Phylogenetic Relationship of Babesia Species Infecting Dogs from Select Regions in Kenya

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Short report

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Abstract

Background

There are over 100 *Babesia* species known to infect vertebrates with some of them being zoonotic. Local dog keeping practices enable extensive and intimate interactions between dogs, livestock, wildlife, and their human owners, thus allowing the possibility of dogs to act as hosts for zoonotic parasites.

Canine babesiosis, known to occur in Kenya causes a severe and debilitating illness in dogs which compromises their welfare and capacity to carry out their role in society. Published data on *Babesia* species circulating among dogs in Kenya is limited. Improved control measures such as vaccines are required against the disease.

Methods

The study design was descriptive and sampling opportunistic. A total of 143 whole blood samples were collected from domestic dogs in Nakuru, Nairobi and Mombasa counties. Total genomic DNA was extracted from each of the samples and screened for *Babesia* parasites using diagnostic PCR. *Babesia* species were identified through bioinformatic analysis of Sanger sequences.

Results

A total of 13 samples were positive for *Babesia* species (95% C.I is 0.0437 to 0.1381). Two were positive for *Babesia canis vogeli*, eleven were positive for *Babesia canis rossi*.77% of the *Babesia* positive samples were from Nairobi county.

Conclusions

The study confirmed that molecular methods can be utilized to detect the presence of *Babesia* species circulating among dogs in Kenya.85% of the *Babesia* positive samples were *Babesia canis rossi* which causes the most severe form of canine babesiosis. Results of the bioinformatic analysis indicate 98.29% to 99.52% sequence identity to *Babesia canis rossi* obtained from black-backed jackals (*Canis mesomelas*).*Babesia canis vogeli* although primarily known as a domestic dog parasite has been shown capable of infecting both domestic and wild felines. This demonstrates the capacity of dogs to serve as hosts for pathogens from wildlife and vice versa.

Background

Babesia species are Apicomplexan parasites that infect vertebrate red blood cells and are transmitted by ticks (1). There are more than 100 species in the genus *Babesia* which infect erythrocytes in different vertebrate hosts (2).*Babesia* species infect livestock, wildlife, and human beings and are found worldwide (3).*Babesia* infections are known to occur in Kenya affecting dogs, other livestock, and wildlife e.g. cattle,

the African buffalo (5, 6, 7, and 8). Published studies on *Babesia* species infecting dogs in Kenya are limited, however similar studies in Africa have been reported (33, 34, 35, 36).

Dogs in Kenya serve a variety of purposes including as livestock guarding and herding dogs, providing security for business premises and homesteads as well as serving as companion animals (6, 9, 10). The role the dog plays in society, as well as local, dog and livestock keeping, practices enable both extensive and intimate interaction between dogs, humans, livestock, and wildlife (11). With some *Babesia* species being zoonotic e.g. *B.microti, B.divergens*, it is important to identify the *Babesia* species circulating among dogs (3). This is especially in light of reports of transmission of the zoonotic *B.venatorum* to human beings where a dog was suspected to be involved in the transmission and source of infection (12).

Canine babesiosis is caused by various species in the genus *Babesia* which have been classified as either small or large piroplasms based on microscopic appearance (4).*Babesia* parasites cause a debilitating illness in dogs that compromises their welfare and reduces their capacity to carry out their role in society (13). Clinical signs of canine babesiosis include fever, tachycardia, lethargy, hemolytic anemia, anorexia, vomiting, jaundice, pallor of mucus membranes amongst others (35, 44). The severity of illness is determined by among other factors, the species of *Babesia* causing infection with *B.canis rossi* known to result in the most serious disease (14). Other *Babesia* species known to infect dogs include *Babesia canis vogeli, Babesia canis canis* and *Babesia gibsoni.* The veterinary treatment required for sick animals is both lengthy and costly further burdening their owners (4).

Current control measures against the disease rely on the use of acaricides against the tick vectors of *Babesia* piroplasms (4). The following tick species are known to be *Babesia* species vectors in dogs; *Haemophysalis elliptica*, *Rhipicephalus sanguineus*, *Dermercentor reticulatus* (43). Unfortunately, owner compliance with the regular washes necessary for tick control is poor with the effective acaricides being toxic chemicals with the potential to harm both owner and animals if not properly used (49). Continuous use of the acaricides also inevitably leads to the development of resistance within the target tick populations (45).

