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Host-adapted *Cryptosporidium* and *Enterocytozoon bieneusi* genotypes in straw-colored fruit bats in Nigeria

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ABSTRACT

Few data are available on the distribution and human infective potential of *Cryptosporidium* and *Enterocytozoon bieneusi* genotypes in bats. In this preliminary study, we collected 109 fecal specimens during April–July 2011 from a colony of straw-colored fruit bats (*Eidolon helvum*) in an urban park (Agodi Gardens) of Ibadan, Nigeria, and analyzed for *Cryptosporidium* spp., *Giardia duodenalis* and *E. bieneusi* using PCR targeting the small subunit rRNA gene, triosephosphate isomerase gene, and ribosomal internal transcribed spacer, respectively. Genotypes of these enteric parasites were determined by DNA sequencing of the PCR products. Altogether, 6 (5.5%), 0 and 16 (14.7%) specimens were positive for *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi*, respectively. DNA sequence analysis of the PCR products indicated the presence of two novel *Cryptosporidium* genotypes named as bat genotype XIV (in 5 specimens) and bat genotype XV (in 1 specimen) and one known *E. bieneusi* genotype (Type IV in 1 specimen) and two novel *E. bieneusi* genotypes (Bat1 in 13 specimens and Bat2 in 2 specimens). In phylogenetic analysis of DNA sequences, the two novel *Cryptosporidium* genotypes were genetically related to Bat genotype II previously identified in fruit bats in China and Philippines, whereas the two novel *E. bieneusi* genotypes were genetically related to Group 5, which contains several known genotypes from primates. With the exception of Type IV, none of the *Cryptosporidium* and *E. bieneusi* genotypes found in bats in this study are known human pathogens. Thus, straw-colored fruit bats in Nigeria are mainly infected with host-adapted *Cryptosporidium* and *E. bieneusi* genotypes.

1. Introduction

Cryptosporidium spp., *Giardia duodenalis* and *Enterocytozoon bieneusi* are parasitic protists, causing diarrhea and other gastrointestinal symptoms in humans and animals (Wright, 2012). They are transmitted by direct contact with infected persons (anthroponotic transmission) or animals (zoonotic transmission) or through consumption of contaminated food or water (foodborne or waterborne transmission) (Checkley et al., 2015; Matos et al., 2012; Ryan et al., 2018).

Genetic diversity exists in each of the three groups of pathogens. Thus far, there are near 40 named *Cryptosporidium* species and as many unnamed species know as genotypes, each with some degree of host specificity (Feng et al., 2018). Similarly, there are at least eight genotypes of *G. duodenalis* known as assemblages A to H, which are likely cryptic species with different host ranges (Feng and Xiao, 2011). There are also over 250 *E. bieneusi* genotypes, forming at least 11 genotype groups with different host preferences (Santin et al., 2018; Zhang et al., 2018; Zhong et al., 2017). Only some of the species or genotypes are major human pathogens, such as *C. parvum* and *C. hominis* among *Cryptosporidium* spp., assemblages A and B in *G. duodenalis*, and Group 1 genotypes in *E. bieneusi* (Feng et al., 2018; Matos et al., 2012; Ryan et al., 2018). Molecular diagnostic tools are needed to differentiate the human-infective species and genotypes from animal-specific ones (Ghosh and Weiss, 2009; Xiao and Feng, 2017). As different human-pathogenic species and genotypes have distinct host range, the use of molecular diagnostic tools in epidemiologic investigations has significantly improved our understanding of the transmission of these pathogens in both industrialized and developing countries (Matos et al., 2012; Xiao and Feng, 2017).

Bats are known to play a major role in the transmission of emerging

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pathogens around the world (Han et al., 2015). This is especially the case with viruses such as coronavirus and rabies virus (Brook and Dobson, 2015). This is largely because of their large numbers, mobile nature, and tolerance to many of the pathogens (O'Connor, 2018; Serra-Cobo and Lopez-Roig, 2017). Their role in the transmission of *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi*, however, remains unclear. There have been a few recent studies on the identity of *Cryptosporidium* spp. in bats in Asia, Australia and Europe, which have identified the occurrence of 12 *Cryptosporidium* genotypes in bats, all of which appear to be bat-specific (Kvac et al., 2015; Murakoshi et al., 2016, 2018; Schiller et al., 2016; Wang et al., 2013). Thus far, there has been no study on *G. duodenalis* in bats, but six *E. bieneusi* genotypes were identified in bats in South Korea recently, one of which belongs to Group 1, with the remaining ones belonging to Group 2, which contains *E. bieneusi* genotypes mostly found in ruminants (Lee et al., 2018).

