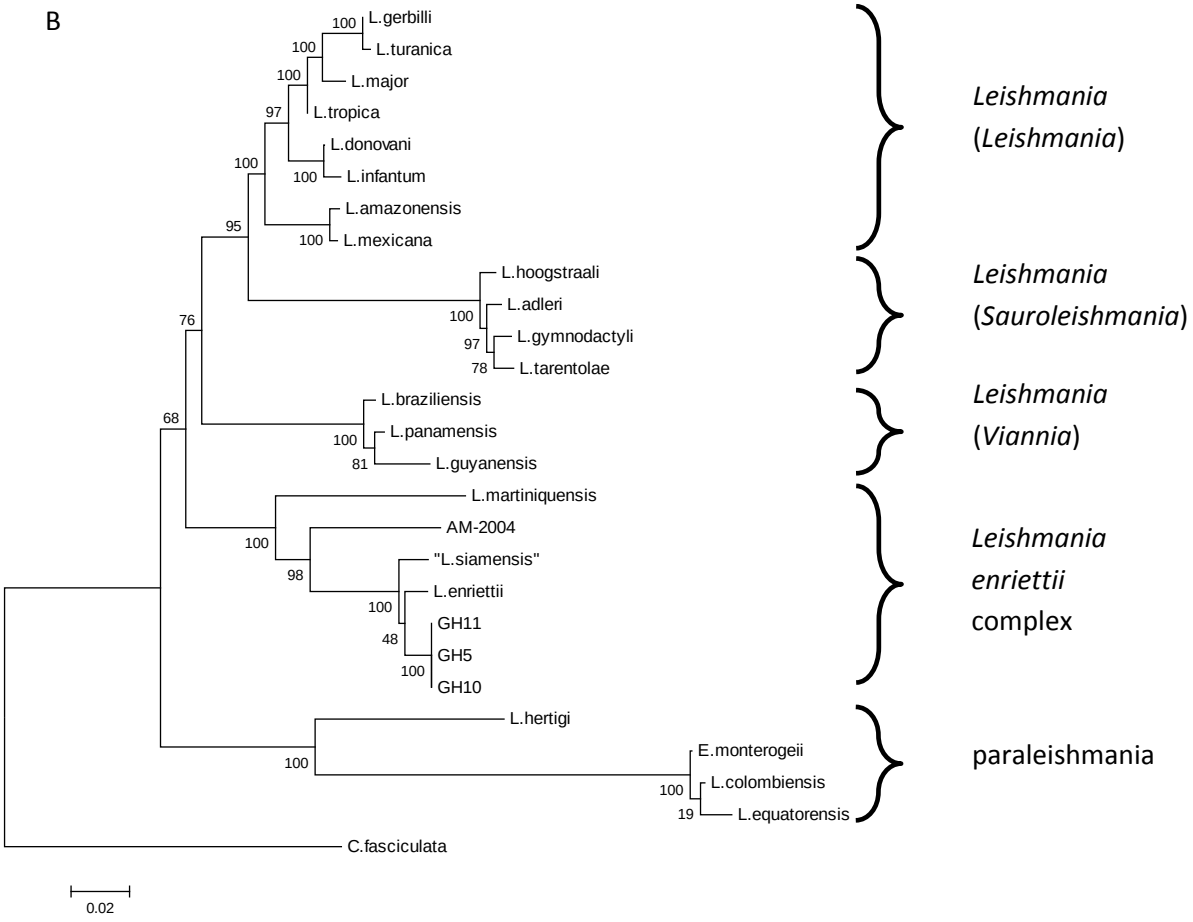


Figure 3

Supplementary Figure 1

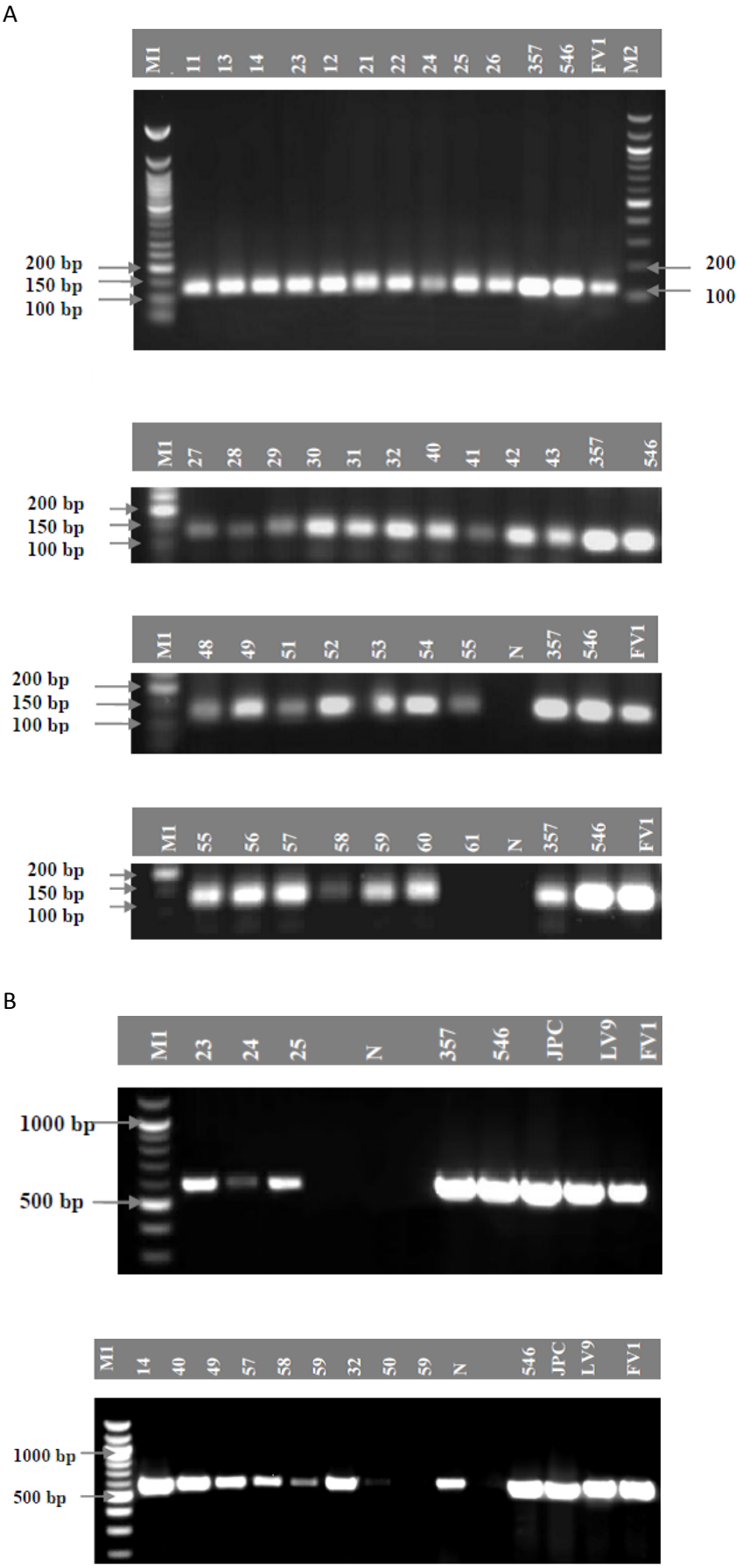


Figure S1

Supplementary Figure 2

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GH5       ATTACACCAAAAAAAAACATACAATCAAAACACGGGGAGGTGTATCTCTCTTTTTGTCAG     60
GH10      ATTACACCAAAAAAAAACATACAATCAAAACACGGGGAGGTGTATCTCTCTTTTTGTCAG     60
GH11      ATTACACCAAAAAAAAACATACAATCAAAACACGGGGAGGTGTATCTCTCTTTTTGTCAG     60
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TAVE      ATAACGCCTTTCCCACATACACACACAACAATATATATGTATATATGTATTGTTATACTC     120
GH5       ATAACGCCTTTCCCACATACACACACAACAATATATATGTATATATGTATTGTTATACTC     120
GH10      ATAACGCCTTTCCCACATACACACACAACAATATATATGTATATATGTATTGTTATACTC     120
GH11      ATAACGCCTTTCCCACATACACACACAACAATATATATGTATATATGTATTGTTATACTC     120
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TAVE      AATTATACAGTAAACAGAAAAACAAAGGCCGGTCGACATATACACACCGCGCGTATATAT     180
GH5       AATTATACAGTAAACAGAAAAACAAAGGCCGGTCGACATATACACACCGCGCGTATATAT     180
GH10      AATTATACAGTAAACAGAAAAACAAAGGCCGGTCGACATATACACACCGCGCGTATATAT     180
GH11      AATTATACAGTAAACAGAAAAACAAAGGCCGGTCGACATATACACACCGCGCGTATATAT     180
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TAVE      ATATATACAACGAAAATGTCCCTTCTTACGGGGGCTTTTCTGGCGGTGTGCTGTGGATAA     240
GH5       ATATATACAACGAAAATGTCCCTTCTTACGGGGGCTTTTCTGGGGGTGTGTTGTGGATAA     240
GH10      ATATATACAACGAAAATGTCCCTTCTTACGGGGGCTTTTCTGGCGGTGTGTTGTGGATAA     240
GH11      ATATATACAACGAAAATGTCCCTTCTTACGGGGGCTTTTCTGGCGGTGTGTTGTGGATAA     240
          ****************************************** ****** *********

