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Tick (Acari: Ixodidae) infestations in cattle along Geba River basin in Guinea-Bissau

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ABSTRACT

Tick infestations are a major problem for animal production in tropical areas where prevention and control remain deficient. The present study sought to assess the awareness of traditional cattle producers towards the importance of ticks and aimed at the identification of tick species infesting bovines within the Geba River basin, Guinea-Bissau. Interviews with producers revealed that the majority directly correlates the presence of ticks with the occurrence of diseases in cattle. However, insufficient or inadequate control approaches prevail. A total of 337 ticks were collected on bovines at 18 different villages (10 during dry season, and 8 during rainy season). The tick species collected during the dry season were *Rhipicephalus* (*Boophilus*) *geigyi* (56.5%), followed by *Amblyomma variegatum* (23.3%), *Rhipicephalus* (*Boophilus*) *annulatus* (17.6%) and *Hyalomma truncatum* (1%). In the rainy season *A. variegatum* was the most collected (88.9%), followed by *R.* (*Boophilus*) *geigyi* (4.2%), *R.* (*Boophilus*) *annulatus* (3.4%), *Rhipicephalus sanguineus* group (2.8%) and *H. truncatum* (0.7%). To support species identification, segments of both cytochrome c oxidase I (COI) and 12S ribosomal RNA (12S) genes were sequenced and the data gathered were analysed by maximum likelihood and parsimony. Morphological and genetic data of individual specimens gathered in this study provide relevant information for future studies on tick population dynamics in the region. In addition, it led to a deeper characterization of *R. sulcatus* and a *R. sanguineus*-like specimen, exploring their genetic relationship with other *R. sanguineus*, which supports their classification as distinct species within *R. sanguineus* group.

1. Introduction

Hard ticks (Acari: Ixodidae) are obligate hematophagous ectoparasites of terrestrial and semiaquatic vertebrates (Guglielmone et al., 2014). Infesting most of the world cattle population, hard ticks are the ectoparasites of livestock with the highest economic impact (Bowman and Nuttall, 2008). Decrease in body weight gain is one of the main consequences of tick parasitism, directly from blood spoliation and indirectly by transmitted tick-borne pathogens. Many of these pathogens are zoonotic and act as important health threats to farming communities, particularly in tropical areas (Jongejan and Uilenberg, 2004). Tick control is thus considered essential in the tropics, where it is largely based on acaricides. More recently, anti-tick vaccines have been investigated (de la Fuente and Merino, 2013). They offer the potential advantages of overcoming tick resistance and environmental constraints related to pollutant residues, but are not yet a real alternative for tick control (Abbas et al., 2014).

The success of any tick control method, either chemical, mechanical or immunological, depends largely on adequate management practices and requires an understanding of tick epidemiology at the local level. In this regard, it is of note that recent records have pointed for changes in tick geographical distribution, mainly attributed to climate changes and increased animal movements (Beugnet and Chalvet-Monfray, 2013). Thus, studies

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providing updated information on tick infestations in a particular region are relevant for the implementation of future rational therapeutic and prophylactic strategies. Not rarely, attempts to introduce more productive animals into tropical areas result, in fact, in the introduction of naïve cattle, highly susceptible to local ticks and tick-transmitted pathogens, leading to disease outbreaks and, consequently, to a loss of investment.

Guinea-Bissau is a country with around 1.5 million inhabitants (UNCTAD, 2011) where cattle production is carried out on family-based extensive production systems. The territory includes an insular archipelago (Bijagós) and mainland, which is a low plateau with four main rivers, Cacheu, Geba, Corubal and Cacine, flowing west from north to south. The basin of river Geba is one of the most important areas for livestock production (cattle, goats and sheep) and has a relatively high population density whose subsistence is based on farming. Thus, the impact of tick infestations on cattle and their ability to act as vectors of pathogens with clinical veterinary relevance make studies on tick populations of particular importance.

Tendeiro's extensive work on the territory, between 1944 and 1959, arises as the first recognition of the importance of ticks which were, until then, considered as simple blood spoliators with no impact on animal health (Tendeiro, 1946). Since then few studies were conducted, but those carried out provided updates on ticks present and on their distribution within different regions of the country (Neves, 1996; Rosa et al., 1998; Terenius et al., 2000). Tendeiro identified 8 different species among ticks infesting bovines. The specimens collected were individually analysed and detailed morphologic and morphometric descriptions of the characterized species were presented: *R. sanguineus* Latreille, 1806 and *R. simus* Koch, 1844 (Tendeiro, 1946); *Amblyomma splendidum* Giebel, 1877, *A. variegatum* Fabricius, 1794 and *Haemaphysalis leachi* Audouin, 1827 (Tendeiro, 1947); *Hyalomma savignyi* Gervais, 1844 (Tendeiro, 1949); *Rhipicephalus senegalensis* Koch, 1844 (Tendeiro, 1956); *R. lunulatus* Neumann, 1907 (Tendeiro, 1959). *H. savignyi* is no longer recognized as a valid species name and Hoogstraal (1956) asserts that the description of the specimens presented by Tendeiro (1949) corresponds to *H. truncatum* Koch, 1844.

Tendeiro makes note to an earlier reference (1929) describing the presence of *Rhipicephalus* (*Boophilus*) *annulatus* Say, 1821 on Guinea-Bissauís bovines (Tendeiro, 1946). Regarding ticks from the *Boophilus* taxonomic group, he was only able to identify one species, which he classified as *R. (B.) decoloratus* Koch, 1844 and, therefore, contested the presence of *R. (B.) annulatus,* assuming that previous descriptions mistook *R. (B.) decoloratus* for *R. (B.) annulatus* (Tendeiro, 1946). A few years later, a new *Boophilus* species was described, *R. (B.) geigyi* Aeschlimann & Morel, 1965 and, as afterwards was confirmed by a re-evaluation of these ticks, the specimens described by Tendeiro as *R. (B.) decoloratus* were, in fact, *R. (B.) geigyi* (Neves, 1996).