There is, therefore, a need for an effective and universal vaccine against the disease, to enhance the available control measures (15). This investigation seeks to determine the *Babesia* species circulating among dogs from the selected counties in Kenya; species identification and phylogenetic analysis to allow for tailored clinical interventions and lay the foundation for the future development of a vaccine against canine babesiosis.

Materials And Methods

Study Areas

Nairobi County

It is a highly urbanized county and the commercial and administrative capital of Kenya. It has an area of 696.3 km^2 with a high human population density of 4800/km^2 (16). There is a high level of farming activities, especially in the peri-urban zones. This includes semi-nomadic pastoralism of cattle, sheep, and goats, intensive rearing of dairy cattle, and poultry (16). Some districts within the county have significant numbers of free-roaming populations of goats,sheep,chicken, and other poultry e.g. ducks. Nairobi National park, located within the county is home to a large and diverse wildlife population, Cape buffalo, Maasai giraffe, Grants zebra, African leopard, lion, eland, impala, cheetah, etc (17). Sections of the park borders are porous allowing constant interaction between wildlife, livestock (including dogs), and human beings (18). Anecdotal reports and published studies indicate that there is a large population of dogs, both stray and owned within Nairobi County. Some of the owned dogs are not kept under confinement and are allowed to roam freely. The dog population comprises both exotic and indigenous breeds with the majority being indigenous (6, 19).

Mombasa County

The county is situated along the Indian Ocean coast of Kenya and consists of Mombasa Island and the immediate surrounding mainland areas. It has an area of 294.9 km^2 of which 65 km^2 is water. It has a human population of about 1.2 million. It is a highly urbanized county and is the 2nd largest city in Kenya (16). Farming activities are carried out in the peri-urban zones and include intensive poultry (broilers & layers) and dairy farming, free-roaming cattle, sheep, goats, and chickens are also observed in the suburban areas (16). There is a large population of dogs both owned and stray, a large percentage of the owned dogs are allowed to freely roam around (20).Commercial/semi-commercial and subsistence fishing is carried out in the Indian Ocean waters around Mombasa. Mombasa also serves as a landing and processing zone for commercial fishing vessels (16, 21).

Nakuru County

The county is located in the Rift valley region. Naivasha town within Nakuru county has a human population of 181,966.Naivasha area is dominated by major geographical features such as Lake Naivasha, a freshwater lake, and Mt. Longonot with an elevation of 2,776 m which is part of and surrounded by the Mt. Longonot National Park. The Park hosts a variety of wildlife including buffaloes, giraffes, plains zebra, Thomson's gazelle, and hartebeest (22).Naivasha is an important livestock farming area that includes intensive dairy cattle, poultry rearing and commercial beef cattle ranching. There is a significant population of free-roaming, and backyard cattle, sheep, goats, chicken and pigs. Nomadic pastoralism of cattle, sheep, and goats is also carried out within the area. There are significant fishing and related activities at L.Naivasha which is also an important wildlife area with hippopotamus and numerous bird species (16). There is a large population of dogs, both stray and owned especially in the peri-urban areas where a significant proportion of the owned animals are allowed to roam freely (23).

Sampling

The study employed a descriptive design, where sampling was opportunistic. A total of 143 dogs were sampled. In all 3 counties, samples were collected from dogs presented at the shelter facilities of the Kenya Society for the Protection & Care of Animals®.The samples were collected from both apparently healthy dogs with others being symptomatic with a variety of ailments.These dogs were of diverse backgrounds including stray, loosely owned, surrendered, and confiscation cases. The dogs were also of varied age, sex, and breed (24). Age assessment was carried out using a combination of criteria, including teeth, size of the dog, hair coat, the state of ocular lens (46, 47). Sex was determined by visual inspection of the external genitalia (48). The breed was established by visually comparing the dog's physical attributes against established breed characteristics (http://www.eastafricakennelclub.com).

