

Ornithodoros savignyi, the Tick Vector of “*Candidatus* *Borrelia* *kalaharica*” in Nigeria

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ABSTRACT Endemic tick-borne relapsing fever (TBRF) has not been documented in Nigeria, yet clinically compatible cases have been described, and soft tick species are endemic in surrounding countries. Consequently, our aim was to investigate if TBRF-associated *Borrelia* is present in Nigeria. To address this, we examined 49 soft tick pools to identify the tick species and to screen for *Borrelia*. The tick species was revealed by 16S rRNA gene amplification and Sanger sequencing to be *Ornithodoros savignyi*, an aggressive, multihost, rapidly feeding species with significant veterinary impact. We detected a *Borrelia* organism in 3 of 49 pooled samples (6%). Molecular analysis of amplified 16S rRNA and flagellin genes and intragenic spacer fragments disclosed that this *Borrelia* organism was synonymous with the recently described organism “*Candidatus* *Borrelia* *kalaharica*,” identified in a tourist returning to Germany from South Africa. Given the widespread endemic range of this tick vector, TBRF should be considered part of the differential diagnosis for patients with fever returning from arid areas of Africa and further afield.

KEYWORDS relapsing fever, *Borrelia*, tick-borne relapsing fever, TBRF, *Ornithodoros savignyi*

Application of molecular approaches has resulted in a knowledge explosion regarding relapsing fever borreliosis. The causative organisms are notoriously challenging to isolate, and clinical cases present without characteristic diagnostic hallmarks that would alert a clinician to consider relapsing fever as part of the differential diagnosis. Many cases are misdiagnosed as other, more commonly encountered conditions, such as malaria (1). Once considered, it has been found that tick-borne relapsing fever (TBRF) can have an alarmingly high prevalence and can result in significant impacts on the health of populations, particularly within developing countries, such as the situation in Senegal (2).

Our knowledge of species diversity and understanding of the ecology and epidemiology of TBRF and its vectors are rapidly expanding (3). Typically, relapsing fever spirochetes are transmitted by soft *Ornithodoros* tick species, though *Borrelia recurrentis*, a louse-borne relapsing fever (LBRF) agent, and the newly recognized species *B. miyamotoi* are transmitted by clothing lice and hard *Ixodes* tick species, respectively, and thus form notable exceptions. Epidemiological knowledge of which species are prevalent in which countries is similarly evolving. As part of this emerging knowledge, new and poorly characterized species are being described, largely through investigation of arthropod vectors and reservoir/accidental vertebrate species hosts. Examples include descriptions of borreliosis in bats and penguins (4–6). Whether these species will have relevance for human health remains to be resolved and might follow the pattern seen for *B. miyamotoi*, where the spirochete was initially described in 1994 but human infections were not recognized until 2011 (7). Conversely, human infection can

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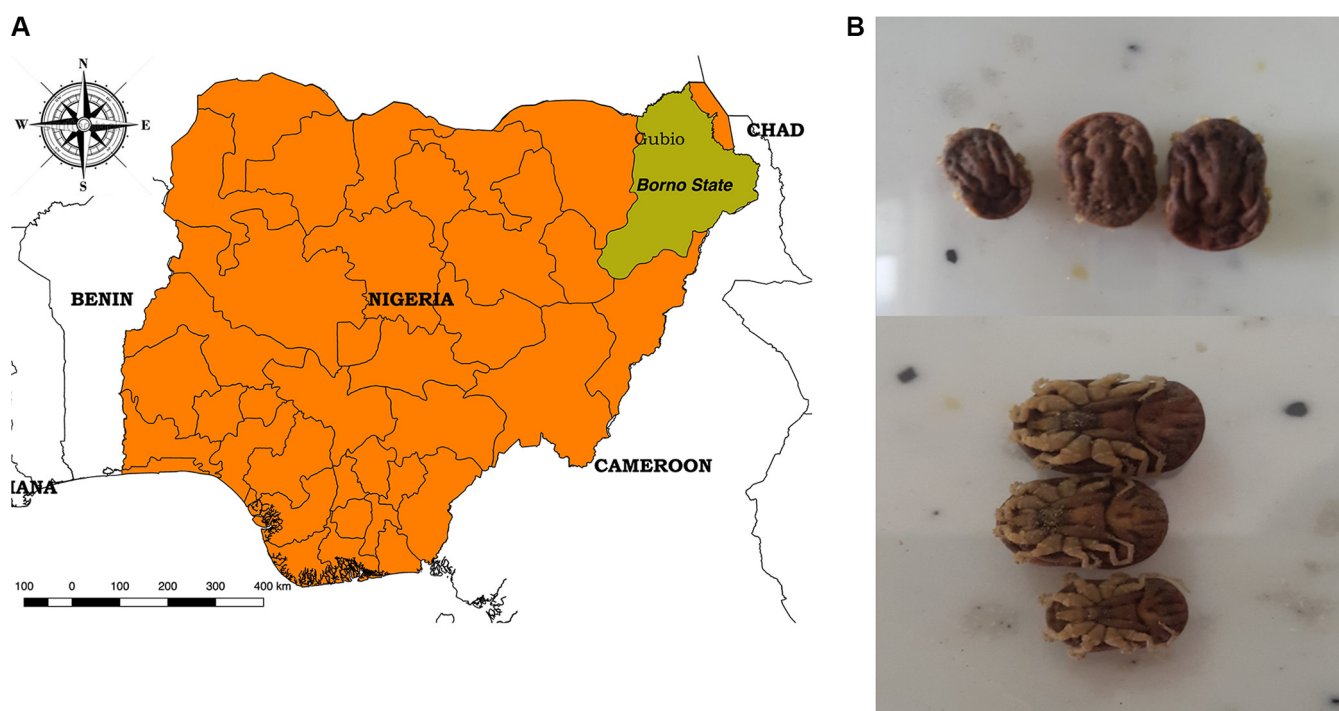