Two additional species have been described in Guinea-Bissau: *R. muhsamae* Morel & Vassiliades, 1965 (Branckaert, 1988) and *R. sulcatus* Neumann, 1908 (GAPTEC, 1993). Terenius et al. (2000) exclude *R. simus* from the list of species occurring in Guiné-Bissau. In fact, these authors do not acknowledge Tendeiro's identification (Tendeiro, 1946) as valid, stating that its description was presented before the taxonomic revision by Pegram et al. (1987b). However, *R. simus* was reported after this species revision (Neves, 1996).

In the present work, we interviewed the local cattle producers about management practices in use and about their opinion on the importance of ticks and their control, and we collected and identified cattle ticks occurring on Geba River basin during dry and rainy seasons. The ticks were identified based on their morphological characteristics and for each species the nucleotide sequences of fragments from the mitochondrial cytochrome c oxidase I (COI) and 12S rRNA (12S) genes were determined, thus corroborating tick taxonomy at the molecular level. The study contributes to the knowledge of the current situation of cattle tick infestation in one of the most important areas for livestock production in Guinea-Bissau.

2. Materials and methods

2.1. Study area

The studied area includes most of the Geba River basin, in the regions of Bafatá and Gabú, extending to approximately 6175 km^2 (Fig. 1). The main landscape is composed of low flat areas, which are temporarily flooded by the Geba River or its tributaries, with meadows and pastures. These flooded lands are surrounded by savannah or forest/savannah, consisting of open bush and oil palm trees or lightly wooded forest interspersed with grasses. It falls into the Sudanese tropical climate zone with two seasons: the rainy season occurring from June to October and the dry season from November to May.

This study covered 19 farmers at 18 villages, one farmer per village, except in Sintchã Quecuta, where a relative present asked to participate as well, thus in this village 2 farmers were included. Data and samples were collected in April 2010 (dry season), when 11 farmers participated, and in September 2012 (rainy season) when 8 farmers participated.

2.2. Interviews and tick sampling

After an initial explanation of the objectives of the study and the acceptance to collaborate, producers were asked about cattle management practices, transhumance, current tick control practices, as well as about their opinion on the importance of tick infestation and its role in cattle diseases. Size of herd and season were also recorded. Interviews were conducted in a semi-structured way, in which issues were addressed during the conversation and answers registered in the questionnaire. Ticks were manually collected from 5 to 10 animals per herd (119 animals in total), by two persons, over a time of approximately 5 min for each animal. Ticks from each animal were preserved in ethanol at 70% (v/v).

2.3. Tick identification

Ticks were cleaned of cattle's hair and debris under a stereomicroscope. Identification was based on morphological keys from Tendeiro (1952) and Travassos Santos Dias (1989), complemented with Hoogstraal (1956) work and confirmed with Walker et al. (2003) updated keys. Specimens belonging to the subgenus *Rhipicephalus* of the genus *Rhipicephalus* were identified based on Pegram et al. (1987a) and Walker et al. (2000). Two of these ticks were dissected under a stereoscope prior to DNA extraction, and the genital apertures as well as one spiracle from each specimen were treated with lactophenol solution and mounted on microscope slides for further analysis and comparison.

2.4. DNA extraction

Molecular identification of at least 2 specimens of each morphology-based identified species was performed, except for the specimens belonging to the subgenus *Rhipicephalus*. In these cases, only one tick of each species was used for DNA extraction and, for each, two independent PCR amplifications were carried out. DNA extraction was performed using a modification of a previously described protocol (Halos et al., 2004). Briefly, ticks were longitudinally sliced in halves with a sterile blade. One half was stored and the other was placed in a 1.5 mL microtube with 180 μL of ATL Buffer (Qiagen, Germany). One sterile stainless bead (Dejay Distribution Limited, UK) was added to each sample, followed by

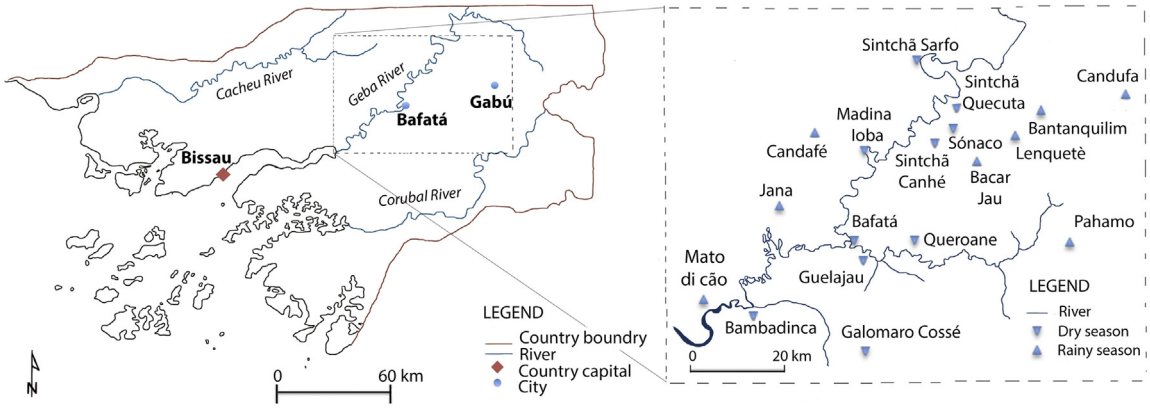

Fig. 1. Geographical area of the study. Guinea-Bissau map with the area of study delimited with a dashed borderline. The area of study is zoomed on the right where the tick collection sites are identified for both dry and rainy seasons.