In this preliminary study, we have examined the occurrence and identity of *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* in straw-colored fruit bats in a popular public park in Ibadan, Nigeria.

2. Materials and methods

2.1. Specimens

The study was conducted with fecal specimens collected from straw-colored fruit bats (*Eidolon helvum*) living in the Agodi Gardens (N 07.40614; E 003.90073), a popular public park in central Ibadan, Nigeria (Fig. 1). It is located between a University College Hospital and a residential area and is 60.7 hectares in size. It had one single colony of bats with no interspecies co-roosting, with thousands of mixed ages of bats on trees with thick canopy. It was estimated that there were about 30,000 straw-colored fruit bats at the time of sampling, with no other species of bats in presence. Fecal droppings from bats hanging on tall forest trees were collected during April–July 2011 at 109 individual points. Only fresh droppings were collected from various locations on two separate occasions. They were stored at −20 °C prior to DNA extraction. There were no direct interactions between sampling personnel and animals at the time of sampling.

2.2. Detection of Cryptosporidium spp., G. duodenalis and E. bieneusi

DNA was extracted from 200 µl of stored fecal specimens using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA). This technique was shown to be better in removing PCR inhibitors in environmental samples than other common commercial DNA extraction kits (Jiang et al., 2005). The extracted DNA was stored at −80 °C before analysis by PCR. To detect *Cryptosporidium* spp., a ~830-bp fragment of the small subunit (*SSU*) *rRNA* gene was amplified by nested PCR, and *Cryptosporidium* genotypes were initially identified by restriction fragment length polymorphism (RFLP) analysis of the secondary PCR products using restriction enzymes *Ssp*I and *Vsp*I (New England BioLabs, Massachusetts, USA) (Xiao et al., 1999). To detect *G. duodenalis*, a ~530-bp fragment of the triosephosphate isomerase (*tpi*) gene was amplified by nested PCR (Sulaiman et al., 2003a). To detect *E. bieneusi*, a 392-bp fragment of the *rRNA* unit containing the entire internal transcribed spacer (*ITS*) was amplified by nested PCR (Sulaiman et al., 2003b). Each specimen was analyzed by PCR twice using 1 µl of

