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# The zoonotic potential of Oesophagostomum bifurcum in Ghana. Epidemiological, morphological and genetic studies

Gruijter, J.M. de

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# CHAPTER 5

# GENETIC SUBSTRUCTURING WITHIN *OESOPHAGOSTOMUM BIFURCUM* (NEMATODA) FROM HUMAN AND NON-HUMAN PRIMATES FROM GHANA BASED ON RANDOM AMPLIFICATION OF POLYMORPHIC DNA ANALYSIS

J. M. de Gruijter, J. Ziem, J. J. Verweij, A. M. Polderman, R. B. Gasser

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**Abstract**

Random amplification of polymorphic DNA (RAPD) was used to study genetic variation within *Oesophagostomum bifurcum* in Ghana. Four different decamer primers were used for the amplification of DNA from individual *O. bifurcum* adults (n = 41) from humans and non-human primates (including the Mona monkey, Patas monkey and Olive baboon) from different geographical regions. Analysis of the amplicons from all 41 nematodes by high resolution, denaturing polyacrylamide gel electrophoresis defined a total of 326 informative RAPD bands. Cluster analysis of the RAPD data (based on pairwise comparison of banding profiles) showed that *O. bifurcum* from humans was genetically distinct from *O. bifurcum* from the Mona and Patas monkey, and from the Olive baboon. These findings clearly demonstrate the existence of population genetic substructuring within *O. bifurcum* from different primate hosts in Ghana, raising interesting questions about host specifity, epidemiology (e.g. zoonotic transmission) and ecology of the different genotypes of *O. bifurcum*.

## Introduction

Human infection with *Oesophagostomum bifurcum* (Nematoda: Strongylida) is recognized as a parasitic disease with major health significance in northern Togo and Ghana.[25,44] The infection causes pathological effects that can result in two distinct clinical presentations.[26,37] The uninodular disease, also referred to as the 'Dapaong tumour', presents as a painful, abdominal mass with a diameter of 2-11 cm, frequently adhering to the abdominal wall. The multinodular disease is associated with hunderds of pea-sized nodules in a thickened, edematous submucosa and subserosa of the large intestine. In spite of the serious health problems caused by *O. bifurcum*, there are major gaps in our knowledge of the epidemiology and transmission of human oesophagostomiasis.[24]

Although it has been proposed that some species of non-human primates can act as reservoir hosts for human oesophagostomiasis,[39] there is a significant difference in the geographical distribution of the infection between human and non-human primates in Ghana (chapter 2). Also, there is evidence that significant variation in morphological characters of the adults of *O. bifurcum* from human and non-human primates exists (chapter 3).[20] These observations have suggested population variation within *O. bifurcum*, and have stimulated investigations into the genetic diversity within *O. bifurcum* from human and non-human primates using molecular tools.

We recently used a PCR-based single-strand conformation polymorphism (SSCP) analysis to scan for nucleotide variability in the ribosomal second internal transcribed spacer (ITS-2) and part of the mitochondrial cytochrome *c* oxidase subunit 1 gene (p*cox*1) (chapter 4) of *O. bifurcum* from human and Mona monkey (*Cercopithecus mona*) hosts.[21,140] While some nucleotide microheterogeneity (representing population variation) was detected in these studies, no genetic differentiation between *O. bifurcum* from humans and the Mona monkey was detectable. Nonetheless, it is possible that genetic variation does indeed exist within *O. bifurcum* from human and non-human primate hosts, but this is not adequately reflected in the ITS-2 and p*cox*1 regions because they represent only a minute part of the nuclear and mitochondrial genomes, respectively, and/or because they are not sufficiently variable in sequence to demonstrate any differentiation. It was thus concluded that another molecular approach, such as the random amplification of polymorphic DNA (RAPD),[99,100] could provide more variable genetic markers.