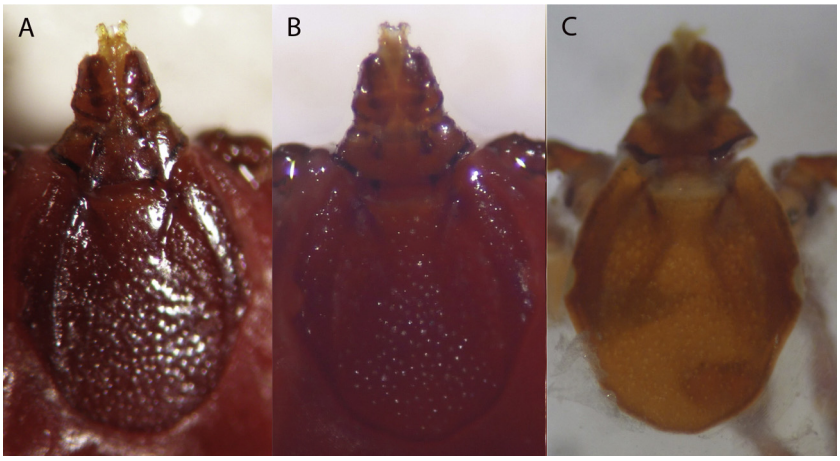

Fig. 2. Morphological characters of *Rhipicephalus sulcatus* female. Scutum, capitulum and palps (A), immersed in 70% ethanol (B) and after DNA extraction (C).

disruption using Tissue Lyser (Qiagen, Germany) and processing with DNeasy Blood & Tissue Kit (Qiagen, Germany). The protocol for animal tissue suggested by the manufacturer was followed except for the incubation period step, which was extended for 6 h.

2.5. PCR amplification

Partial sequences of two different genes were amplified by separate PCR protocols. Amplification of the COI fragment was done using the primers F1 and R1 (Kushimo, 2013) and for the amplification of the 12S fragment, conserved T1B and T2A primers (Beati and Keirans, 2001) were used. The amplification protocols proposed by Kushimo (2013) and by Beati and Keirans (2001) were optimized following Phusion DNA polymerase (ThermoScientific) manufacturer instructions, resulting in annealing temperatures of 57 °C and 51 °C for COI and 12S amplification, respectively. PCR products were visualized in 1–1.25% agarose gels stained with ethidium bromide.

2.6. Cloning and sequencing

DNA fragments were excised from agarose gels, purified using QIAquick Gel extraction Kit (Qiagen, Germany) and ligated to vector pJET1.2/blunt (ThermoScientific). Competent DH5α cells were transformed with the ligation products and grown overnight in agar plates containing 100 μg/mL of ampicillin. Randomly-selected colonies were tested using pJET1.2/blunt-specific primers and GreenTaq DNA polymerase (ThermoScientific). Amplified fragments were visualized in 1% agarose gels with ethidium bromide. Selected colonies were grown overnight in LB medium and the plasmids were purified using the QIAprep spin miniprep Kit (Qiagen, Germany), quantified and sequenced (STABVIDA-Portugal) using pJet1.2/blunt primers (Forward and Reverse) (STABVIDA-Portugal).

2.7. Molecular analysis

Partial sequences of mitochondrial COI and 12S were obtained from sequencing or retrieved from GenBank (see Supplementary file 1). Alignments of the sequences were performed using ClustalOmega (Larkin et al., 2007) with default parameters, and due to differences in size the sequences were trimmed, resulting in partial DNA sequences of 424 bp for COI and 326–332 bp for 12S. Analysis and comparison of multiple sequence alignments were performed using Jalview (Waterhouse et al., 2009) and Base-By-Base (Hillary et al., 2011). The jModelTest 2.1.7 (Darriba et al., 2012) was used to select the best model for maximum likelihood (ML) tree construction, with models GTR + I + G and GTR + I being selected for COI and 12S, respectively. ML trees, with 1000 bootstrap replicates, were constructed using PhyML 3.0 (Guindon et al., 2010) and the tools seqboot, consense and retree from the PHYLIP package version 3.965 (Felsenstein, 1989). Maximum parsimony (MP) trees were constructed using the tools dnapars, seqboot, consense and retree from the PHYLIP package version 3.965 (Felsenstein, 1989), with 10000 bootstrap replicates. Trees were edited using the program MEGA 6 (Tamura et al., 2013).

3. Results and discussion

3.1. Farmers interviews

Inquiries were used to ascertain farmers' awareness towards the importance of tick infestations, to identify the methods used for tick control and to portray some characteristics of the production system itself. In all the villages visited cattle are all N'Dama breed and are raised under similar conditions, in an extensive production system feeding natural vegetation (bushlands) in the rainy season and the remnants of crops, such as rice, maize, peanuts and others, in the dry season. Water sources are usually rivers, natural pools and wells. Cattle are herded by men and young boys and kept at night near the villages, tied by ropes. Cows are milked, once a day, for fermented milk production. Disease prophylaxis is restricted to vaccination against anthrax and black leg, when farmers apply for the vaccination. Sick animals receive treatment either with folk remedies or with the use of veterinary medicines (antibiotics, anthelmintics and acaricides).