Approximately 2 ml of blood was collected from the cephalic vein of each dog and loaded into 4 ml EDTA tubes and refrigerated at 4°C as previously described (25)

Table 1
Total collected samples indicating sex, age and breed

Sex		Age			Breed	
Male	Female	J	A	O	Local	Exotic
94	49	41	79	23	112	31

DNA extraction and characterization

Total DNA was extracted using the TanBead Automated DNA extractor. For extraction process optimization, two protocols were used; viral DNA extraction protocol and optipure protocol for blood DNA.300 µl of whole blood were drawn from 6 randomly selected samples, and 10 µl of Proteinase K was then added to each of the 6 samples. This mixture was then transferred to the TanBead extractor for total DNA extraction.DNA yield determined through agarose gel electrophoresis and Nanodrop Spectrophotometry indicated the better quantity and quality obtained from the optipure protocol. Therefore the rest of the samples were extracted using the optipure protocol. The extracted DNA was stored at -20̊C. (26)

Polymerase Chain Reaction (PCR)

To optimize PCR conditions for the screening PCR, total DNA was extracted according to the manufacturer's instructions (using QIAGEN DNeasy kit® for blood and tissue using the nucleated tissue protocol) from blood drawn from a dog clinically diagnosed with babesiosis, the protocol was altered after adding lysis buffer, where the incubation temperature was set at 56°C at 1000 rpm for 12hrs. The extracted DNA sample was then diluted to 25 ng/µl using triple distilled Milli-Q water (26, 27).

Gradient PCR was used to determine optimum diagnostic PCR conditions. Master mix was as follows 2x AccuPower master mix 5 µl, pF 0.33 µl, pR 0.33 µl,g DNA 2.5 µl,double distilled water 1.84 µl for a total volume of 10.0 µl.Diagnostic PCR conditions were as follows 95°C for 5 min,94°C for 30sec,52°C for 1 min,72°C for 1 min,72°C for 10 min,15°C ∞ .A 1.5% gel was prepared to run the PCR products at 135V

for 30 min. The results were visualized using a gel documentation system. (27)All the DNA samples were screened using the established diagnostic PCR conditions. The primers used were as outlined in Table 2

Table 2
Primers used, their sequence, length, target gene and species

Primer	Oligo Sequence	Gene	Target Species
18S_rDNA_BTH_F	5'-CCT GMG ARA CGG CTA CCA CAT CT-3' (23mer)	18S rDNA	*Babesia* genus
18S_rDNA_BTH_R	5'-TTG CGA CCA TAC TCC CCC CA-3'(20mer)	18S rDNA	*Babesia* genus
18S_rDNA_GF1	5'-GTC TTG TAA TTG GAA TGA TGG-3'(21mer)	18S rDNA	*Babesia* genus
18S_rDNA_GR2	5'-CCA AAG ACT TTG ATT TCT CTC-3'(21mer)	18S rDNA	*Babesia* genus

PCR products were visualized using agarose gel electrophoresis (28). The positive PCR products were purified using the QIAGEN quick gel purification kit according to the manufacturer's instructions and the products submitted for sequencing.

Sequencing and analysis of PCR products

Sequencing was done by the Sanger Dideoxymethod at the International Livestock Research Institutes' SegoliLab (29). The obtained sequences were analyzed using the CLC Genomics Workbench Version 20.0.2 where the sequences were trimmed, conflicts resolved using the forward and reverse sequences, and a consensus sequence generated (30). The consensus sequence was analyzed using the BLASTn program of the National Center for Biotechnology Information to identify closely related sequences on GenBank (31). Multiple sequence alignment and a phylogenetic tree were constructed using Geneious Prime 2020.1.2, Build 2020-04-07 08:42, Java Version 11.0.4 + 11(64bit) using the MUSCLE program ver 3.8.425 by Robert C.Edgar and Geneious tree builder. The genetic distance model used was Jukes-Cantor, while the tree build method was Neighbor joining; the number of bootstrap replicates was 1000. The following GenBank accession no's were used in analyzing the sequences, AJ404608.1, HQ895984.1, HQ289870.1, AY150061.1, KT333456.1, LC331058.1, EU084681.1, MN067708.1, AB303076.1, MN625891.1, AY077719.1, DQ111765.1, LC331056.1, KY463434.1, DQ111760.1, JN982353.1, MH143395.1, MF040149.1, HM585429.1, AY618928.1, KF928958.1, KU204792.1 (32)