FIG 1 (A) Study location (Gubio, Borno State, Nigeria). (B) Dorsal and ventral views of ticks collected during this study.

serve as a sentinel for detection of novel species. Indeed, this was recently the case for detection of a novel TBRF species endemic to Iran (8, 9) and, more recently, with the report of a new TBRF species, "*Candidatus* *Borrelia kalaharica*," in a tourist returning from a holiday in southern Africa (10). This patient from Germany had clinical signs compatible with relapsing fever, and raised clinical awareness in this region, probably through recent introductions of LBRF among the influx of African refugees into Germany (11, 12), potentially aided diagnosis. What had not been determined was the tick vector and, consequently, our understanding of the potential epidemiological spread of this newly described *Candidatus* species.

Clinical descriptions of potential TBRF cases have emerged from Nigeria (local newspapers/personal communication); however, diagnostic methods in this resource-poor setting were not able to substantiate these claims. Extensive studies of *Ornithodoros* tick species have not included Nigeria (13), thus leaving a knowledge gap regarding the presence (if any) of *Ornithodoros* ticks in Nigeria. Local Nigerian studies have described an abundance of what was believed to be *Ornithodoros moubata*, the East African vector of TBRF, describing this tick as infesting up to 80% of households/animal dwellings and markets (14). Others have reported the presence of soft ticks belonging to the species *Argas persicus* or *Argas walkerae*, in addition to both *O. moubata* and *Ornithodoros savignyi* ticks, infesting poultry in Nigeria (15). Collectively, the presence of an *Ornithodoros* vector and the occurrence of compatible clinical cases prompted this study to investigate whether TBRF is present in Nigeria.

MATERIALS AND METHODS

Tick samples. Ticks were collected by sieving earth, by use of standard kitchen food sieves, from around human and animal shelters and market areas in Gubio Town, Gubio LGA, in the Borno State region of Nigeria (Fig. 1A and B). Collected ticks were pooled by life stage, with 47 pools containing 1 to 5 ticks each and the remaining 2 pools containing unspecified numbers of nymphal ticks. These were surface cleansed with sterile saline prior to homogenization using a mortar and pestle. Samples were vortexed and subjected to overnight digestion with proteinase K and DNeasy ATL buffer (20 μ l and 180 μ l, respectively; Qiagen) at 56°C. Samples were again vortexed and DNA extracted according to the DNeasy kit protocol (Qiagen). DNA extracts from 50 pooled tick samples were then shipped to the University of East London for molecular analysis. One sample had leaked in transit, leaving 49 for analysis. Upon receipt, sample purity was checked using NanoDrop spectrophotometry (Thermo Scientific), which

revealed that the extracts still contained excessive protein, and samples were consequently reextracted using DNeasy kits prior to analysis.

Tick identification. Because DNA was received, morphological identification of ticks was not possible, and consequently, molecular approaches were used. Various primers used previously to characterize tick identity, targeting the tick 16S and 18S ribosomal genes, COI and Cox1 genes, and internal transcribed spacer ITS2, were employed and used according to published methods (16–20). Details of the primers and their use are given in Table 1. Primers described by Dupraz and team (18) were used at a final concentration of 500 nM together with MgCl₂ at 2.5 mM, whilst those described by Lv and coworkers (20) were used at 300 nM together with MgCl₂ at 1.75 mM. DNA extracted from an *Ixodes ricinus* tick was used as a positive control, while nucleotide-free water served as a negative control. Buffer, magnesium chloride, deoxynucleoside triphosphates (dNTPs), and recombinant *Taq* polymerase were all supplied by Invitrogen (Fisher Scientific, United Kingdom). Amplifications were done using conventional PCR in Bio-Rad T100 thermocyclers, with amplicons resolved in 1% agarose gels stained with SybrSafe (Invitrogen), and results were captured by use of an imaging system (ChemiDoc; Bio-Rad). Amplicons were cleaned by use of a PCR cleanup kit (Qiagen) prior to being submitted for Sanger sequencing at DBS, Durham University Sequencing Service. All amplicons were sequenced in both directions.

Screening and identification of *Borrelia*. Tick DNA extracts were screened using a real-time genus-specific PCR targeting the conserved 16S rRNA gene of *Borrelia* (21). Briefly, primers were used at 700 nM, while the HEX- and BHQ-labeled probe was used at 100 nM; dNTPs were used at 0.2 mM (each), while 5 mM MgCl₂ was used with single-strength buffer and recombinant *Taq* polymerase (Invitrogen). Reaction mixtures were made up to a final volume of 25 µl, which contained 2 µl of template DNA. Amplification was performed using an Aria Mx1.2 (Agilent) thermocycler, using a hot start of 95°C for 10 min and 40 cycles of 95°C for 15 s and annealing at 60°C for 30 s.