All interviewed farmers reported the presence of ticks infesting their cattle. Of the 19 farmers interviewed, 15 mentioned severe infestations but only 9 of them correlated these infestations to the occurrence of diseases in cattle; other 5 admitted a correlation with sporadic diseases and 1 reported no knowledge of diseases in cattle due to ticks. Three other farmers considered that infestations were mild; nevertheless, 2 of them were able to correlate these mild tick infestations to severe cattle diseases. Only 1 of the farmers didn't consider ticks to be a problem for his animals as he reported their presence to be minimal and that the animals presented no diseases. In fact, this was the only farmer who didn't take any action for tick control or prevention. All other farmers reported some type of control, which included weekly manual tick removal, tick perforation with a needle, topical application of liquid from old batteries or *Baygon,* as well as a variety of traditional local medicines based on salt and local plants as bissilon (*Khaya senegalensis*). Only 4 farmers used ivermectin on a regular basis and all of them reported severe tick infestation. It was unclear if the high infestation levels were the reason for the repeated use of ivermectin or if their persistence was a consequence of inadequate use of the acaricide. The reported control measures address infestations at individual animal level. It is expected that cattle production at the Geba River basin will expand, due to its potential, and a herd-based approach to tick control, with less hand-labour requirement, would be desirable.

The knowledge of contact areas between animals during grazing and drinking times is useful to understand vector biodynamics and transmission capacity. Five of the nineteen farmers reported that their cattle have regular contact with herds from other villages by entering or exiting of transhumant animals. The knowledge of these animal contacts alerts to the possibility of introduction of new cattle tick species. This assumes a threat of particular importance for the tick *R.* (*Boophilus*) *microplus*, already described as the most successfully invasive tick species in West Africa (Madder et al., 2011).

3.2. Tick collections and morphological studies

In the dry season (year 2010), 109 cows were screened and 193 ticks were collected. No ticks were found on 35 animals. In 2012, during the rainy season, a total of 144 ticks were collected from 40 screened cows, of which 2 had no ticks.

The specimens collected during the dry season belong to 3 different genera (*Amblyomma*, *Rhipicephalus* and *Hyalomma*). Within these genera, different species were found (Table 1). The species with the highest number of collected individuals was *Rhipicephalus* (*Boophilus*) *geigyi* with 109 ticks (56.5%), followed by *Amblyomma variegatum* (45; 23.3%), *Rhipicephalus* (*Boophilus*) *annulatus* (34; 17.6%) and *Hyalomma truncatum* (2; 1%). There were also 2 females and 1 nymph (1.6%) identified as belonging to the *Rhipicephalus* (*Boophilus*) subgenus, but with mouth parts and legs degraded and/or missing, not allowing their identification to the species level.

Table 1
Tick species collected from cattle in the dry season.

Tick species	No. of individuals	Life stage		
		Nymphs	Females	Males
Amblyomma variegatum	45	25	19	1
Hyalomma truncatum	2	0	0	2
Rhipicephalus (Boophilus) sp.	3	1	2	0
Rhipicephalus (Boophilus) annulatus	34	0	32	2
Rhipicephalus (Boophilus) geigyi	109	0	104	5
Total of ticks	193			

Table 2
Tick species collected from cattle in the rainy season.

Tick species	No. of individuals	Life stage		
		Nymphs	Females	Males
Amblyomma variegatum	128	0	36	92
Hyalomma truncatum	1	0	0	1
Rhipicephalus (Boophilus) annulatus	5	0	4	1
Rhipicephalus (Boophilus) geigyi	6	0	5	1
Rhipicephalus sanguineus Group	4	0	4	0
Total of ticks	144			

During the rainy season, the specimens found belonged to the same genera as above (*Amblyomma*, *Rhipicephalus* and *Hyalomma*). However, in this collection *A. variegatum* was clearly the most abundant tick with 128 ticks (88.8%), followed by *R.* (*B.*) *geigyi* (6; 4.2%), *R.* (*B.*) *annulatus* (5; 3.5%), 4 specimens (2.8%) identified as members of *Rhipicephalus sanguineus* group and one specimen (0.7%) of the species *H. truncatum* (Table 2).

Tick's life cycle is seasonal and even though this study did not intend to be a systematic survey, the relative proportion of the more abundant species in the two collections suggest differences in the species infesting cattle in the dry and rainy seasons. While the predominant species in the rainy season collection was *A. variegatum* (88.8%), ticks from the subgenus *Boophilus* were 75.6% of the dry season collection. If adequate humidity and temperature are provided during the rainy season, *Boophilus* ticks are able to complete their on-host life cycle in about 21 days, allowing them to quickly multiply leading to a peak of ticks later at the beginning of the dry season (Dipeolu, 1975). It is of note that the latest published tick survey in Guinea-Bissau (Terenius et al., 2000) did not register the presence of any specimen of the subgenus *Boophilus*. Further studies are needed to clarify if this divergence reflects a change in tick populations or simply the fact that in the mentioned survey ticks were collected only in the rainy season when these species are less frequent. Differently, *Amblyomma* spp., as three-host life cycle ticks, remain inactive in the pastures for long periods when moulting. *Amblyomma variegatum* nymphs feed during the dry season (consistent with this, 55.5% of the *Amblyomma* ticks collected in this study at the dry season were nymphs), detach from hosts and moult, but adults only resume activity at the beginning of the rainy season when temperature and humidity are adequate (Pegram et al., 1986). In the present study, all the *Amblyomma variegatum* specimens collected in the rainy season were adults.

Species found in both seasons are a threat to animal production. *Boophilus* ticks are competent vectors of apicomplexan parasites of the genus *Babesia*, known to cause bovine babesiosis (redwater), and of *Anaplasma marginale*, the etiological agent of bovine erythrocytic anaplasmosis. So far, *B. bovis* is yet to be found in the region,

but *Babesia bigemina* and *Anaplasma marginale* have been described in Guinea-Bissau (Rosa et al., 1998; Tendeiro, 1946).