TAVE      CGGCTCACAT    250
GH5       CGGCTCACAT    250
GH10      CGGCTCACAT    250
GH11      CGGCTCACAT    250
          **********
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Figure S2

Supplementary Figure 3

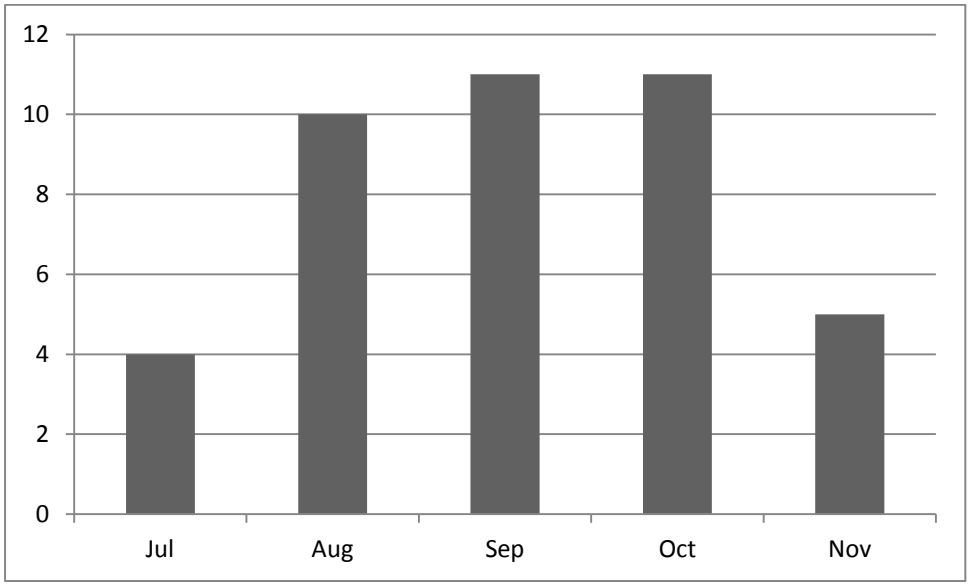

Figure S3

Supplementary Data S1

Sample collection

Skin scrapings were collected from the site of an active lesion using a single-use sterile scalpel. The surfaces and about 30 mm around the diameter of the lesions were cleaned with 70 % alcohol. The scrapings obtained from the borders and the centre of the ulcerated lesions were transferred into 150 µl of phosphate buffered saline (PBS) contained in 1.5 ml microfuge tubes. The PBS and their contents were then spotted onto FTA cards (Whatman BioScience WB12 0205) and air-dried. Samples were stored in closed plastic bags and kept under dry conditions at room temperature to preserve DNA for later PCR analysis. Alternatively, lesions were sterilized with 70 % alcohol, anaesthetized with 1 % lidocaine and sampled by needle aspiration. Aspirates were placed into bijou tubes containing 2 ml of Hank's balanced salt solution (Life Technologies 14170-112) and 25 µg/ml gentamicin sulphate, and stored on ice until processing in the laboratory. Any bleeding was controlled and a sterile dressing applied. All patients received treatment against secondary infections with topical antibiotics and antifungal creams.