Following amplification, samples yielding positive results were subjected to conventional PCR for the 16S rRNA gene, the flagellin gene *flaB*, and *uvrA*, and intragenic spacer (IGS) assays were performed as previously described (10, 22–24). Purified DNA from *Borrelia burgdorferi sensu stricto* (strain B31) was used as a positive control, while nuclease-free water served as the negative control. The PCR products were subsequently prepared for Sanger sequencing as described above.

Analysis of data. Resulting sequences were analyzed using MEGA7 software to assess the quality of sequencing, produce multiple-sequence alignments, and undertake phylogenetic comparisons by use of the neighbor-joining algorithm with a 1,000-bootstrap test of confidence (25). Comparisons of resulting sequences and other sequences from the GenBank repository were performed by using the similarity search tool BLAST with its default settings.

Accession number(s). A representative *O. savignyi* 16S rRNA gene sequence has been deposited in GenBank under accession number [MG256662](#).

RESULTS

Most assays used for tick identification remained negative, despite successful amplification of the positive control, an *I. ricinus* tick (collected in the United Kingdom) that produced an amplicon of the expected size for all assays. NanoDrop assessment of samples revealed poor purity, which might account for the variable amplification success. The exception was the tick 16S rRNA gene assay described by Lv and coworkers, which successfully produced amplicons for 23 of the 49 pools (47%) (20).

Of these tick pools, a convenience sample of eight was prepared for Sanger sequencing of the 16S rRNA gene. All sequences were found to be identical over the 431 bp generated. Similarity BLAST searches demonstrated that the tick identity was *Ornithodoros savignyi* (sand tampan). The compatibility of our sequences with that of *O. savignyi* is shown in Fig. 2.

In total, 3 of the 49 pooled tick DNA samples were positive for *Borrelia* (6.1%) by the genus-specific 16S rRNA gene real-time screening PCR (21). Removing the two pools with unspecified numbers of nymphs from analysis, and based on actual numbers of ticks within the pools, the minimum prevalence was 2.7% (95% confidence range, –0.36% to 5.76%), and the maximum prevalence was 7.4% (95% confidence range, 2.46% to 12.34%). Attempts to further characterize this *Borrelia* organism by conventional PCR generated amplicons from the 16S rRNA gene (555 bp from 3 samples; representative sequence [MG255295](#)) (Fig. 3), the flagellin gene (749 bp from 2 samples; sequences [MG257488](#) and [MG257489](#)) (Fig. 4; also see Fig. 6), and the IGS (726 bp from 2 samples; representative sequence [MG257909](#)) (Fig. 5). A synonymous mutation was detected between the two flagellin sequences, at position 177, encoding lysine (AAA/AAG). None of the *Borrelia*-positive DNA extracts produced an amplifiable product for *uvrA*. BLAST searches of *flaB* for similarity revealed that the *Borrelia* species was highly similar to "*Ca. Borrelia kalaharica*," recently described for a German traveler returning from South Africa (10). The IGS sequences were highly similar to three sequences

TABLE 1 Primers, probes, and thermocycling conditions used for this study

	Primer or probe sequence (5'–3')			
Gene target	Forward	Reverse	Thermocycling conditions	Reference(s)
Tick targets				
16S rRNA	CTG CTC AAT GAT TTT TTA AAT TGC	CCG GTC TGA ACT CAG ATC ATG TA	94°C for 3 min; 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 45 s; final extension at 72°C for 10 min	17, 18
18S rRNA	GCA AGT CTG GTG CCA GCA GCC	CTT CCG TCA ATT CCT TTA AG	94°C for 3 min; 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 45 s; final extension at 72°C for 10 min	16, 18
COI	GGA GGA TTT GGA AAT TGA TTA GTT CC	ACT GTA AAT ATA TGAT GAG CTC A	94°C for 3 min; 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 45 s; final extension at 72°C for 10 min	18, 19
ITS2	ACA TTG CGG CCT TGG GTC TT	TCG CCT GAT CTG AGG TCG AC	94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 68°C for 120 s; final extension at 68°C for 7 min	20
Cox1	GGA ACA ATA TAT TTA ATT TTT GG	ATC TAT CCC TAC TGT AAA TAT ATG	94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and elongation at 68°C for 60 s; final extension at 68°C for 7 min	20
COI	ATC ATA AAK AYH TTG G	GGG TGA CCR AAR AAH CA	94°C for 5 min; 5 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 30 s; 5 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 30 s; 5 cycles of 94°C for 30 s, 48°C for 30 s, and 68°C for 30 s; 25 cycles of 94°C for 30 s, 46°C for 30 s, and 68°C for 30 s; final extension at 68°C for 7 min	20
16S rRNA	TTA AAT TGC TGT RGT ATT	CCG GTC TGA ACT CAS AWC	94°C for 5 min; 5 cycles of 94°C for 30 s, 49°C for 30 s, and 68°C for 30 s; 5 cycles of 94°C for 30 s, 47°C for 30 s, and 68°C for 30 s; 5 cycles of 94°C for 30 s, 45°C for 30 s, and 68°C for 30 s; 25 cycles of 94°C for 30 s, 43°C for 30 s, and 68°C for 30 s; final extension at 68°C for 7 min	20
<i>Borrelia</i> targets				
16S rRNA	AGC CTT TAA AGC TTC GCT TGT AG	GCC TCC CGT AGG AGT CTG G	95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 30 s	21
16S rRNA (probe)	HEX-CCG GCC TGA GAG GGT GAA CCG-BHQ			21
FlaB (Bor1 and Bor2)	TAA TAC GTC AGC CAT AAA TGC	GCT CTT TGA TCA GTT ATC ATT C	94°C for 3 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and elongation at 72°C for 30 s; final extension at 72°C for 7 min	24
uvrA	GCG TTA TCT TWC AAC TGA ATC	TCT AGA CTC TGG AAG CTT	94°C for 3 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, and elongation at 72°C for 90 s; final extension at 72°C for 7 min	10
IGS (first round of nested PCR)	GTA TGT TTA GTG AGG GGG GTG	GGA TCA TAG CTC AGG TGG TTA G	94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 60 s; final extension at 72°C for 7 min	22, 23
IGS (second round of nested PCR)	AGG GGG GTG AAG TCG TAA CAA G	GTC TGA TAA ACC TGA GGT CGG A	94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 60 s; final extension at 72°C for 7 min	22, 23