Amblyomma variegatum, a tick with high impact on animal health in the tropics (Bournez et al., 2015; Rahajarison et al., 2014) acts as one of the main vectors of *Ehrlichia ruminantium*, responsible for heartwater in cattle as well as in goats and sheep. This disease is endemic in most of sub-Saharan Africa (Allsopp, 2015; Camus and Barré, 1988; Provost and Bezuidenhout, 1987) and was reported in Guinea-Bissau for the first time by Tendeiro (1945). *A. variegatum* may also be responsible for *E. bovis* transmission (bovine ehrlichiosis), not yet registered within the territory. Heavy *A. variegatum* infestations are highly immunosuppressant to cattle and the saliva from this species was already associated as a predisposing factor to severe dermatophilosis (Walker and Lloyd, 1993).

Even though in low numbers, *Hyalomma truncatum* was present in both seasons. The three males collected were compared to the descriptions of *H. nitidum* Schulze, 1919 from Tomassone et al. (2005) and Apanaskevich and Horak (2008) since this species has been confused and synonymized with *H. truncatum* and occurs in Guinea (Conakry) and Senegal (Apanaskevich and Horak, 2008; Tomassone et al., 2005), the Guinea-Bissau neighbouring countries. However, the three specimens present distinct ivory-coloured bands distally and small dorsal spots proximally in their leg segments and, therefore, were diagnosed as *H. truncatum*. Besides its direct role in pathogen transmission, the relevance of this species resides also in its saliva toxins, capable of inducing sweating sickness (Cabezas-Cruz and Valdes, 2014).

The 4 specimens presented above as belonging to the *R. sanguineus* group, collected during the rainy season (Table 2), although different, are morphologically close to the classical description of *Rhipicephalus sanguineus* specimens occurring in Portugal. Their identification down to the species level required a deeper analysis of morphologic descriptions of species belonging to this group. In fact, this has been the most controversial group within the genus *Rhipicephalus* (Pegram et al., 1987a) and more assembled information on both their morphological and genetic data is needed to take us closer to a consensus among the scientific community (Hekimoglu et al., 2016; Moraes-Filho et al., 2011; Nava et al., 2015). The *R. sanguineus* group includes 17 species (Camicas et al., 1998), so we addressed them individually, looking for candidate species descriptions suitable for these collected specimens. We were able to compare our specimens to sixteen of the seventeen published descriptions (Hoogstraal, 1956; Tendeiro, 1959; Walker et al., 2000). Unfortunately, we were not able to find descriptions of *Rhipicephalus aurantiacus* Neumann, 1907. This species status has been quite controversial. Theiler and Robinson (1954) declared this species as being the same as *Rhipicephalus ziemanni*, while Walker et al. (2000) described it as a junior synonym of the latter. More recently, Guglielmone et al. (2014) included it again as a valid species name. These two species must be very close in morphology to generate such lack of consensus and so we have considered that if *R. ziemanni* was not coincident with our findings, *R. aurantiacus* would not be as well.

The above-mentioned four specimens were all engorged females, which hindered the visual evaluation of individual morphology. Based on their elongated scutum with very dense punctuations, cervical fields in inverted V-shape and equally densely punctuated and an apparent U-shape genital aperture we considered these specimens as being close to *Rhipicephalus sulcatus*, in accordance with previous descriptions (Pegram et al., 1987a). However, some divergence was noticed in one of these ticks. The scuta from one of the 3 identical specimens (Fig. 2) and from the dissimilar specimen (Fig. 3) are presented. In the first case, after cleaning, the punctuation pattern appeared tracery (Fig. 2a) but when immersed in ethanol this was not clear. In addition, the cervical fields were found to be deeper (Fig. 2b). This scutum mounted after DNA extraction shows the cervical fields depression and allows to better perceive its posterior margin (Fig. 2c). The slight variation observed in the intact dissimilar specimen (Fig. 3a) could be accounted as normal individual variation. However, after DNA extraction, the shallower cervical fields and a more sinuous scutum posterior margin are evident (Fig. 3b). Subsequently, we proceeded with mounting genital apertures and spiracles from these 2 ticks. In the first case, genital apertures (Fig. 4a) and spiracles (Fig. 4b) are compatible with *R. sulcatus*, and, therefore, from now on the 3 similar ticks will be referred to as *R. sulcatus*. The dissimilar specimen shows a more opened U-shaped genital aperture (Fig. 5a) and a more marked comma-shaped spiracle (Fig. 5b), all supporting that this specimen belongs to a different species. Other morphologically similar species would be *R. turanicus*. However, characters such as the distance between porose areas, the shape of the scutum, punctuation distribution and spiracles design are different from *R. turanicus* description (Pegram et al., 1987a). In addition, this species has never been reported in Guinea-Bissau. Therefore, this specimen, as member of the *R. sanguineus* group, will be hereafter referred to as *Rhipicephalus sanguineus*-like.

3.3. Molecular studies

Genetic markers were used in this work not only for species confirmation but also as a way to provide information regarding Ixodid tick species infesting cattle in the studied area, since there are no molecular data available regarding ticks from Guinea-Bissau. Two partial gene sequences (COI and 12S) were determined and used to infer genetic relationships between specimens collected in this study and voucher specimens' sequences available at GenBank. The mitochondrial COI gene has been elected as a standard barcoding marker for tick species identification (Lv et al., 2014a,b) and hence it was the chosen gene for identity confirmation. Genes with faster substitution rates as the 12S rRNA gene are useful to perceive genetic variation among closely related species and, for this reason, they are of particular importance to address the specimens within the genus *Rhipicephalus* (Beati and Keirans, 2001; Cruickshank, 2002; Latrofa et al., 2013; Murrell et al., 2000).

For these studies, at least two specimens of each morphologically identified *A. variegatum*, *H. truncatum*, *R. (B.) annulatus* and *R. (B.) geigyi* species were used. In the case of *R. sulcatus* and *R. sanguineus*-like, due to a very low number of specimens collected, only one tick was used and two independent PCR amplifications were carried out for each specimen. In all the cases, the two PCR products showed nucleotide sequences 100% identical.