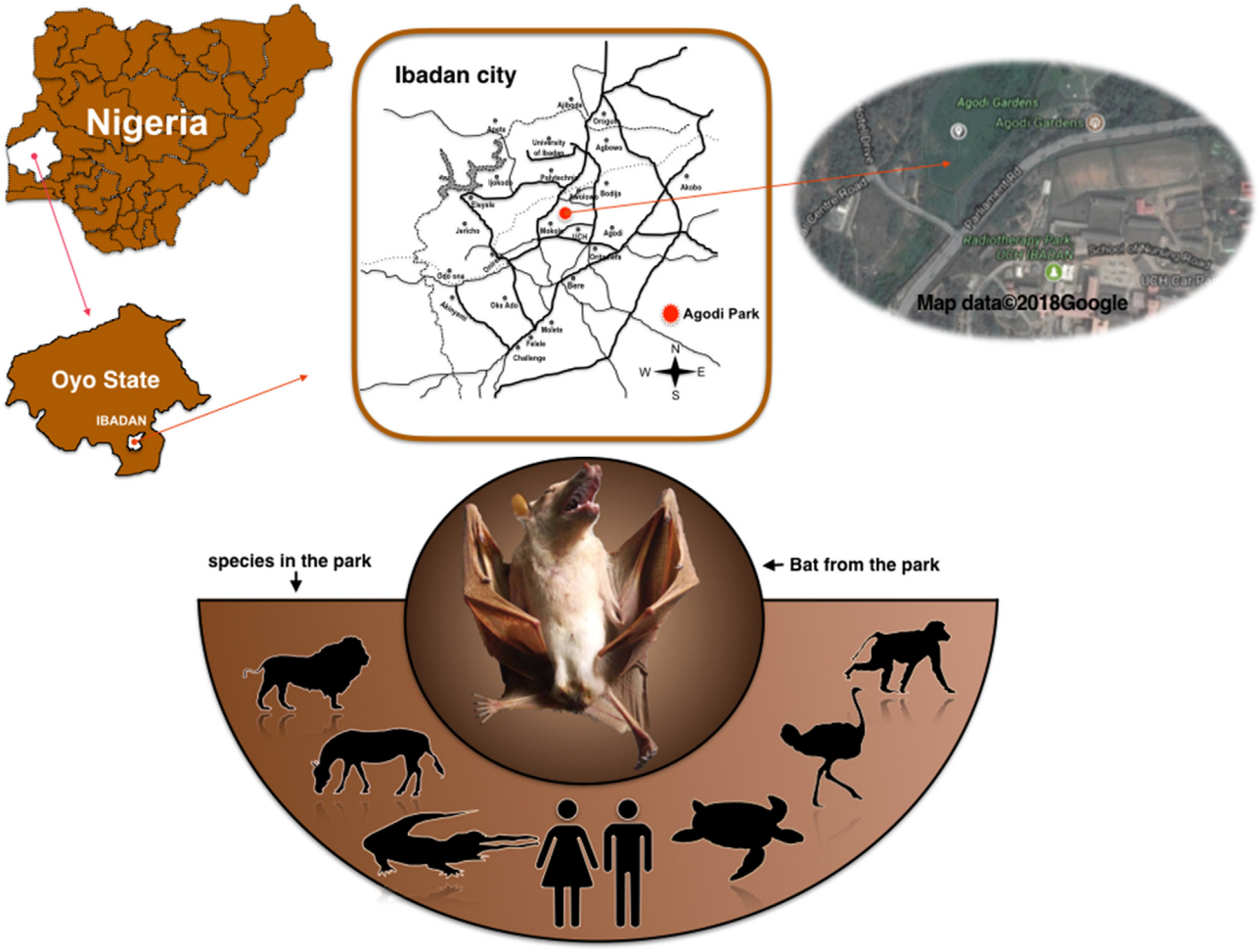

Fig. 1. Location of the straw-colored fruit bats examined in the study.

Table 1
Occurrence of *Cryptosporidium* spp., *Giardia duodenalis* and *Enterocytozoon bieneusi* in straw-colored fruit bats in Ibadan, Nigeria.

Pathogen	No. of specimens	No. positive (%)	Genotype
Cryptosporidium spp.	109	6 (5.5%)	Bat genotype XIV (5), Bat genotype XV (1)
G. duodenalis	109	0	–
E. bieneusi	109	16 (14.7%)	Type IV (1), Bat1 (13), Bat2 (2)

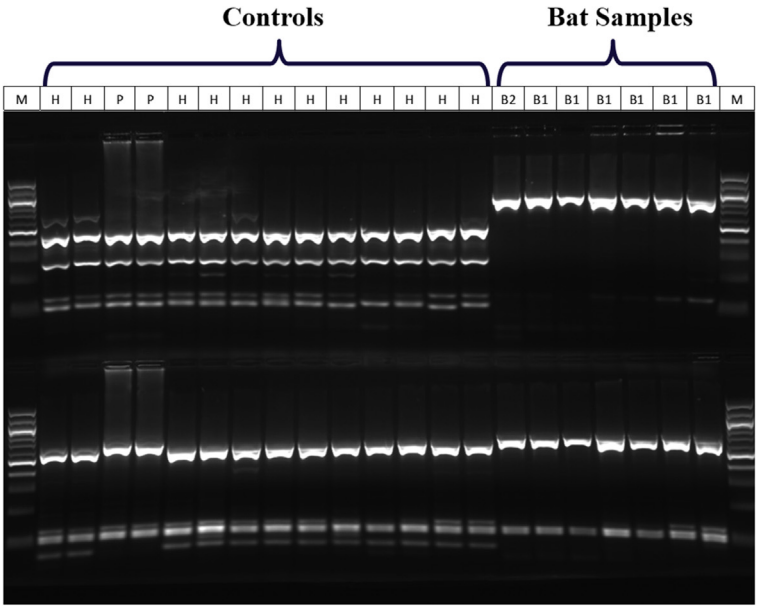

Fig. 2. Genotyping of *Cryptosporidium* spp. in straw-colored fruit bats by small subunit rRNA-based PCR-RFLP. Upper panel: *Ssp*I RFLP patterns; lower panel: *Vsp*I RFLP patterns; M: 100-bp molecular markers; H: *C. hominis* positive control; P: *C. parvum* positive control; B1: *Cryptosporidium* bat genotype XIV; B2: *Cryptosporidium* bat genotype XV.