previously reported from *O. moubata* ticks in Tanzania (accession numbers [DQ000284](#) to [DQ000286](#)) (tick extracts IM/16, IM/19, and IK/23) (Fig. 5) (23). These observations were further corroborated by the notable similarity to flagellin sequences from ticks and patients in Tanzania (Fig. 6) (26, 27). The 16S rRNA gene sequences showed poor

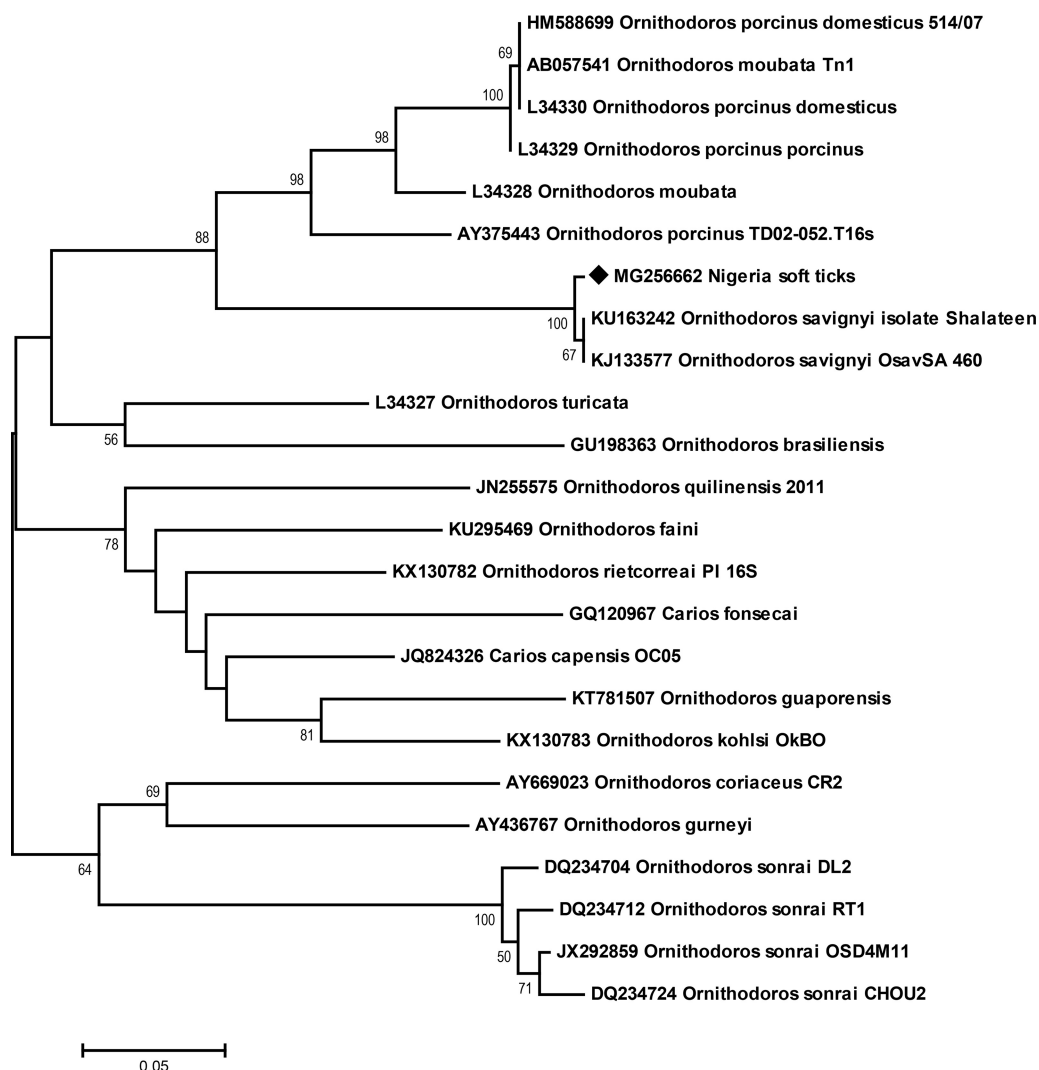


FIG 2 Neighbor-joining tree of tick-derived DNA sequences for the 16S rRNA gene (431 bp), comparing the Nigerian ticks with ticks of other *Ornithodoros* species. The optimal tree, with a sum branch length of 1.74331329, is shown. The bootstrap value was set to 1,000 replicates. Evolutionary distances were computed using the maximum composite likelihood method within MEGA7. The diamond identifies the Nigerian tick sequence.

discriminatory values and were thus phylogenetically less informative (Fig. 3). The relationships of amplicons produced in this study to those previously deposited are shown in Fig. 2 to 6. Collectively, our results indicate that the ticks were *O. savignyi* and that the *Borrelia* species found associated with these was "*Ca. Borrelia kalarharica*."