The sequence of the amplified COI gene fragment from *Amblyomma variegatum* [KU568507 and KU568508] was 100% identical to the only corresponding sequence available for this species, GU062743, from ticks collected in neighbouring Senegal (Mediannikov et al., 2010). The sequence of the 12S gene fragment [KU568495 and KU568496] matched entirely (100%) with a specimen from Nigeria, JF949801 (direct submission).

For *Hyalomma truncatum*, the COI gene fragment [KU568509] only shared a 97% identity with a specimen from Ethiopia, AJ437084 (Rees et al., 2003). The same identity level (97%) was observed for the 12S partial gene fragment between our specimen [KU568497] and a corresponding sequence from Zimbabwe, AF150031 (Beati and Keirans, 2001).

Regarding *Rhipicephalus* (*Booplilus*) *annulatus*, the sequences determined for the COI gene fragments [KU568510 and KU568511] showed maximum identity levels (98%) with specimens from distant locations, Romania KC503256 (Burger et al., 2014) and Iran KM888754 (direct submission). For *Rhipicephalus* (*Boophilus*) *geigyi* COI sequence [KU568512 and KU568513], a match of 99% identity was observed with specimens from Burkina Faso, KC503263 (Burger et al., 2014), and from unknown origin AY008680 (Murrell

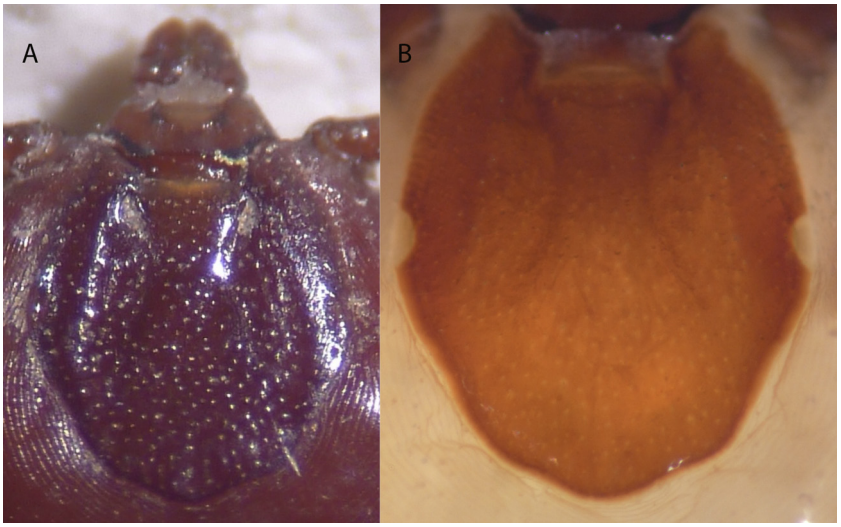

Fig. 3. Morphological characters of *Rhipicephalus sanguineus*-like female. Scutum, capitulum and palps (A) and scutum mounted after DNA extraction (B).

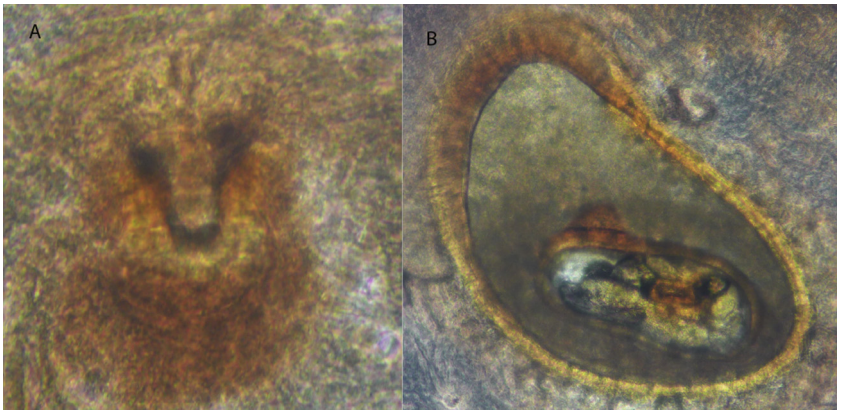

Fig. 4. Morphological characters of *Rhipicephalus sulcatus* female. Dissected genital aperture (A) and spiracle plate (B) after treatment with lactophenol.

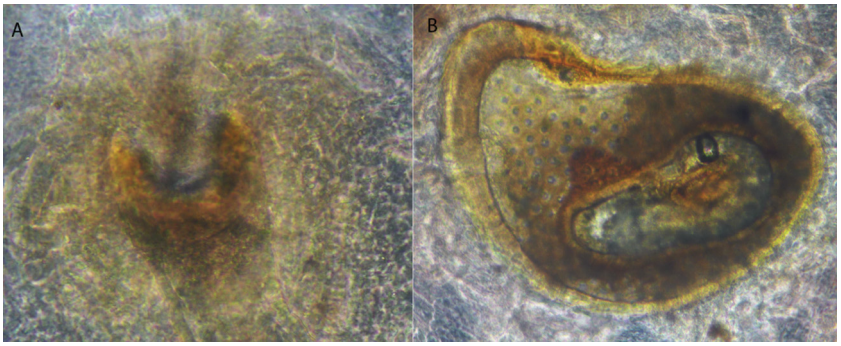

Fig. 5. Morphological characters of *Rhipicephalus sanguineus*-like female. Dissected genital aperture (A) and spiracle plate (B) after treatment with lactophenol.

et al., 2001). The 12S gene fragments of *R.* (*Booplilus*) *annulatus* [KU568498, KU568499 and KU568500] share 99% identity with geographically distant specimens from Italy AM410573 (Epis et al., 2008) and from Egypt EU921773 (direct submission) while the *R.* (*Boophilus*) *geigyi* sequences [KU568501, KU568502 and KU568503] show 99% identity with specimens from Burkina Faso KC503263 (Burger et al., 2014) and from Mali KF569939 (McCoy et al., 2014).

