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Succinctus

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, Ghana that identified individuals with active cutaneous leishmaniasis. Three isolates were successfully cultured and DNA sequences from these were analysed (ribosomal RNA internal transcribed spacer 1; ribosomal protein L23a intergenic spacer; RNA polymerase II large subunit), showing them to be *Leishmania*, identical to each other but different from all other known *Leishmania* spp. 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.

Human 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 271,881 (Ghana Statistical Service, 2010 Population and Housing Census http://www.statsghana.gov.gh/docfiles/2010phc/2010_POPULATION_AND_HOUSING_CENSUS_FINAL_RESULTS.pdf). From clinical signs, 8,533 cases were recorded in the Ho District in 2002 and 2003, which represents approximately 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 remains uncertain. One PCR-confirmed case from a biopsy was identified as *Leishmania major* by rRNA internal transcribed spacer 1 (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* sp. (Villinski 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* spp., which are not generally regarded as likely vectors of human leishmaniasis (Ready, 2013). However, low numbers of the possible vectors, *Phlebotomus rodhaini* and *Phlebotomus duboscqi*, were found (Fryauff et al., 2006). It has been recently reported that *Leishmania tropica* DNA was found in *Sergentomyia*

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hamoni and *Sergentomyia 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 characterise 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. To our knowledge, these parasites are the first new human-infective *Leishmania* spp. to be isolated in Africa for over 40 years.

Fifteen villages in the Ho District with previous records of suspected or reported cases of CL were initially visited, and of these five villages 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, Ghana, 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 three cases by successful culture and DNA sequence analysis (see Supplementary Data S1 for further details of sampling and other methods). Typical households included 10–14 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* spp. minicircle DNA of the parasite kinetoplast, generating a product of 120 bp (Degrave et al., 1994; da Silva et al., 2004); primers 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 with ~10,000 copy number of the minicr1/minicr2 targets (Degrave et al., 1994). The three 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 (Whatman Bioscience Ltd), 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 of NaCl, 0.42 g of KCl, 0.4 g of CaCl, 0.2 g of $NaHCO_3$, 1 g of glucose and dH_20 to 1 L) with 1.3 g of Agar No 1, 2 g of bacteriological peptone, and 0.2 g of beef extract (Bovril, UK), and autoclaved.