DISCUSSION

Epidemiological maps of *Ornithodoros* tick species overlaid with those carrying borreliae indicate an absence of both soft ticks and relapsing-fever-causing spirochetes in Nigeria (13). Despite this, clinical presentations compatible with TBRF occur. The ticks assessed were amplified by only one of the sets of primers used, those for the 16S rRNA gene (20), and only 47% of samples were successfully amplified with these primers. Whether the failure of other targets to be amplified rests in sequence heterogeneity or was a result of the poor DNA quality of the samples received remains to be resolved. The positive-control DNA, extracted from a single *I. ricinus* tick, demonstrated that the reagents and cycling conditions were appropriate.

Analysis of eight samples clearly demonstrated the tick identity to be *O. savignyi*, an aggressive, rapidly feeding, multihost tick species with a wide distribution in arid areas

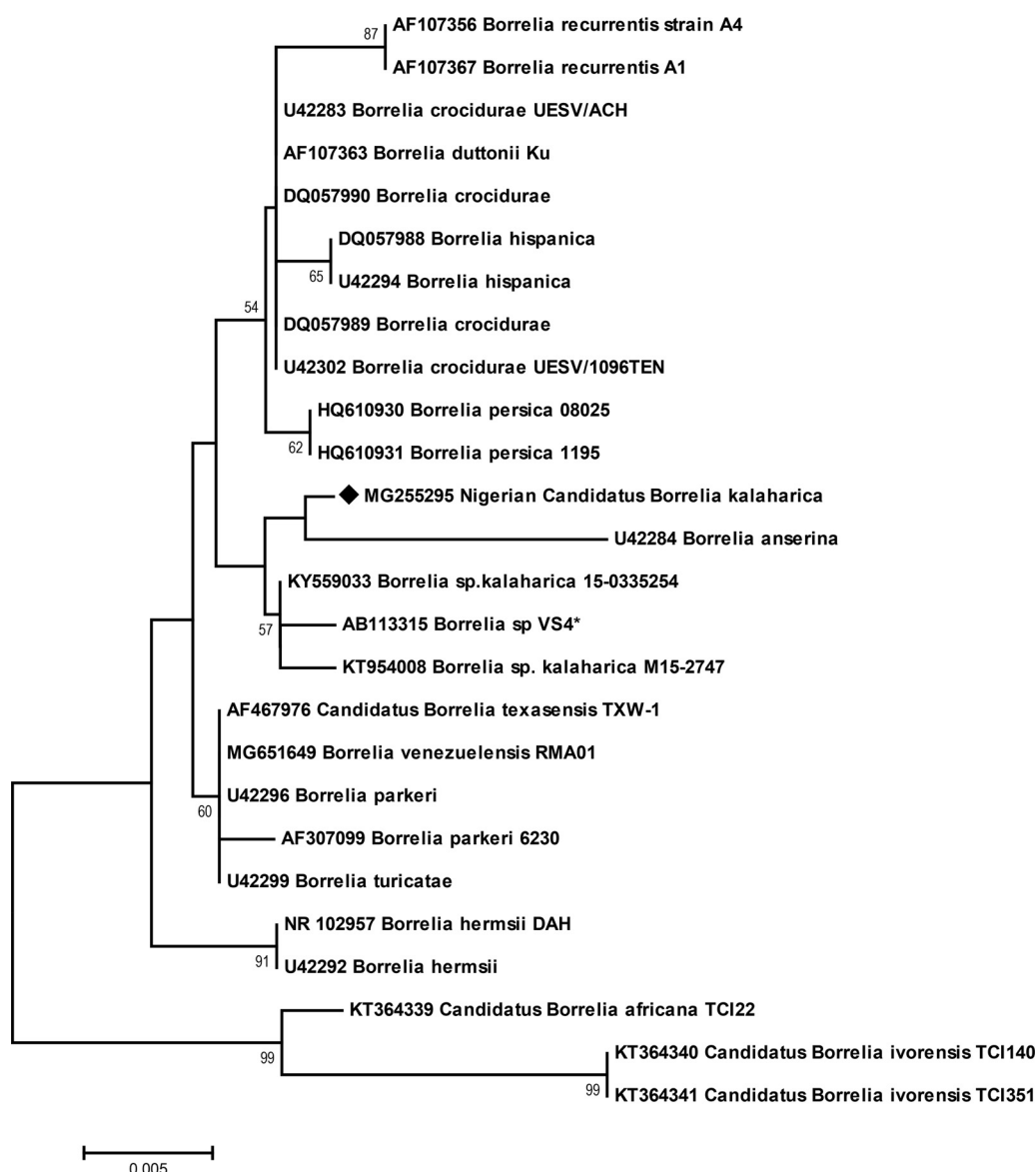


FIG 3 Neighbor-joining phylogenetic analysis of *Borrelia* 16S rRNA gene nucleotide sequences (475 bp). The optimal tree, with a sum branch length of 0.07300324, is shown. The bootstrap value was set to 1,000 replicates. Evolutionary distances were computed using the maximum composite likelihood method within MEGA7. The diamond identifies the Nigerian *Borrelia* sequence. *, *Borrelia* species erroneously deposited as *B. duttonii* in GenBank.