There are no sequences available at GenBank for the *R. sulcatus* COI gene to compare with the one determined in this study [KU568514]. However, the 12S gene fragment here determined [KU568504] matched the two *R. sulcatus* sequences available, FJ536565 and FJ536564 (direct submission), with 99% and 98% respectively, from ticks collected in Zambia. These high identity levels support the morphological identification of our specimen as a *R. sulcatus*.

The partial COI gene sequence from the specimen identified as *R. sanguineus*-like [KU568515] is closest to GenBank KC243896 (97%) and the 12S partial sequence [KU568505] to GenBank KC243810 (99%). Both of these GenBank sequences derive from the same specimens collected on cattle in Nigeria and were classified as *Rhipicephalus* sp. morphotype IV (Dantas-Torres et al., 2013). The authors

included these sequences on a phylogenetic analysis of *Rhipicephalus sanguineus* sensu lato and concluded that this morphotype IV, although morphologically different, is genetically related to *R. turanicus*. Unfortunately, specific morphological data, such as images of scutum, spiracle and genital aperture, were not included in the publication, as were for the other three morphotypes presented and, thus, a comparison with our specimen is not possible.

To better understand the genetic relationship between the specimens classified in the present study as *R. sulcatus* and *R. sanguineus*-like and others within the *Rhipicephalus sanguineus* group we analysed both COI and 12S rRNA gene fragments. In order to perceive morphological divergences among *R. sanguineus* sensu lato, Dantas-Torres et al. (2013) studied specimens from all five continents taking as references the *Rhipicephalus sanguineus* description from Walker et al. (2000) and the *R. turanicus* original description by Filippova (1997). The ticks that diverged from these morphological descriptions were assigned in 4 distinct groups named *Rhipicephalus* sp. I to IV. Therefore, we decided to use a representative sample of sequences from each of these 6 groups for our analysis, in addition to the sequences determined in this work. For the COI study we included the following sequences: *R. sanguineus* KC243872, KC243874, KC243877, KC243878 and KC243880; *R. turanicus* KC243910, KC243912 and KC243914; *Rhipicephalus* sp. I KC243883 and KC243884; *Rhipicephalus* sp. II KC243885 and KC243887; *Rhipicephalus* sp. III KC243893 and KC243894; *Rhipicephalus* sp. IV KC243896. Also, some other sequences were added to facilitate the analysis within a larger framework, namely a Portuguese *R. sanguineus* tick determined in this work [KU568516] and others retrieved from GenBank (*R. annulatus:* AF132825 (Murrell et al., 2000), KM494917 (direct submission); *R. geigyi*: AY008680 (Murrell et al., 2001), KC503263 (Burger et al., 2014); *R. decoloratus*: AF132826 (Murrell et al., 2000)). For the 12S study, we included sequences from the same 6 groups used for COI analysis (Dantas-Torres et al., 2013): *R. sanguineus* KC243786, KC243787, KC243788, KC243789 and KC243790; *R. turanicus* KC243816, KC243818 and KC243826; *Rhipicephalus* sp. I KC243791 and KC243797; *Rhipicephalus* sp. II KC243802, KC243804 and KC243807; *Rhipicephalus* sp. III KC243808 and KC243809; *Rhipicephalus* sp. IV: KC243810. Here, the Portuguese *R. sanguineus* 12S sequence determined in this work [KU568506] and others retrieved from GenBank (*R. annulatus:* EU921773 (direct submission), U95866 (direct submission), AM410573 (Epis et al., 2008); *R. decoloratus*: KF569940 (Mattioli et al., 1997), EU921774 (direct submission), AF150044 (Beati and Keirans, 2001); *R. geigyi*: KF569939 (McCoy et al., 2014); *R. sulcatus*: FJ536564, FJ536565, direct submission) were again used. The selection of the subgenus *Boophilus* sequences to include in these studies was based on the criteria of overlapping the fragments chosen for the *R. sanguineus* group study (Dantas-Torres et al., 2013).

In the analysis performed, the trees obtained by MP and ML showed identical topology and those obtained by the latter method are presented in Figs. 6 and 7. For both COI (Fig. 6) and 12S (Fig. 7) gene fragments, the subgenus *Boophilus* and *Rhipicephalus* originate distinct branches, as expected. *R.* (*B.*) *annulatus*, however, separates earlier than *R.* (*B.*) *decoloratus* from *R.* (*B.*) *geigyi*. *R.* (*B.*) *annulatus* is grouped with specimens from distant locations. *R.* (*B.*) *geigyi*, limited to an African distribution, is closer to a Burkina Faso specimen on COI (99.29%) and to a Mali specimen 12S (100%).

There are no published sequences for *R. sulcatus* COI gene so we were only able to analyse the sequences from the specimen identified as *R. sulcatus* by comparison with the other *Rhipicephalus* species on the tree of this gene (Fig. 6). The tree points to a closer relationship between these specimens and *R. turanicus* and *Rhipicephalus* sp. III. However, the highest identity levels observed were relatively low: 91.51% with *Rhipicephalus* sp. III from Pakistan and 91.27% with *R. turanicus* from Italy KC243910 (see Supplementary file 1). On the 12S analysis (Fig. 7), although close to morphotype