extracted DNA per PCR, with DNA from *C. canis* as the positive control for the *SSU rRNA* PCR, DNA from *G. duodenalis* assemblage C as the positive control for the *tpi* PCR, and DNA from *E. bieneusi* genotype PtEb IX as the positive control for the *ITS* PCR. Two negative controls (reagent-grade water) for primary PCR and secondary PCR were further used in each PCR run. Non-acetylated bovine serum albumin (Sigma-Aldrich, St, Louis, MO, USA) was used at the concentration of 400 ng/μl in the primary PCR to neutralize residual PCR inhibitors in DNA, as previously described (Jiang et al., 2005).

2.3. Genotyping pathogen by sequence analysis

All positive secondary PCR products in this study were sequenced in bi-directionally using the Big Dye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA) on an ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences were assembled using ChromasPro 1.5 (http://technelysium.com.au/ChromasPro.html) and compared with reference sequences in NCBI database using ClustalX (http://clustal.org/) to determine the genotypes of pathogens of *Cryptosporidium* spp. and *E. bieneusi* in fecal specimens. Phylogenetic trees were constructed from the nucleotide sequence alignments using the Bayesian inference and Monte Carlo Markov Chain (MCMC) methods in MrBayes 3.2.6 (http://nbisweden.github.io/MrBayes), with the posterior probability (pp) values calculated by running 1,000,000 generations. Nucleotide sequences generated from the study were submitted to GenBank under accession numbers MK007969-MK007974.

3. Results

3.1. Occurrence of Cryptosporidium spp., G. duodenalis and E. bieneusi

Among the 109 specimens analyzed, 6 (5.5%) were positive for *Cryptosporidium* spp. by PCR analysis of the *SSU rRNA* gene and 16 (14.7%) were positive for *E. bieneusi* by PCR analysis of the *ITS* (Table 1). In contrast, none of them were positive for *G. duodenalis* by *tpi*-based PCR.

3.2. Cryptosporidium genotypes in bats

RFLP analysis of the *SSU rRNA* PCR products revealed a unique banding pattern for the *Cryptosporidium* spp. detected. All *SSU rRNA* products produced the same banding pattern, with the *Ssp*I restriction enzyme failing to digest the PCR product, while the *Vsp*I restriction enzyme producing a banding pattern that was similar to *C. parvum* (Fig. 2). DNA sequence analysis revealed the presence of two novel *Cryptosporidium* genotypes that were closely related to each other and to *Cryptosporidium* bat genotype II (Fig. 3). They were named as bat genotypes XIV and XV. The former was found in five specimens while the latter was found in one specimen. They differed from each other by about 28 single nucleotide polymorphisms (SNPs). Within *Cryptosporidium* bat genotype XIV, one specimen (No. 32604) produced a sequence that had one two-nucleotide insertion, one four-nucleotide deletion and one two-nucleotide deletion in two polymorphic areas of the *SSU rRNA* gene compared with other bat genotype XIV specimens.

3.3. E. bieneusi genotypes in bats

DNA sequence analysis showed the presence of three genotypes among the 16 *E. bieneusi*-positive specimens, including one known one