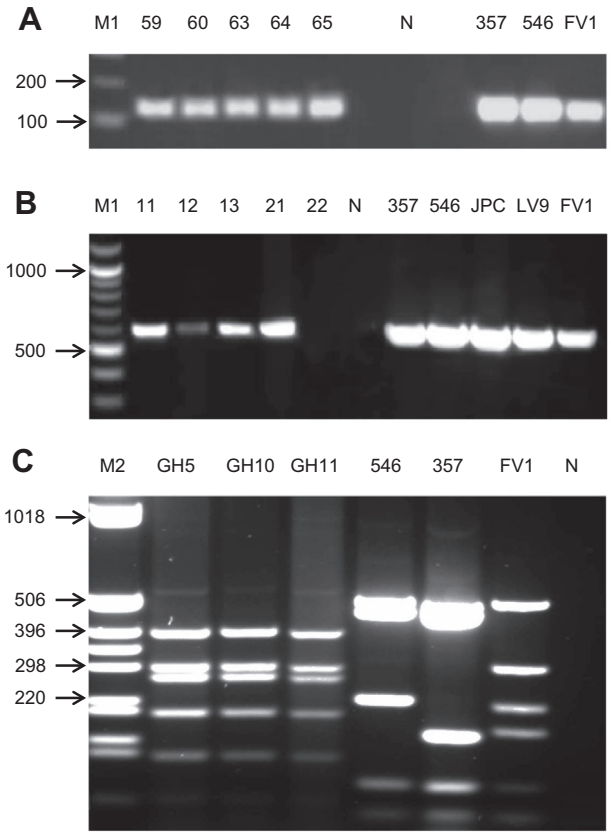

Fig. 1. PCR diagnosis and restriction fragment length polymorphism analysis of DNA extracted from human isolates of *Leishmania*. (A) An example of diagnosis using kinetoplast (k)DNA minicircle primers. Lesions were cleaned with 70% alcohol and scrapings stored on FTA® cards (Whatman BioScience, UK). Three 2 mm discs from each FTA® sample were processed for PCR and the products examined by agarose gel electrophoresis. Numbers 59–65 refer to participant sample numbers; M1 is a 100 bp ladder marker; N is a negative control; 357, 546 and FV1 are positive controls for *Leishmania tropica*, *Leishmania aethiopica* and *Leishmania major*, respectively. (B) An example of diagnosis using 18S rRNA primers. Numbers 11–22 refer to participant sample numbers; M1 is a 100 bp ladder marker; N is a negative control; 357, 546, JPC, LV9 and FV1 are positive controls for *L. tropica*, *L. aethiopica*, *Leishmania infantum*, *Leishmania donovani* and *L. major*, respectively. (C) Analysis of isolates by PCR-restriction fragment length polymorphism. 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. M2 contains size markers as indicated in bp; 546, 357 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 Fig. S1.

Defibrinated sterile rabbit blood (50 ml) was added, mixed and 2 ml aliquots were dispensed into sterile Bijou tubes. Lesion aspirates were transferred into 2 ml volumes of Sloppy Evans medium, incubated at 26 °C and checked every 48 h for up to 1 month by phase contrast microscopy. Upon observing promastigotes, aliquots were transferred into liquid culture medium comprised of Medium 199 (12350-039, Life Technologies, UK) supplemented with 20% (v/v) FCS, BME vitamins (B6891, Sigma–Aldrich, UK) and 25 μg/ml of gentamicin sulphate. Cultures were expanded and sub-passaged as required and promastigotes cryopreserved in 7.5% glycerol at −80 °C and liquid nitrogen. The World Health organisation (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 ages ranged from infants to adults above 50 years (Supplementary Table S1). 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, six had two lesions and two 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 S2). 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 it may be due to some behavioural 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