The method of RAPD is based on the screening of the entire genome without the need for any prior sequence information. Although there has been some reservation about the reproducibility of results using this method,[101,102,104] it has been applied effectively to investigate genetic variation within and among a range of species of parasitic nematodes.[141-143] Importantly, RAPD results have proven to be reproducible when an increased stringency (i.e., an annealing temperature of > 45°C) is used in the PCR and amplicons are subjected to denaturing polyacrylamide gel electrophoresis.[144] In the present study, RAPD analysis was

employed for investigating genetic diversity among individual adults of *O. bifurcum* from humans, the Patas monkey (*Erythrocebus patas*), the Mona monkey (*Cercopithecus mona*) and the Olive baboon (*Papio anubis*) from different geographical regions in Ghana, in order to establish whether population genetic substructuring exists within *O. bifurcum*.

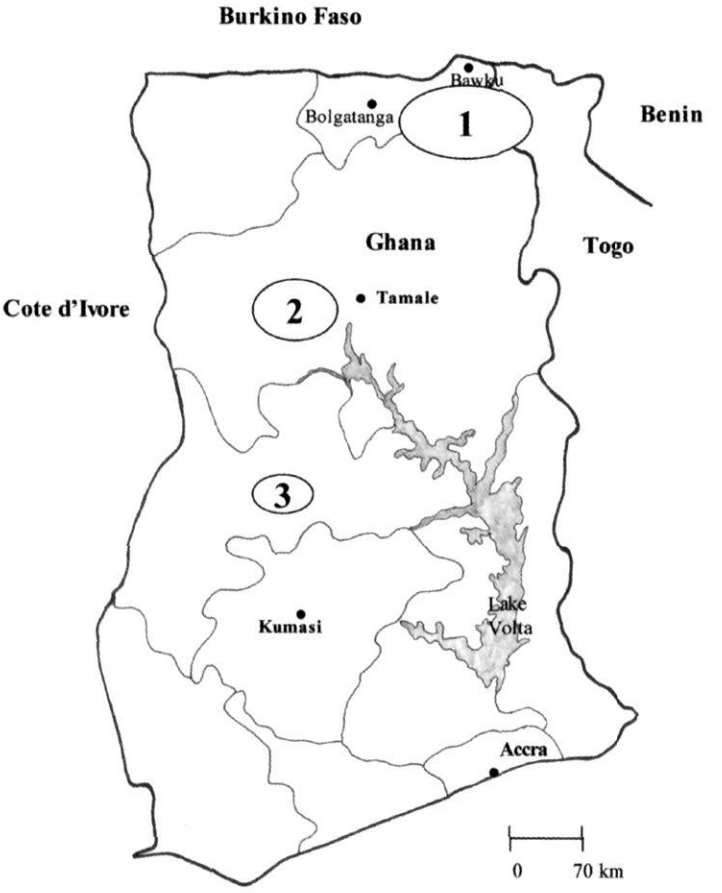
## Materials and Methods

*Parasites and isolation of genomic DNA*

Adult worms (n = 41) of *O. bifurcum* were obtained from human and non-human primate hosts from three different geographical regions in Ghana (see Fig. 1). The study was approved by the Ministry of Health in Bolgatanga (Ghana) and the Wildlife Division in Accra (Ghana). Informed consent for participation was obtained from all human adult participants and from parents of children less than 15 years of age. Worms were obtained from the faeces of infected patients after treatment with pyrantel pamoate, as described previously,[44] whereas worms from non-human primate hosts were removed from the large intestine at necropsy. The worms were washed extensively in physiological saline and then stored in 70% ethanol until required for DNA isolation. Each specimen of *O. bifurcum* was identified morphologically using published keys and descriptions.[12,20,127] Genomic DNA was isolated from individual worms by sodium dodecyl-sulphate/proteinase K digestion,[134] purified over spin columns (Wizard™ DNA Clean-Up; Promega, Madison, WI, USA) and eluted into 50 µl of $H_2O$. Purification and isolation of DNA from the large intestinal content from non-infected hosts (i.e., control DNA samples) were carried out as described previously.[45]