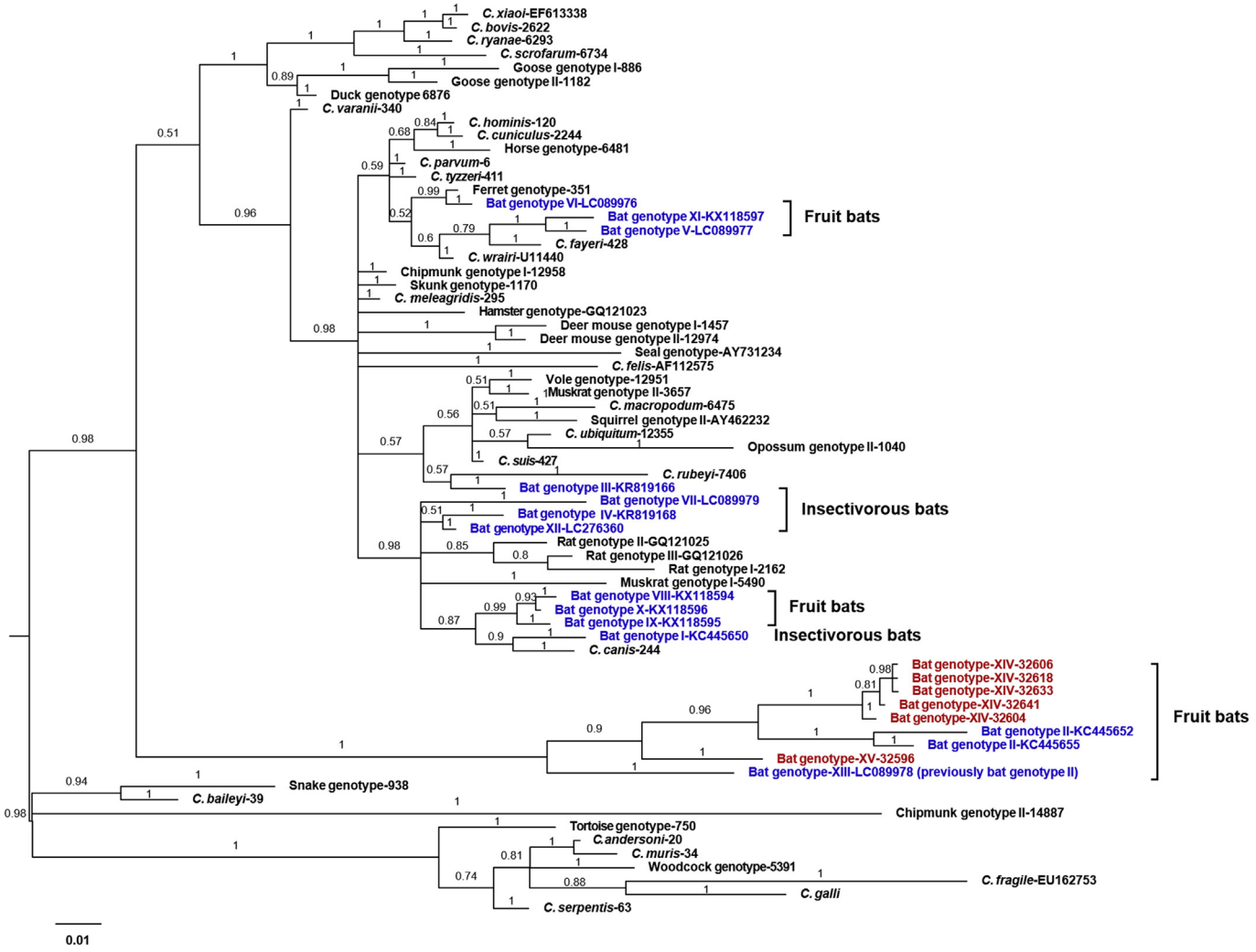

Fig. 3. Phylogeny of *Cryptosporidium* genotypes in bats based on Bayesian inference of sequences of the small subunit rRNA gene. The posterior probability values are indicated on the branches. Red ones are *Cryptosporidium* genotypes identified in straw-colored fruit bats in the present study, while blue ones are known *Cryptosporidium* genotypes previously reported in bats. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and two novel ones. The former included Type IV, which was found in one specimens, while the latter were represented by two closely related genotypes named as Bat1 and Bat2, which differed from each other by 6 SNPs and were found in 13 and 2 specimens, respectively. Phylogenetically, the two new *E. bieneusi* genotypes belonged to Group 5, which contains several known genotypes from primates, such as CAF4, PtEb XII and KB-6 (Fig. 4).