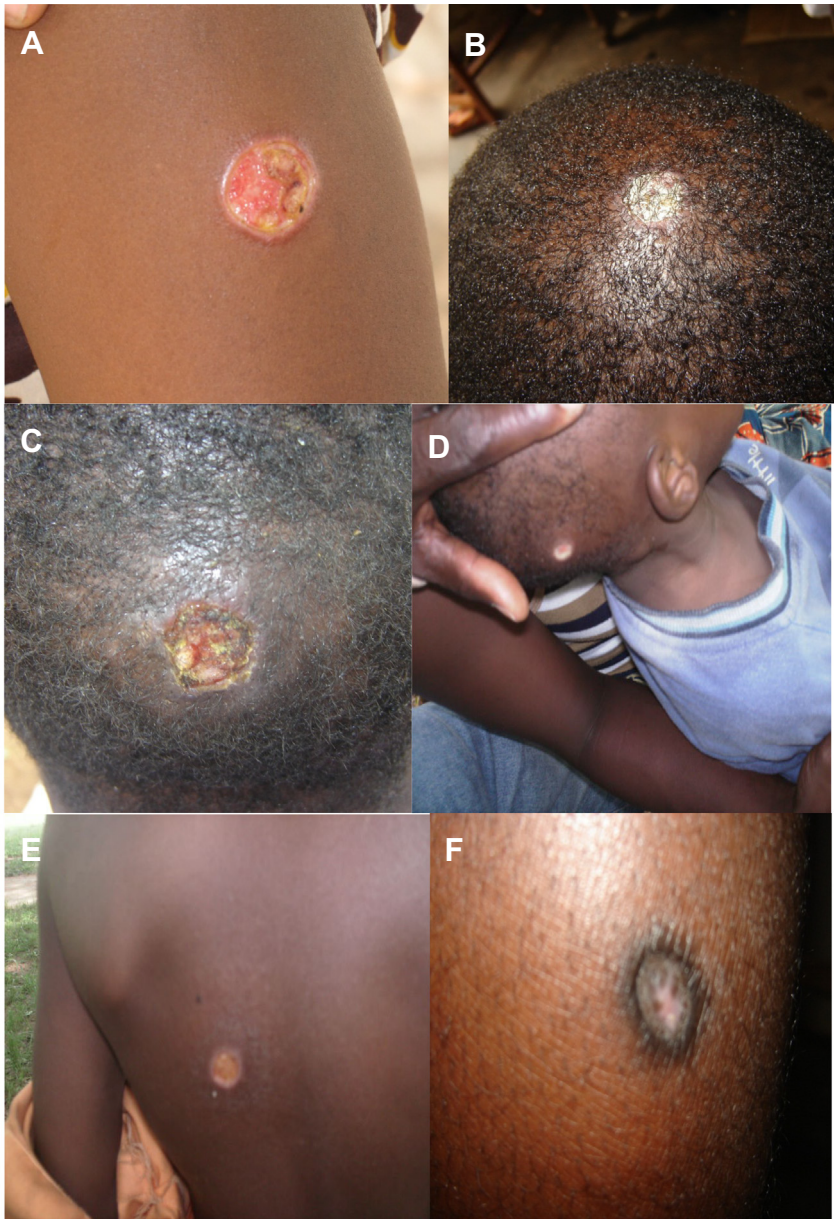

Fig. 2. Appearance of lesions from confirmed cases of human cutaneous leishmaniasis. Examples of typical active lesions are shown on several participants: (A) arm; (B–D) head; and (E) back. (F) An example of the scar developing in a healing lesion.

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 2012, peaking in September and declining to November (Supplementary Fig. S3). This peak in recruitment is approximately 3 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 on 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 that we 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* spp. 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 from those found with reference strains (Fig. 1C). In addition to the data shown, the banding pattern of the Ghanaian isolates was different from that seen in a wide range of additional reference strains examined, including *Leishmania infantum*, *Leishmania donovani*, *Leishmania amazonensis*, *Leishmania mexicana*, *Leishmania braziliensis* and *Leishmania 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 450–500 bp between two ribosomal protein L23a (RPL23a) genes (*L. major* chromosome 6), followed by sequencing, as used previously for phylogenetic analysis (Dougall et al., 2011). Each isolate generated a 468 bp product of identical sequence. Analysis of these sequences and comparison with the homologous sequences from a range of other *Leishmania* spp. in a maximum likelihood (ML) tree is shown in Fig. 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), *Leishmania martiniquensis* (Desbois et al., 2014), a new as yet un-named species from Australia (AM-2004) (Rose et al., 2004) and "*Leishmania 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 Fig. 3B. This tree is topologically very similar to Fig. 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 is *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* spp. 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 *Leishmania 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*. *Leishmania 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; Liautaud et al., 2014; Pothirat 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 is not regarded as *Leishmania* spp. 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* sp. in Ghana. Given the previous report of one case of