*Enzymatic amplification and single-strand conformation polymorphism (SSCP) analysis*

Since the sequence of the ITS-2 of ribosomal DNA (rDNA) allows the specific identification of *O. bifurcum*,[79,86] all individual worms (n = 41) used in this study were subjected to mutation scanning analysis of this rDNA region[21] to verify their identity prior to RAPD analysis. To do this, the ITS-2 was amplified by PCR using primers NC1 (forward: 5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3'). Primers were end-labelled with [$\gamma$-$^{33}$P]ATP (NEN Life Science Products, Boston, MA) using T4 polynucleotide kinase (Promega) prior to PCR. The PCR amplification was performed in 50 µl volumes using 25 pmol of each primer, 250 µM of each dNTP, 3 mM $MgCl_2$ and 2 U *Taq* polymerase (Promega) under the following conditions: after an initial denaturation at 94°C for 5 min, 30 cycles at 94°C, 30 sec (denaturation); 55°C, 30 sec (annealing); 72°C, 30 sec (extension), followed by a final extension at 72°C for 5 min in a thermocycler (Perkin Elmer




| Name of region | Sample code | Host (individual) |
|---|---|---|
| Region 1 | H30, H31, H38, H39 | Human (A) |
| (Endemic area for human | H32, H33, H49, H50 | Human (B) |
| Oesophagostomiasis) | H34-H37 | Human (C) |
| | H40-H43 | Human (D) |
| | H45-H48 | Human (E) |
| | PMN2, PMN6, PMN8, PMN9 | Patas monkey (F) |
| Region 2 | PMD1-PMD3, PMD7 | Patas monkey (G) |
| (Mole National Park) | B2-B5 | Olive baboon (H) |
| Region 3 | M4-M8, M10, M16, M18 | Mona monkey (I) |
| (Baobeng-Fiema) | | |

**Figure 1** Map of Ghana showing the geographical regions where *Oesophagostomum bifurcum* was collected from different primate hosts.

Cetus, Norwalk, CT). Samples without genomic DNA (no-DNA controls) were included in each amplification run. Also, control-DNA samples derived from the contents of the large intestine from a non-infected human, Mona monkey, Patas monkey and Olive baboon were subjected to the same amplification procedure as used for parasite DNA. In no case were products detectable in these control-DNA samples.

A volume of 10 µl of each PCR product was mixed with 3 µl of loading buffer (an aqueous solution of 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanole and 30% glycerol) and examined on ethidium bromide-stained 2% agarose-TBE (65 mM Tris-HCl, 27 mM boric acid and 1 mM EDTA, pH 9.0; Bio-Rad, Hercules, CA) gels using ΦX-174-*Hae*III (Promega) as a size marker. Subsequently, SSCP analysis was carried out as described previously.[97] Ten µl of each PCR product were mixed with an equal volume of loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanole). After denaturation at 95°C for 5 min and snap-cooling on a freeze block (-20°C), 2 µl of each sample was loaded into the wells of a 0.4 mm thick, non-denaturing gel (0.5 x MDE, mutation detection enhancement; FMC BioProducts, Rockland, ME), and subjected to electrophoresis in a conventional sequencing apparatus (BaseRunner; IBI, New Haven, CT). The conditions for electrophoresis (35 W for 5 h at 18°C) were standardised for optimum resolution of bands, and the gel concentration was as recommended by the manufacturer. After electrophoresis, gels were dried on to blotting paper and subjected to autoradiography using RP1 film (Agfa, Mortsel, Belgium).