Results And Discussion

From the 143 samples, 13 were positive for *Babesia canis* species giving a prevalence of 9.0%, (95% C.I is 0.0437 to 0.1381). Out of the13 positive samples, 2 were positive for *Babesia canis vogeli* (1.4%; 95% CI: 0.0138 to 0.0142) which were assigned GenBank accession numbers, *B.vogeli* Nrb – MT740261, B.vogeliNrbB10 – MT740272.While 11 were positive for *Babesia canis rossi* (7.69%; 95% CI: 0.033 to 0.12),which were assigned the following GenBank accession numbers, *B.rossi* nvsa – MT740262,*B.rossi*

NrbA1 – MT740263,*B.rossi* NrbB2 – MT740264,*B.rossi* NrbC3 – MT740265,*B.rossi* NrbD4 – MT740266,*B.rossi* NvsaB5 – MT740267,*B.rossi* NrbE6 – MT740268,*B.rossi* Msa7 – MT740269,*B.rossi* NrbF8 – MT740270,*B.rossi* NrbG9 – MT740271,*B.rossi* NrbB10 – MT740272,F12_BTH_F – MT740273

Table 3
Results with a breakdown for *Babesia* species detected, age, sex, and breed.

		Sex		Breed		Age		
	No.	M	F	L	E	J	A	O
Babesia canis rossi	11	6	5	9	2	3	7	1
Babesia canis vogeli	2	1	1	2	-	-	2	-
Totals	13	7	6	11	2	3	9	1
Totals as %	-	53.85	46.15	84.61	15.38	23.08	69.23	7.69

*J-Juvenile (< 1 year), A-Adults (between 1–5 years), O-Older (> 5 years), M-males, F-Females, L-Local breed, E-Exotic breed

Of the 13 positive 23.08% were juveniles < 1 year old, while 69.23% were adult animals > 1 year ≤ 5 years old and 7.69% were older dogs 5 years old.

Of the 13 positive individuals, 53.85%were males and 46.15% were females.

Table 4
Positive results with a breakdown of age, sex, breed and the county of origin

		Age			Totals	Sex		Totals	Breed		Totals
		J	A	O		M	F		L	E	
Nairobi	B.canis rossi	2	5	1	8	5	3		6	2	8
	B.canis vogeli	-	2	-	2	1	1		2		2
Naivasha	B.canis rossi	-	2	-	2	1	1		2	-	2
	B.canis vogeli	-	-	-	-	-	-		-	-	-
Mombasa	B.canis rossi	-	1	-	1	-	1		1	-	1
	B.canis vogeli	-	-	-	-	-	-		-	-	-

*J-Juvenile (< 1 year), A-Adults (between 1–5 years), O-Older (> 5 years), M-males and F-Females L-local breed, E-exotic breed

Key

J	Juvenile	≤ 1yr
A	Adult	1 year ≤ 5yrs
O	Old	5yrs
M	Male	-
F	Female	-
E	Exotic breed	-
L	Local breed	-

Oyamada M et al (2005) determined the prevalence for *B.canis vogeli* and *B.canis rossi* in a study from South Sudan to be 9.0% which is comparable to the results from this study which was 9.09% (Table3,33). In both studies, the prevalence of *B.vogeli* was significantly lower than that of *B. rossi*.

Co-infections of *Babesia* species and *Hepatozoon canis* were detected in South Sudan with a prevalence of 3.4% compared to none from this study (33).

In Uganda, *B.rossi* was detected in dogs with a prevalence of 7.8% which compares well to the prevalence observed in Kenya. In contrast, *B.vogeli* was not detected in the Ugandan study (34).

In addition *B.rossi* was detected in a Zambian study at a prevalence of 8.0% which compares well to the findings from our study. Interestingly, *B.gibsoni* was as well detected in Zambia and there was a high level of co-infections of *B.gibsoni* and *B.vogeli* with a frequency of 59.7% (35).*Babesia gibsoni* is known to occur in Africa,(13)some reports indicate wide distribution on the continent (13),other studies point to a more limited regional occurrence (14, 53, 54).Using molecular studies, *B.gibsoni* has been detected in the following countries (35, 38, 53).Similar studies failed to detect *B.gibsoni* in the following countries (33, 34, 36, 39).Due to the high prevalence of the parasite in Zambia (35) and the fact that this country shares a border with a core East African state like Tanzania, it would seem probable that the parasite is present in the region and will eventually be detected. This *Babesia* species was not detected in our study.