of Africa (including Egypt), Saudi Arabia, the Persian Gulf, India, and Sri Lanka. Five of these samples were derived from single ticks, whereas the remaining three were pools of three or four ticks, and thus it is not inconceivable for these to have contained mixed species. The sequence reads were of good quality and gave no evidence of mixed species, suggesting that our conclusions regarding identity are valid.

The *O. savignyi* tick frequents areas where livestock and humans seek shade, often hidden in the sandy earth under trees or in cracks and crevices of animal housing or surrounding areas where livestock congregate, such as markets. This tick is believed to have a life span of some 15 to 20 years and to survive periods of starvation of 5 to 6 years (28). To date, the *O. savignyi* tick has largely been overlooked as a vector of pathogens, with focus instead being placed on its toxic potential. This tick species has a major impact upon animal husbandry, with reports of mortality particularly among lambs and calves. More recently, it was proposed as a potential vector for Alkhurma hemorrhagic fever virus (29).

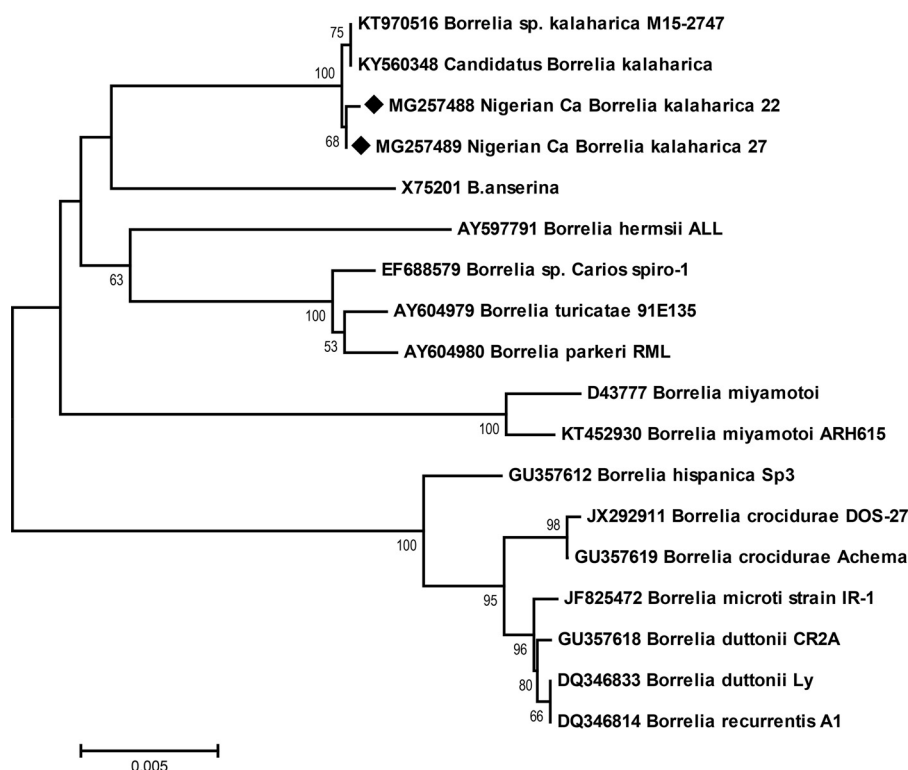


FIG 4 Neighbor-joining phylogenetic analysis of *Borrelia* flagellin gene (*flaB*) DNA sequences (655 bp). The optimal tree, with a sum branch length of 0.09695016, is shown. Bootstrap validation was conducted using 1,000 replicates. The diamonds identify Nigerian *Borrelia* sequences.

The question pertaining to the role of *O. savignyi* as a vector of relapsing fever is old, with prior reports believed to have erroneously attributed *O. savignyi* as the vector of TBRF in Africa. Investigation of 2,000 ticks and use of animal inoculation failed to substantiate these early claims, with *O. moubata* subsequently being established as the vector for TBRF (30). Interestingly, elegant infection studies of *O. savignyi* with *B. crocidurae* demonstrated successful transstadial transmission but not transovarial transmission, unlike the case for *Ornithodoros erraticus*, which was also able to show efficient transovarial infection rates (31).

More recently, a few reports of *Borrelia* associated with *O. savignyi* emerged from Egypt (32–34). Elegant studies by Shanbaky and Helmy demonstrated that the *Borrelia* species present in *O. savignyi* showed successful transstadial and transovarial transmissions analogous to those seen with the sympatric tick species in Egypt, *O. erraticus*, and *B. crocidurae* (33). Cross infection of *O. savignyi* with *B. crocidurae* and the *Borrelia* species of *O. savignyi* showed the ability of the organism to survive transstadially and to be infectious to hamsters, but less efficiently than the naturally associated tick-spirochete relationship, and failed to demonstrate transovarial transmission. These studies did not further characterize the *Borrelia* species. A later study suggested, somewhat surprisingly, that the *Borrelia* species present in *O. savignyi* belongs to the *B. burgdorferi sensu lato* complex (34). This has not subsequently been confirmed by others.