Amplicons representing variable SSCP profiles were purified over spin columns (Wizard™ PCR-Prep, Promega) and sequenced in both orientations by automated sequencing (Big Dye chemistry, ABI; Foster city, CA) using primers NC13 (forward: 5'-ATCGATGAAGAACGCAGC-3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3'). Sequences were aligned manually and compared with the ITS-2 sequence of *O. bifurcum* from Togo and Ghana recorded previously.[79,145]

*Random amplification of polymorphic DNA (RAPD)*

The RAPD analysis was performed in 25 µl reaction volumes containing 125 pmol $\gamma^{33}$P end-labelled primer (Operon Technologies, Alameda, CA), 250 µM of each dNTP, 3 mM $MgCl_2$, 2 U *Taq* polymerase (Promega), and ~1 ng of genomic DNA. The cycling profile consisted of 35 cycles at 94°C, 2 min (denaturation); 48°C, 2 min (annealing); 72°C, 2 min (extension) in a thermocycler (Perkin Elmer Cetus). Control samples without DNA were included in each PCR run, and DNA isolated from the large intestinal content from non-infected hosts (i.e., human, Mona monkey, Patas monkey and Olive baboon) was subjected to the same amplification procedure as employed for parasite DNA. For agarose gel electrophoresis, individual RAPD products (10 µl) were loaded on 2% agarose gels and subjected to electrophoresis at 80 V for 2 h using TBE buffer (65 mM Tris-HCl, 27 mM boric acid, 1 mM EDTA, pH 9), stained with

ethidium bromide, and then detected using an Imago Compact Imaging System (Isogen Life Science, Maarssen, The Netherlands). A 100 bp ladder (Promega) was included on all gels. For denaturing gel electrophoresis (which achieved a substantially higher resolution than agarose gel electrophoresis), RAPD amplicons (25 µl) were mixed with 15 µl of loading dye, denatured at 94°C for 15 min, and snap cooled on ice. Subsequently, samples (1.3 µl) were loaded on to a 5% denaturing polyacrylamide gel and subjected to electrophoresis at 55 W for 2 h and 15 min at 55°C using TBE buffer. Gels were dried on to blotting paper and exposed to RP1 film (Agfa). The pGEM marker (Promega) was used as a size standard on every gel.

Statistical and cluster analyses were carried out using the software program FreeTree, (available at www.natur.cuni.cz/flegr/programs).[146] Similarity coefficients were calculated according to the method of Nei and Li,[147] and an unrooted dendrogram was constructed using the unweighted pair group method using arithmetic averages (UPGMA). Statistical support for the dendrogram was obtained by bootstrapping using 200 re-samplings, and bootstrap values of > 80% were considered significant.

**Results**

*Verification of the identity of individual O. bifurcum by SSCP analysis*

Agarose gel electrophoresis showed that there was no detectable size difference in the ITS-2 amplicons within or between *O. bifurcum* from human and non-human primates. Autoradiographic exposure of the agarose gels indicated the specificity of the PCR products and conditions in that each product appeared as a single band and no non-specific background bands could be detected. The SSCP analysis of the 41 *O. bifurcum* samples revealed 14 different profiles, each consisting of 2-4 strong bands and 1-4 additional weak bands. Subsequent sequencing of amplicons representing each of the 14 profiles revealed 5 polymorphic nucleotide positions (99, 105, 112, 117 and 162) that were consistent with some recorded previously for the ITS-2 of *O. bifurcum* from humans from Togo (accession number Y11733).[79] Thus, there was no unequivocal sequence difference in the ITS-2 between any of the 41 *O. bifurcum* individuals from Ghana and those from previous studies in Togo and Ghana,[55,79] providing genetic evidence that all specimens represented *O. bifurcum*.