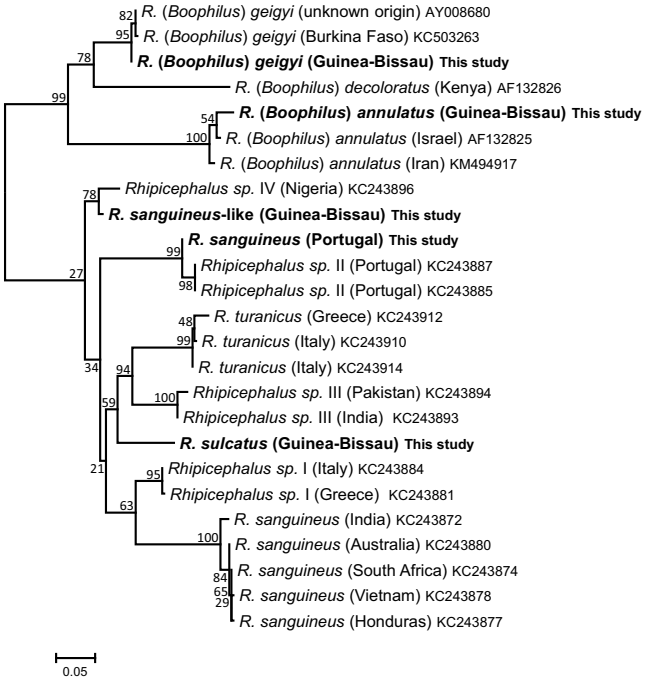

Fig. 6. Genetic relationships of *Rhipicephalus* spp. inferred from COI gene sequences. Maximum likelihood (ML) phylogenetic tree construction with 1000 bootstrap replicates.

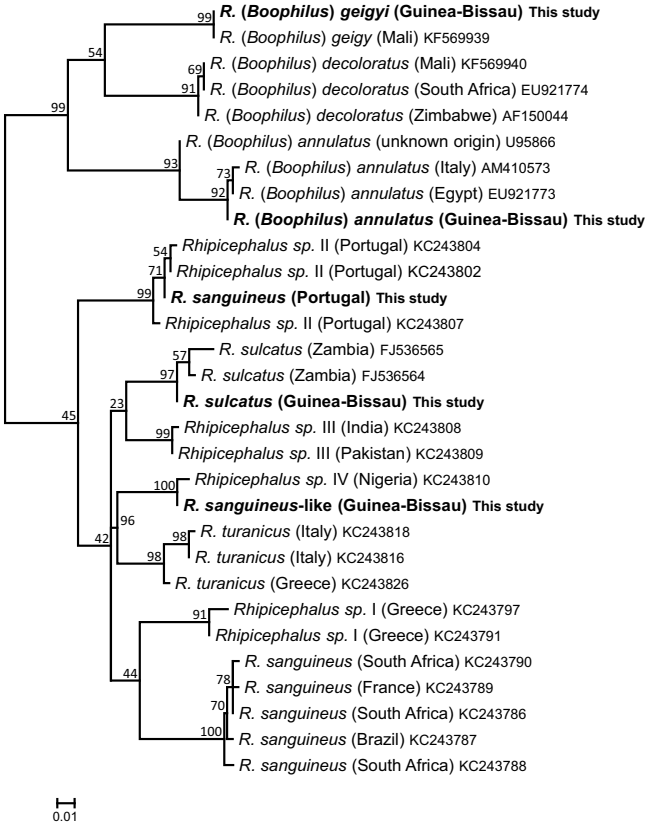

Fig. 7. Genetic relationships of *Rhipicephalus* spp. inferred from rDNA 12S gene sequences. Maximum likelihood (ML) phylogenetic tree construction with 1000 bootstrap replicates.

III (collected in India and Pakistan), the specimen identified as a *R. sulcatus* forms a separate group together with the other *R. sulcatus* sequences, from Zambia. These results support the morphological classification of these specimens as *R. sulcatus* and, altogether, the data presented support *R. sulcatus* as a valid species within the *R. sanguineus* group.

In the trees for both COI and 12S, the specimen morphologically classified as *R. sanguineus*-like branches together with morphotype IV (Figs. 6 and 7) and the identity matches are as high as 99.39% for 12S and 97.17% for COI, suggesting that these ticks belong to the same species. More specimens from the group *R. sanguineus* are needed to clarify if this group represents a valid individualized species, but, so far, the data support this hypothesis.

For the species in the group *R. sanguineus*, the clade distribution shows differences between the COI and the 12S trees, which does not happen in the subgenus *Boophilus*. In the COI tree, all classified species form homogenous groups and, therefore, our findings reinforce COI gene to be suitable for tick identification. However, COI seems less suitable for proximity inference among close related species such as in the *R. sanguineus* group and, for these purposes, 12S seems to be more informative and in accordance to the morphological data.

4. Conclusions

In the present study, we identify ticks collected from cattle within Geba River basin in two occasions, in the dry season of 2010 and in the rainy season of 2012. In the first collection, a relatively high number of *Boophilus* ticks (*R.* (*B.*) *geigyi* and *R.* (*B.*) *annulatus*) was found while in the rainy season collection *Amblyomma variegatum* was the dominant tick. Other ticks were found but with much less expression, *Hyalomma truncatum* (in both seasons) and specimens belonging to the *Rhipicephalus sanguineus* group (only in rainy season).

This is the first report on molecular information on tick species infesting cattle in Guinea-Bissau. The analysis of two gene fragments (COI and 12S) supports the morphological identification of the ticks collected and provides data with value for tick taxonomy. In this respect, combined morphology and molecular data here presented support *R. sulcatus* as a valid species within *R. sanguineus* group. Nevertheless, taxonomic revisions to this group remain crucial to name or to restore ancient taxons allowing a correct classification of specimens belonging to its subgroups.

Overall, the tick species found are *per se* a reason to support implementation of integrated prevention measures in cattle due to their role as vectors of important pathogens. Although more studies are needed to identify the presence and distribution of tick-borne pathogens they may harbour, the present study alerts to the possibility of disease outbreaks brought with the desirable future intensification and diversification of cattle production systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ttbdis.2016.10.013.

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