4. Discussion

Results of this preliminary study have shown the occurrence of *Cryptosporidium* spp. and *E. bieneusi*, but not *G. duodenalis*, in fruit bats living in an urban public park in Nigeria. The 5.5% infection rate for *Cryptosporidium* spp. is in line with the 2.1–8.9% infection rates of *Cryptosporidium* spp. reported in previous studies of several species of bats in Australia, USA, Czech Republic, China and Philippians (Kvac et al., 2015; Murakoshi et al., 2016; Schiller et al., 2016; Wang et al., 2013). The 14.7% detection rate of *E. bieneusi* in fruit bats examined in this study was significantly higher than the 1.9% detection rate of *E. bieneusi* in eight species of bats analyzed recently in South Korea (Lee et al., 2018). *Giardia duodenalis* was not examined in any of the previous studies of enteric protozoa in bats. Despite the use of a PCR assay that is designed to detect divergent *Giardia* species (Sulaiman et al., 2003a), we failed in obtaining any expected PCR products, indicating that *G. duodenalis* is not common in the bat species examined in Nigeria.

The two *Cryptosporidium* genotypes found in bats in this study are not known human pathogens. This agrees with previous characterizations of *Cryptosporidium* spp. in bats in several countries, where mainly novel *Cryptosporidium* genotypes were detected in both fruit bats and insectivorous bats (Kvac et al., 2015; Murakoshi et al., 2016, 2018; Schiller et al., 2016; Wang et al., 2013). Human-pathogenic *C. hominis* and *C. parvum*, however, were identified in four bats in Australia, Czech Republic, and Australia (Kvac et al., 2015; Schiller et al., 2016). Similarly, the two common *E. bieneusi* genotypes found in fruit bats in this study are not known human pathogens, only with one fruit bat positive for Type IV, a common zoonotic *E. bieneusi* subtype in humans (Matos et al., 2012). Thus, in contrast to their role in the transmission of emerging pathogens, bats can be only minor reservoirs of human-pathogenic *Cryptosporidium* spp. and *E. bieneusi*.

The two novel *Cryptosporidium* genotypes identified in straw-colored fruit bats in Nigeria appear to be host-adapted *Cryptosporidium* spp. Phylogenetically, the two *Cryptosporidium* genotypes in this study formed the basal branch of the *SSU rRNA*-based Bayesian inference tree, together with *Cryptosporidium* bat genotypes II and XIII previously identified in fruit bats in China and Philippines (Murakoshi et al., 2016; Wang et al., 2013). Similarly, bat genotypes V and XI from fruit bats in

Fig. 4. Phylogeny of *Enterocytozoon bieneusi* genotypes in bats based on Bayesian inference analysis of sequences of the internal transcribed spacer of the rRNA gene. The posterior probability values are indicated on the branches. Red ones are *E. bieneusi* genotypes identified in straw-colored fruit bats in the present study. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Australia and Philippines formed another cluster (Murakoshi et al., 2016; Schiller et al., 2016), while bat genotypes VIII, IX, and X from fruit bats in Australia formed a third cluster (Schiller et al., 2016). In contrast, other six *Cryptosporidium* genotypes identified in insectivorous bats are mostly dispersed over the *SSU rRNA*-based tree, probably as a results of the more diverse nature of their hosts (Fig. 3). As only a small number of bat species have been examined in a few countries, more novel *Cryptosporidium* genotypes are likely to be identified in future.

The two novel *E. bieneusi* genotypes identified are very divergent from common human-pathogenic *E. bieneusi* genotypes, which are mostly belong to Group 1. In phylogenetic analysis of the *ITS* sequences, they formed a cluster with several *E. bieneusi* genotypes from primates. They, however, have 10–13 SNPs compared to other genotypes in the group, thus could be bat-adapted *E. bieneusi* genotypes rather than related genotypes from primates. The host specificity of the various *E. bieneusi* genotype groups described thus far appears to be less strict than previously believed (Guo et al., 2014).

In conclusion, this preliminary study has shown the occurrence of *Cryptosporidium* spp. and *E. bieneusi*, but not *G. duodenalis*, in straw-colored fruit bats in an urban public park in Nigeria, mostly with the host-adapted *Cryptosporidium* and *E. bieneusi* genotypes. More genetic characterization of the pathogens in divergent host species, geographic areas, and environmental settings are needed to have better understanding of the prevalence, host specificity and evolution of *Cryptosporidium* spp., *Giardia* spp., and *E. bieneusi* in bats.

Declaration of interest

We have no conflict of interest to declare with this work.

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