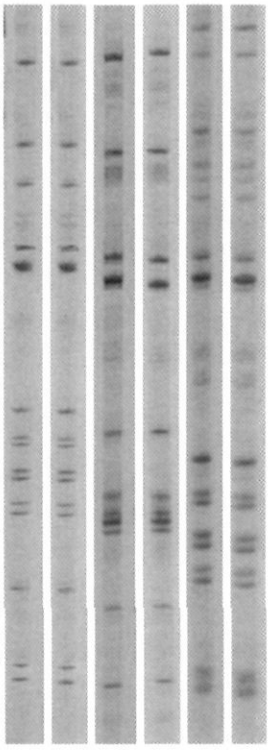
*Evaluation of RAPD primers and analysis on agarose gels*

Originally, 40 individual decamer primers (OPA1-OPA20 and OPB1-OPB20) were originally tested for their ability to amplify PCR products from genomic DNA (~1 ng) from individual adults of *O. bifurcum*. Different annealing temperatures (45°C, 48°C, 50°C, 52°C and 55°C) were tested, and 48°C achieved the highest amplification efficiency. Increasing the annealing temperature beyond 50°C resulted in a substantially decreased efficiency. Four primers (OPA-

10, OPB-1, OPB-6 and OPB-8) that gave reproducible and discrete banding patterns on agarose gels (using a PCR annealing temperature of 48°C) were selected for further RAPD analysis of *O. bifurcum* individuals. Amplicons for each of these 4 primers consisted of 2-7 strong bands ranging from 0.2-1.2 kb in size. Profiles for each primer (using 10 selected DNA samples) were shown to be reproducible on different days and in different laboratories (i.e., in the Department of Parasitology, Leiden University Medical Center, The Netherlands, and the Department of Veterinary Science, The University of Melbourne, Australia).

*RAPD analysis on high resolution denaturing polyacrylamide gels*

The RAPD analysis was performed on all 41 individual adults of *O. bifurcum*, (20 from human, 9 from the Mona monkey, 8 from the Patas monkey and 4 from the Olive baboon) from different geographical locations in Ghana (Fig. 1). Analysis (using each primer OPA-10, OPB-1, OPB-6 and OPB-8) on denaturing polyacrylamide gels gave reproducible results (i.e., banding profiles) for all individuals on consecutive days, using the same amplicons and different amplicons produced on different days. An example of the reproducibility of RAPD banding profiles for primer OPB-1 is shown in Figure 2.




No products were amplified from the no-DNA samples. Some amplicons were produced from samples containing DNA isolated from large intestinal contents from non-infected hosts (i.e., human, Mona monkey, Patas monkey or Olive baboon), but none of them were the same in their position on gels as those produced from any of the *O. bifurcum* individuals examined. Using primers OPA-10, OPB-1, OPB-6 and OPB-8, 320 polymorphic and 6 monomorphic bands (i.e., bands present in all 41 *O. bifurcum* samples analysed) ranging in size from 50-700 bp could be detected. Amplification with primer OPA-10 produced the most bands (n = 109) compared with OPB-1 (n = 79), OPB-6 (n = 74) and OPB-8 (n = 64). Using primer OPB-1, a polymorphic band of ~300 bp was detected that was common to all 20 *O. bifurcum* individuals from humans, but was absent from all 21 *O. bifurcum* specimens from non-human primates examined.

**Figure 2** An example of the reproducibility of RAPD profiles for three adult specimens (A-C) of *O. bifurcum*. Primer OPB-1 was used. Denaturing polyacrylamide gel electrophoresis of amplicons produced on different days (1-3) was carried out on different days.

Subsequently, for each of the 41 *O. bifurcum* samples subjected to analysis, the presence or absence of each of the 326 polymorphic bands was recorded, and a binary data matrix of the data was constructed. Based on these data, similarity coefficients were calculated and a dendrogram was constructed (see Fig. 3). Bootstrap values (greater than 80%) supported 3 main clusters. Cluster I comprised all 20 *O. bifurcum* individuals from humans, cluster II included all 9 *O. bifurcum* from the Mona monkey as well as all 8 from the Patas monkey, and cluster III comprised all 4 *O. bifurcum* individuals from the Olive baboon. Similarity coefficients among individual *O. bifurcum* within clusters I, II and III ranged from 0.33-0.63, 0.39-0.73, and 0.39-0.51, respectively. With the exception of the grouping of samples M16 and M18 (representing *O. bifurcum* from the Mona monkey) and that of PMD1 and PMD7 (representing *O. bifurcum* from the Patas monkey) within cluster II, there was no significant bootstrap support for subclustering within either cluster I, II or III. Also, there was no evidence for clustering according to geographical origin of each host species.