In agreement with the findings from the Kenyan study, *B.rossi* and *B.vogeli* were detected in Nigeria (36). Both *B.rossi* and *B.vogeli* were detected at a lower prevalence compared to *other studies in Africa at* 2.0% and 0.3% respectively. In studies from African countries, *B.vogeli* had a prevalence ranging between 1.4–5.8%. (37, 38).One of the most important factors determining infection rates with canine babesiosis is presence of competent tick vectors (13).The authors in the Nigerian study speculated that the higher infection rate observed in some African countries was probably due to greater levels of infestation with tick vectors e.g. South Africa (39) and South Sudan (33). There are no reports on the rate of tick infestation of dogs in Nigeria. From a Namibian study, *B.vogeli* was detected but from only one sample which had initially been diagnosed microscopically (37).

From a similar Angolan study and in agreement with this study, *B.vogeli* was detected at a prevalence of 1.0%.In contrast, *B.rossi* was not detected, but *B.gibsoni* was identified at a prevalence of 1.0% as well (38)

In agreement with the Kenyan study, *B.vogeli* and *B.rossi* were detected from a South African study with the prevalence of *B.vogeli* at 1.58%. Also, co-infections for these two *Babesia* species was only observed in one sample. In contrast, *B.rossi* was detected at a much higher prevalence of 36.93% compared to this and other similar studies in Africa (39).

The query sequences obtained from this study, B.vogeliNrb and B.vogeliNrbB had a 99.0% percentage identity (P.I) to a *B.vogeli* sequence from a pet cat in China (42).

The two *Babesia canis vogeli* sequences detected, GenBank accession no's MT740272.1 (B.vogeliNrbB) and MT740261.1 (B.vogeliNrb), on the phylogenetic tree clustered closely to each other and with *B.vogeli* sequences from Africa. *B.vogeli* from Nigeria AB303076.1 with a percent identity P.I of 98.89% (36), *B.vogeli* from Egypt MN625891.1 with percent identity of 99.42% (52).Other closely related sequences include *B.vogeli* from Japan AY077719.1 with P.I of 99.42% (51)

Eleven *Babesia canis rossi* sequences were detected in the study. They were detected from all the counties. On the phylogenetic tree, the Kenyan sequences clustered closely to each other and *B.rossi* sequences from the African continent. Of the Kenyan sequences, MT740273.1 (F12) appeared most different from the others with P.I ranging between 97.88% for MT740267.1 (Nvsa_B) to 99.39% for MT740262.1 (Nvsa).

Other *B.rossi* sequences closely related to the Kenyan sequences include KY463431.1 obtained from black-backed jackals in sub-Saharan Africa with a P.I of 99.52% (40), DQ111760.1 obtained from dogs in Sudan with a P.I of 99.52% (33), LC331056.1 obtained from dogs in Zambia with a P.I of 99.36% (50).

Conclusion And Recommendation

This study to the best of my knowledge constitutes the first report of molecularly detected cases of *B.canis rossi* and *B.canis vogeli* in dogs from Kenya.

The *B.rossi* sequences obtained from this study had at least 98.29% percentage identity to sequences from black-backed jackals in South Africa (*Canis mesomelas*). This is a strong indication of the wildlife origin of this *Babesia* species (38).

The bioinformatic analysis indicates the ability of *Babesia canis vogeli* although considered a dog pathogen can infect felines both domestic and wild.The fact of which should inform relevant authorities and stakeholders on the management of wildlife living in close proximity to domestic dogs.

The most commonly detected *Babesia canis rossi* is known to cause the most clinically severe form of babesiosis in dogs. As such, more emphasis should be placed on preventative measures, early diagnosis,

and treatment to reduce losses from the disease. A study aimed at collecting a larger sample size from more diverse species (livestock & wildlife) including tick vectors, living in close proximity to wildlife ecosystems would allow further clarification of the *Babesia* species circulating in these closely interacting animal and human populations.