During the current study, we similarly detected *Borrelia* in *O. savignyi* ticks, albeit at a much lower prevalence rate (6.1% of pools, with a potential range of 2.7 to 7.4% among individual ticks). This might have reflected a genuinely low infection prevalence at the collection site or may be a consequence of the poor quality of DNA received for analysis, with only 23 of the 49 samples yielding tick 16S rRNA gene amplicons. The three samples positive for *Borrelia* were also ones demonstrating amplicons for the tick 16S rRNA gene, and thus the prevalence among these samples was 13% (3/23 samples).

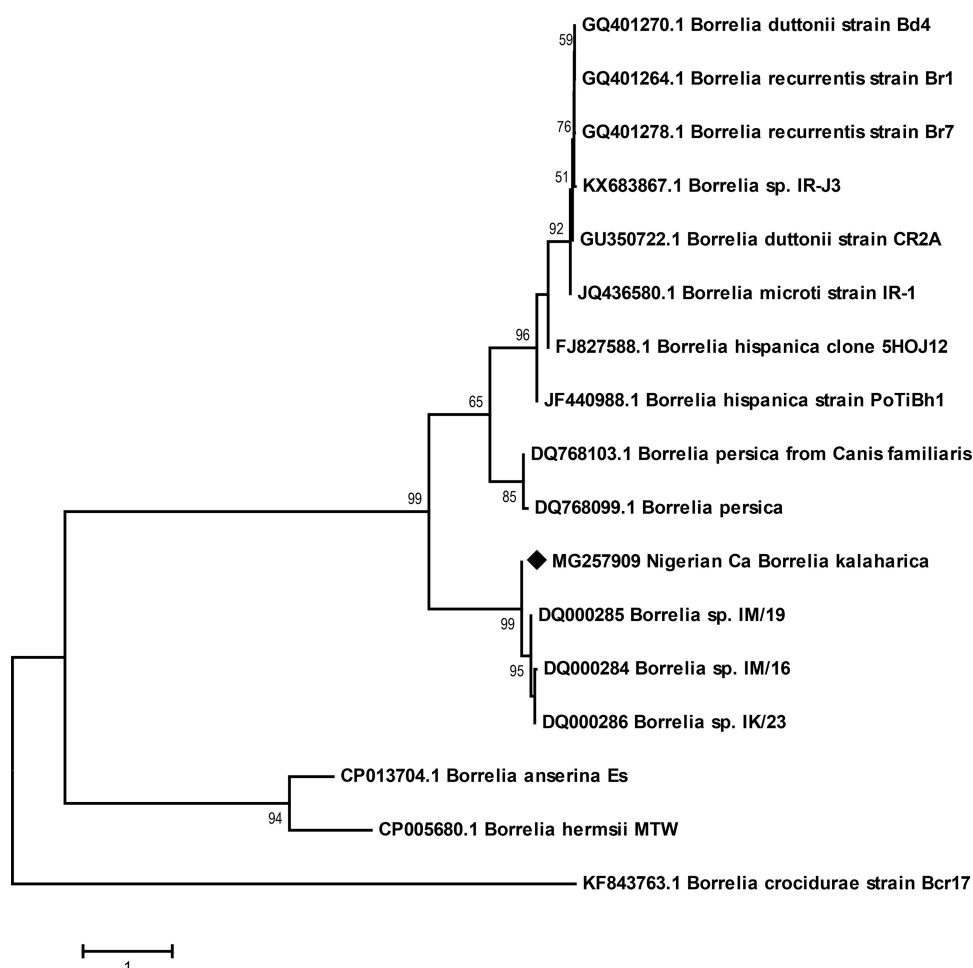


FIG 5 Neighbor-joining phylogenetic analysis of *Borrelia* intragenic spacer (IGS) DNA sequences (622 bp). The optimal tree, with a sum branch length of 18.18022296, is shown. Bootstrap validation was conducted using 1,000 replicates. Evolutionary distances were computed using the maximum composite likelihood method within MEGA7. The diamond identifies the Nigerian *Borrelia* sequence.

Analysis of the 16S rRNA gene highlighted the similarity of *Borrelia* to Nearctic species but was poorly discriminatory, as demonstrated by low bootstrap values, and thus was not able to accurately identify bacteria to the species level. Others have reported the poor discriminatory value of the 16S rRNA gene for identification of borreliæ (35). Both flagellin gene (*flaB*) and intragenic spacer (IGS) sequencing assays were more informative. Flagellin gene sequencing revealed a single polymorphism between the two sequences, but both were most like the proposed “Ca. *Borrelia kalaharica*” isolate, detected in a tourist returning from a holiday in South Africa (10). This patient was bitten by a “mite-like” arthropod from an area where *O. savignyi* is known to be endemic, and thus it is entirely possible that this tick species might have been the undocumented culprit in this case. Our sequence analysis, as in the report on “Ca. *Borrelia kalaharica*,” highlighted the similarity to Nearctic species and *B. anserina*, known to be present in the widely distributed *Argas* tick species (36). This closer link to *B. anserina* and Nearctic species was further confirmed by IGS sequencing. Surprisingly, the results showed the greatest similarity to sequences found among presumed *O. moubata* ticks from Ikombolinga and Iringa Mvumi, Dodoma Rural District, Tanzania (23). These ticks were collected from dwellings heavily infested by *O. moubata* and with a high infection prevalence of *B. duttonii*. The identities of ticks collected during that study were not confirmed by molecular methods or by entomological keys; however, similar strains were reported by *flaB* sequencing, whereby the ticks were confirmed to