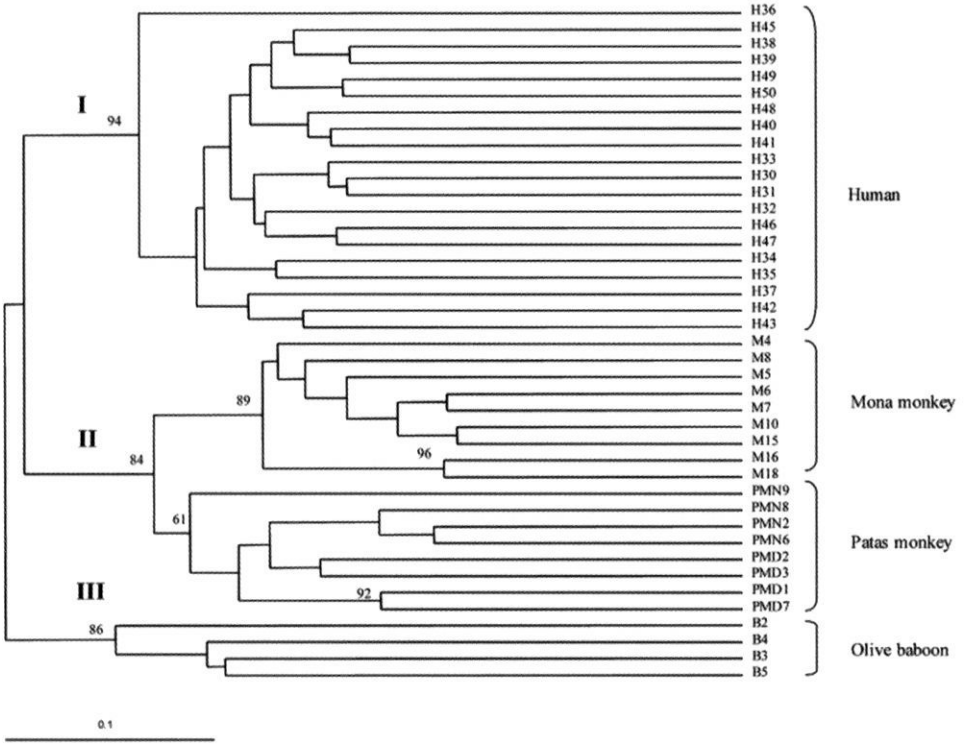



**Figure 3** Dendrogram based on cluster analysis of RAPD data for 41 individuals of *O. bifurcum* from humans (cluster I), the Mona or Patas monkey (cluster II) and the Olive baboon (cluster III) from Ghana. Similarity coefficients were calculated according to Nei and Li.[147] The branch lengths represent the genetic distances between the individuals, and the numbers on branches are bootstrap values (using 200 re-samplings).

**Discussion**

RAPD has been widely used as a genetic screening method[107,108,116,148] because it is rapid, relatively simple to perform and requires only a small amount (5-20 ng) of genomic DNA. In addition, it requires no genome sequence information prior to analysis, and can be applied to complex DNA of any origin.[99,100] The main limitation described for RAPD has been the lack of reproducibility. RAPD banding patterns can be affected by a number of factors, for example, the quality and quantity of template DNA, concentration of reagents, use of different thermocyclers and/or co-migration of non-homologous fragments.[101-104,149,150] However, the effect of these factors on the resultant banding patterns is largely due to the low annealing temperatures (25-35°C) used in the PCR. Thus, the use of increased annealing temperatures (45-55°C), as is the case in the present study, increases the stringency of the PCR reaction and thus substantially improves the reproducibility of RAPD results.[144,151] Also, the analysis of single-stranded RAPD products on denaturing polyacrylamide gels achieves a much better resolution of DNA fragments compared with analysis of double-stranded RAPD products on agarose gels, and improves reproducibility of banding patterns.[144,152,153] These findings are supported by the results of the present study.