Declarations

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Author Contributions

ITN-conceived the study, collected the samples, extracted DNA, carried out lab experiments including PCR, submitted samples for DNA sequencing, analyzed the data and drafted the manuscript, RP-supervised the lab work and analysis of the data, MK-participated in and supervised the lab work and analysis of the data, KM-supervised the entire project as well as participated in the refining of the study idea and design, PJ-refined the study idea and design, DO-participated in refining the study idea and drafting of the manuscript, All the authors read the manuscript suggested their adjustments and approved publication

Competing Interests

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

Study involved use of blood samples collected for other purposes

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Consent for Publication

Not applicable

Availability of Data & Materials

All the data obtained through this study was deposited with the National Centre for Biotechnology Informations' GenBank public DNA sequence repository

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Figures

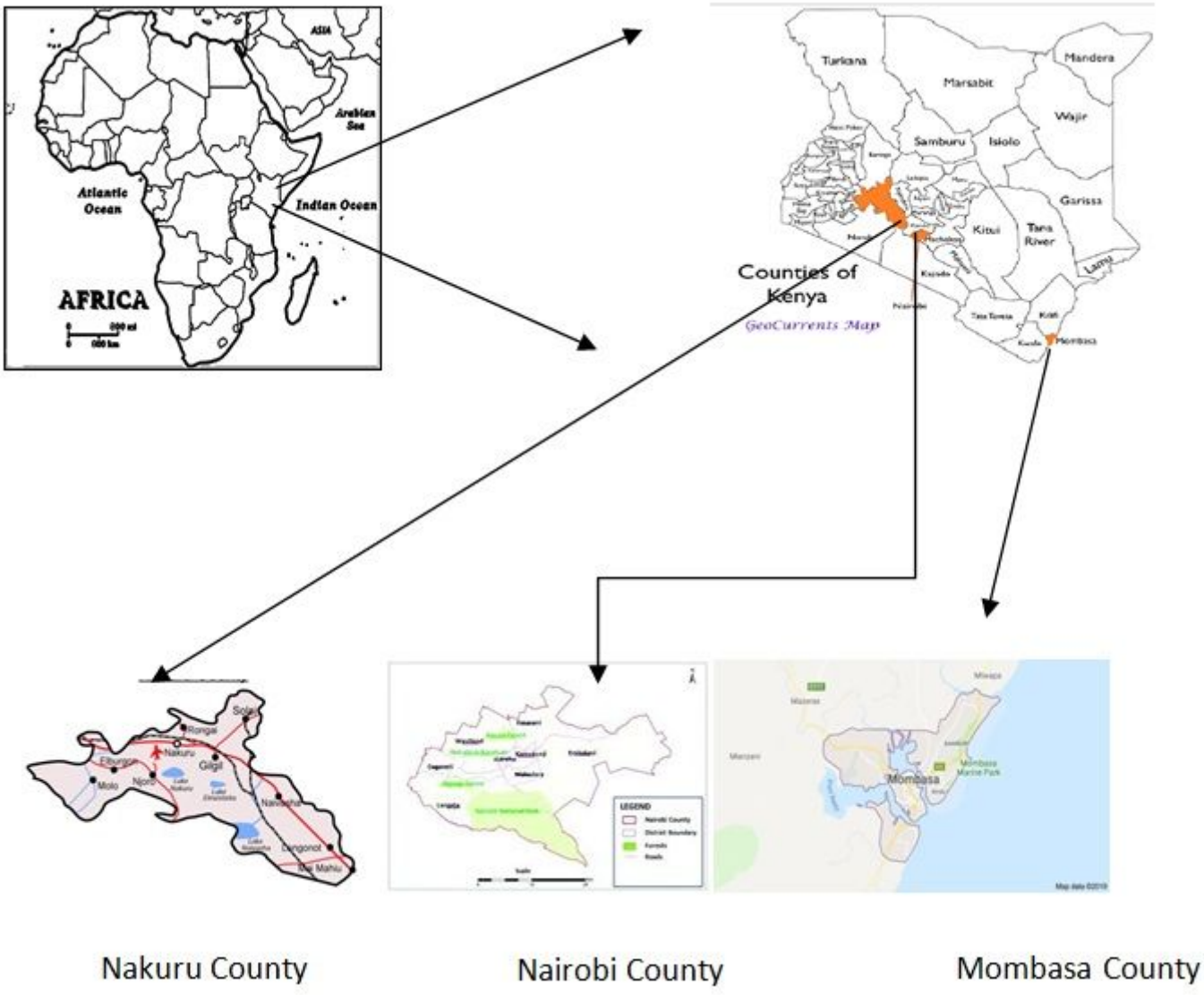

Figure 1

Map of Africa showing the position of Kenya in East Africa and the counties the samples were collected from. Note: The designations employed and the presentation of the material on this map do not imply the

expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

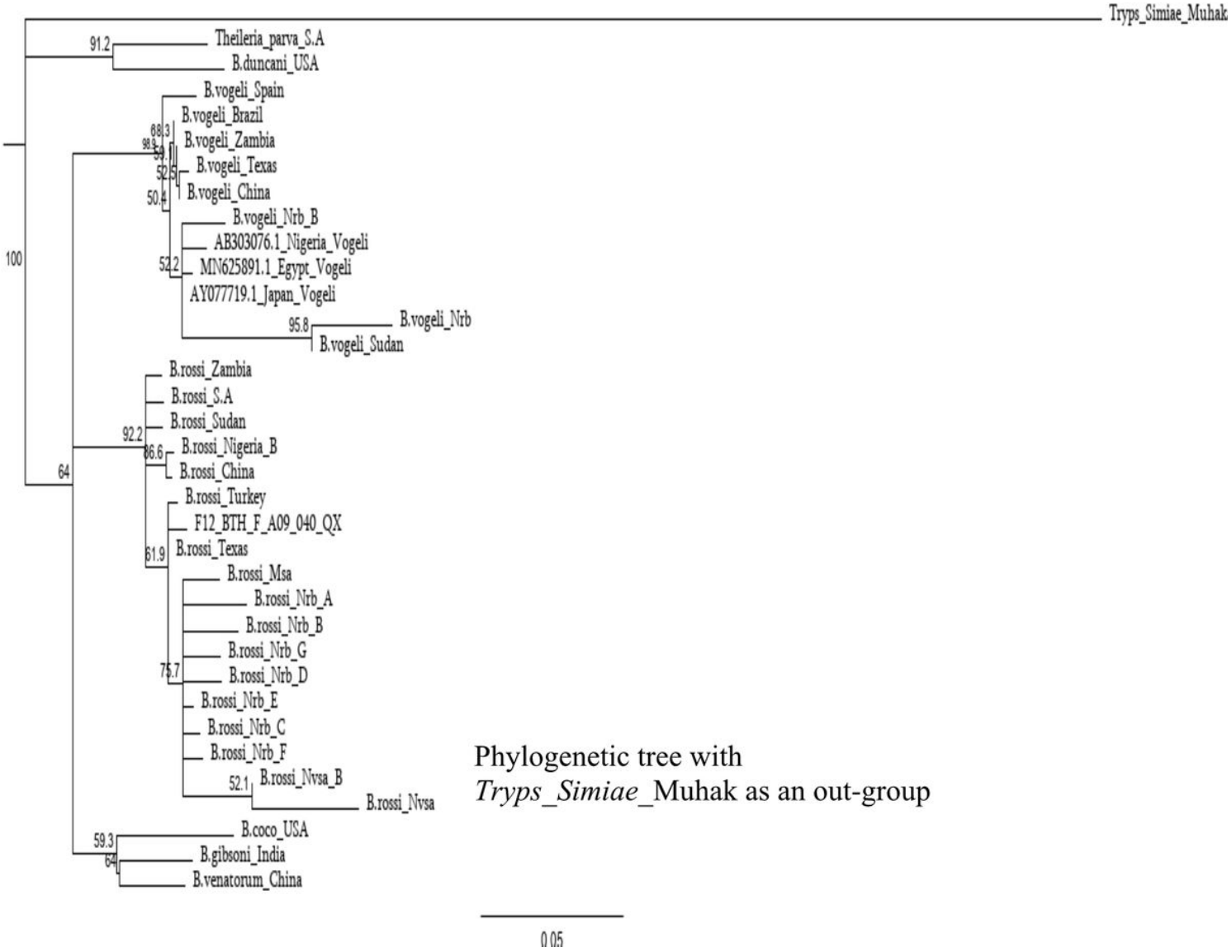

Figure 2

Phylogenetic Tree,the Kenyan samples e.g.B.rossi_Msa,B.rossi_Nrb_A,B.rossi_Nrb_B,B.rossi_Nrb_G clustered around each other, the 2 samples from Naivasha formed a subcluster.B.rossi most closely related were samples from Turkey, China, Nigeria, Sudan, South Africa, and Zambia. The B.vogeli samples were closely related to samples from Sudan, Japan, Egypt, and Nigeria.