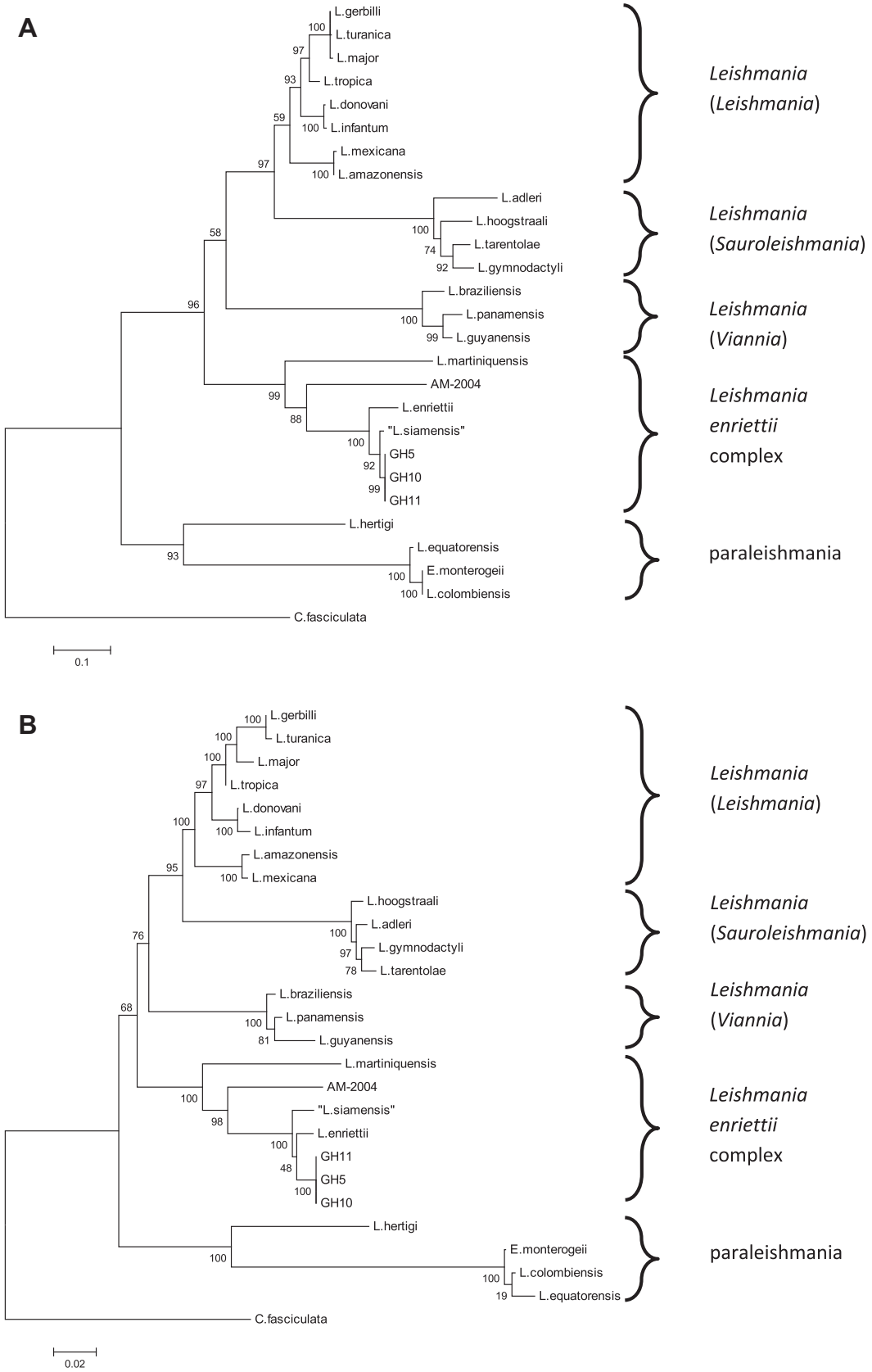

Fig. 3. Phylogenetic analysis of Ghanaian *Leishmania*. (A) Maximum likelihood tree based on ribosomal protein L23a intergenic sequences, with 22 species of *Leishmania* and *Endotrypanum monterogeii*, and using *Crithidia fasciculata* as an outgroup, based on alignment of 405–547 homologous nucleotide sequences. AM-2004 is an un-named species of *Leishmania* from Australia. The accession numbers for the sequences used are given in Supplementary Table S3 and the various subgenera and other groups are as indicated. Bootstrap values from 1000 replicates are given at the nodes. (B) ML tree based on RNA Polymerase II large subunit (RNAPolII) gene sequences, with 22 species of *Leishmania* and *E. monterogeii*, using *C. fasciculata* as an outgroup, based on alignment of 1191–1200 homologous nucleotide sequences. Accession numbers are given in Supplementary Table S3. Further technical details are given in Supplementary Data S1.

L. major infection (Fryauff et al., 2006), we cannot exclude the possibility that other *Leishmania* spp. 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 District 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2015.05.001.

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