PCR using FTA discs

Samples were prepared according to the manufacturer's instructions. At least three 2 mm discs from different sites on the same sample spot of the FTA card were excised using a carbon steel surgical blade and placed in PCR amplification tube. A single blade was used for each sample to prevent cross contamination between samples. The excised discs were then suspended in 200 µl of FTA purification reagent (Whatman Bioscience WB12 0204) in the PCR tube and incubated for 5

minutes at room temperature. The FTA Purification Reagent was then removed and discarded. This washing step was repeated for a total of 3 washes with FTA purification reagent. After the third wash, the discs were re-suspended in 200 µl of TE buffer (10 mm Tris-HCl, 0.1 mM EDTA, pH 8.0) and incubated for 5 minutes at room temperature, after which the TE buffer was removed and discarded. The washing procedure with TE buffer was repeated three times. Finally, the discs were allowed to dry at room temperature for 1 hour. PCR reagents were then added directly to the discs in the tube. Primers Mincr2 (5′ GGG GAG GGG CGT TCT GCG AA 3′) and Mincr3 (5′ CGC CCC CTA TTT TAC ACA ACC CC 3′) were used in an optimized PCR reaction mixture with conditions as follows: 25 µl volumes were prepared using 2 x Reddy Mix^{TM} PCR Master Mix (ABgene AB-0575/DC/LD) (1.5 mM $MgCl_2$, 1.25 units *Taq* DNA polymerase, 75 mm Tris-HCl (pH 8.8 at 25 ºC), 20 mM $(NH_4)_2 SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP, dTTP, and Precipitant and red dye for electrophoresis), 100 pmol of each primer and 2 mm incised discs of the FTA card. Initial denaturation was at 94 ºC for 5 minutes, followed by 35 cycles of 94 ºC for 30 seconds, annealing at 60 ºC for 30 seconds, extension at 72 ºC for 1 minute 30 seconds, and a final extension at 72 ºC for 3 minutes. Primers R221 (5′ GGT TCC TTT CCT GAT TTA CG 3′) and R332 (5′ GGC CGG TAA AGG CCG AAT AG 3′) were used in an optimized PCR reaction mixture on FTA discs with conditions as above, except that a 1.1 x master mix with 2.5 mM $MgCl_2$ was used (Abgene AB-0619/LD) and a 63 ºC annealing temperature.

PCR on cultured parasites

DNA extraction from parasites was performed using a QIAamp DNA Mini Kit (Qiagen), following the manufacturer's instructions. Amplification of the ITS1

sequence was performed using primers L5.8S (TGA TAC CAC TTA TCG CAC TT) and LITSR (CTG GAT CAT TTT CCG ATG). Amplification of the RPS7 intergenic sequence was performed using primer pairs AM1 (CGC GTG TCG TTC GGC TTT ATG TG) and AM2 (CTT ACG GAG CTT GCT GAG GTG AGG), followed by digestion with restriction enzyme *Msp*I. Amplification of the RPL23a intergenic sequence was performed using primers BN1 (GAA GGT CAA CAC CCT GAT CC) and BN2 (CTT CTT GGC GGT CTT CTG AG). Amplification of RNA Polymerase II was performed with several primer pairs: RPOF1 (GAC ACA GCC GTC AAG AC) and RP0R1 (GCA GCC GCA CAA TGC GCT); PolIIN5 (GCA CTT CAT GTT GGA CGA CT) and PolIIN6 (GTA CTT GGT GCG GAT CTC CT); PolIIN7 (AGG AGT ACA GGC TGA ACG AC) and PolIIN8 (TGT CGT CCA CTT GCC GGA). Amplification was performed with proof-reading DNA polymerase (Qiagen HotStar HiFidelity Polymerase).

Agarose gel electrophoresis

PCR products were loaded onto 8 cm 1.5 % - 2 % agarose gels containing 5 ng/µl ethidium bromide and Tris-acetate (TAE) buffer and electrophoresed for 45 minutes to 1 hour at 75 – 100 V. DNA bands were examined under ultraviolet trans-illumination and their size determined by comparison with markers. Alongside test samples, positive controls were run including products generated from *L. donovani* (MHOM/ET/67/HU3; LV9); *L. infantum* (MCAN/ES/98/LEM-935; JPC; M5); *L. tropica* (MHOM/IR/60/LV357); *L. major* (MHOM/IL/80/Friedlin; FV1); and *L. aethiopica* (MHOM/ET/72/LRC-L147/546).

DNA sequencing and phylogenetic analysis

Products were directly sequenced using commercial services. Results were checked for quality using Chromas Lite 2.1.1 (http://technelysium.com.au/). Initial alignments and analyses were performed using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). For phylogenetic analysis, alignment and tree building programmes in MEGA version 6 were used (http://www.megasoftware.net/). Testing of alternative models was performed, for RPL23a sequences the Hasegawa-Kishino-Yano model and for RNA polymerase the Tamura-Nei model were the best fitting models of sequence evolution, respectively, and these were used for tree construction using the maximum likelihood method. Bootstrapping was performed on all trees with 1000 replicates.