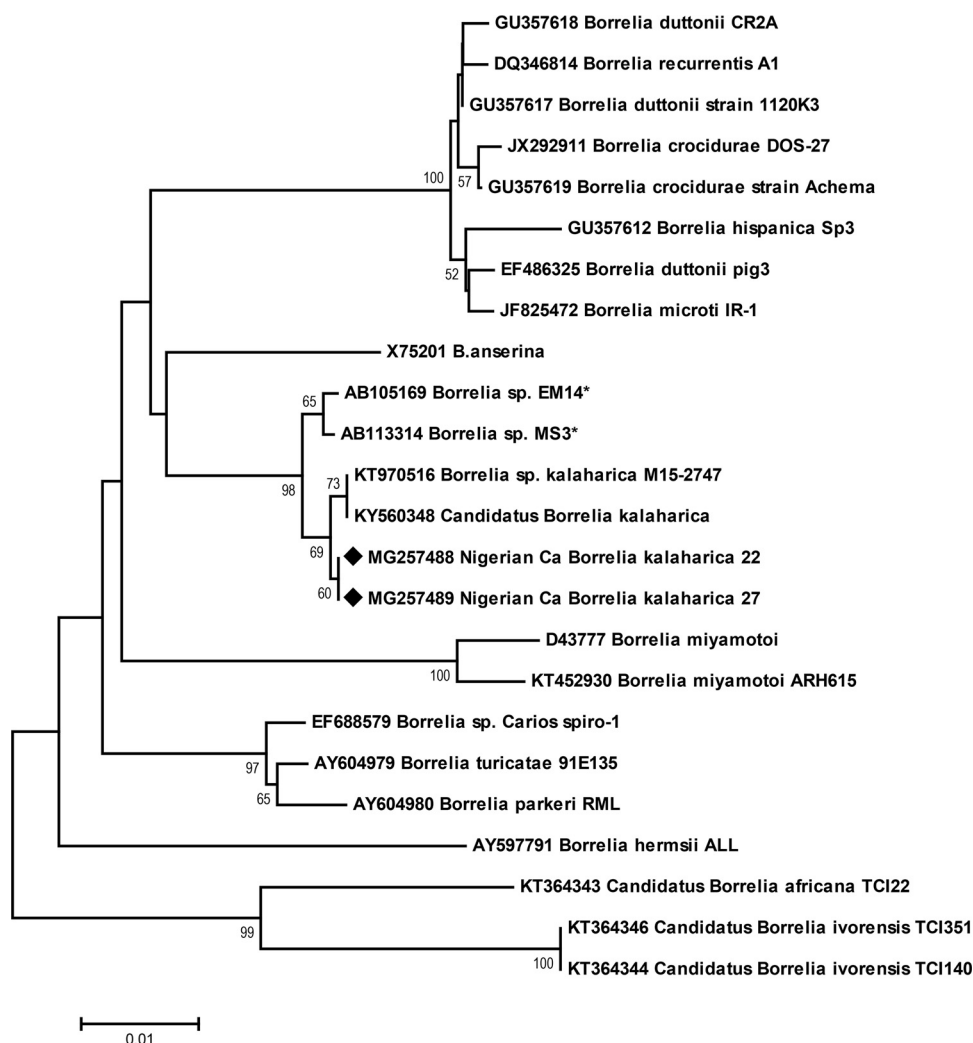


FIG 6 Neighbor-joining analysis of the flagellin gene DNA sequence of the Nigerian *Borrelia* organism, trimmed to 287 bp for comparison with newly described variant strains from Africa. The optimal tree, with a sum branch length of 0.26956810, is shown. Bootstrap validation was conducted using 1,000 replicates. Evolutionary distances were computed using the maximum composite likelihood method within MEGA7. The diamonds identify Nigerian *Borrelia* sequences. *, *Borrelia* species erroneously deposited as *B. duttonii* in GenBank.

be members of the *Ornithodoros porcinus* complex (which includes *O. moubata*) (37). Significantly, these *Borrelia* species have been documented from human cases in Tanzania (26, 27).

In conclusion, we have demonstrated the presence of *O. savignyi* ticks in Nigeria with evidence of infection with a *Borrelia* organism. This spirochete is highly related to "Ca. *Borrelia kalaharica*," suggesting that *O. savignyi* ticks are the vector for this species. The similarity to species present in *O. moubata* ticks in Tanzania is intriguing, as *Borrelia* species generally show strict vector associations. Whether both tick vectors are sympatric in this region or if indeed these *Borrelia* species are less vector specific than previously appreciated remains to be addressed. Importantly, the *Borrelia* organism reported here is capable of producing TBRF and has a geographically wide distribution, from Africa through the Middle East, and possibly beyond. Consequently, greater consideration of TBRF as part of the differential diagnosis among febrile patients from regions where *O. savignyi* ticks are present is essential for diagnosing this treatable infection.

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