The main objective of this study was to investigate the genetic make up of *O. bifurcum* from human and different species of non-human primates from Ghana by RAPD analysis. Together with a morphological study, SSCP-based analysis of the ITS-2 region demonstrated that all individuals included in this investigation represented *O. bifurcum*. Subsequent RAPD analysis, using primers OPA-10, OPB-1, OPB-6 and OPB-8, revealed a relatively high degree of polymorphism (320 polymorphic bands) among the individuals of *O. bifurcum* (n = 41) examined. Cluster analysis of the RAPD profile data (including a total of 326 RAPD bands) showed that *O. bifurcum* represented three distinct groups, namely those from humans, those from the Patas or the Mona monkey, and those from the Olive baboon. This result demonstrates clearly the existence of population genetic substructuring within the species *O. bifurcum* according to host species, and that *O. bifurcum* from human and non-human primates represent genetically distinct groups. The fact that *O. bifurcum* from humans (e.g., samples H30-H43) and from the Patas monkey (e.g., samples PMN2, PMN6 and PMN8) from geographical region 1 grouped into different clusters (i.e., clusters I and II, respectively) (Fig. 1 and 3) showed that there was no association between *O. bifurcum* genotype and the geographical origin of the host species based on the RAPD data. This was also indicated for *O. bifurcum* from the Patas monkey (e.g., samples PMD1-PMD3, and PMD7) and from the Olive baboon (samples B2-B5) from region 2, which related to clusters II and III, respectively (Fig. 1 and 3).

Interestingly, the infection of humans with *O. bifurcum* appears to be restricted to the extreme north of Togo and Ghana, where at least 250 000 people are infected.[23,26,44] The non-human primates in this geographical area are also infected but have significantly decreased in numbers over the last decades. In other locations further south in Ghana (e.g., Mole National Park and Baobeng-Fiema; Fig. 1), non-human primates remain numerous and in close contact with human settlements. There, the prevalence of infection in the non-human primates is high but humans have been found not to be infected.[45] To date, there is no explanation for this fascinating observation. The results of the present study (i.e., the existence of genetically distinct groups within *O. bifurcum* according to host species) suggest that the parasite of non-human primate hosts in Togo and Ghana may be unable to infect or inefficient in infecting the human host. Although the latter proposal is supported (to some extent) by an experimental study,[138] showing that non-human primates appear to be poorly susceptible to infection with *O. bifurcum* from humans, it cannot yet be ruled out that transmission of the parasite does not occur between humans and non-human primates in Togo and Ghana, and/or that at least some non-human primates may represent a natural reservoir for human infection in these countries. These proposals still require testing. Also, it remains unclear why *O. bifurcum* from humans is restricted to the extreme northern regions of Togo and Ghana.

The definition and characterisation of genetic markers for the differentiation of human *O. bifurcum* from non-human primate *O. bifurcum* is of significance for addressing epidemiological and ecological questions. In this study, RAPD analysis using primer OPB-1 showed one polymorphic band that was specific to *O. bifurcum* from humans. In future work, DNA of this band should be cloned and sequenced. Primers designed specifically to this band sequence will be evaluated in the PCR for the specific identification of *O. bifurcum* from humans. Such a specific PCR assay could be used to assess (by amplifying *O. bifurcum* egg DNA from the faeces of the host) whether non-human primates from Ghana can harbour the 'human genotype' of *O. bifurcum* and/or to undertake ecological studies of this genotype. Clearly, a better understanding of the transmission patterns of *O. bifurcum* could assist in the effective control of the parasite. In addition, AFLP™ analysis [119] will be conducted to examine genetic substructuring within *O. bifurcum*, in order to define additional genetic markers specific for *O. bifurcum* from different species of non-human primate hosts and from humans.