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# Development and validation of a PCR–RFLP test to identify African *Rhipicephalus* (*Boophilus*) ticks

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ABSTRACT

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* has recently invaded West Africa and caused anxiety amongst farmers in Ivory Coast, as livestock production was severely affected. The introduction of this tick species has remained unnoticed for several years, as all the members of this genus are very similar in appearance. To overcome the cumbersome morphological identification of the four closely related *R.* (*Boophilus*) spp. in the region, a PCR–RFLP test, based on a part of the second internal transcribed spacer ribosomal DNA (ITS2), was developed.

The molecular tool was successfully validated with a large number of ticks recently collected from West Africa and that were identified both morphologically and genetically. The tool developed is simple, fast, reliable and reproducible; hence it can be routinely applied for species identification.

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## 1. Introduction

Ticks of the genus *Boophilus*, recently grouped as a subgenus of *Rhipicephalus*, are some of the most important tick species in the world from an economical point of view (Estrada-Peña et al., 2006). Five species compose this genus: *R.* (*Boophilus*) *annulatus*, *R.* (*Boophilus*) *decoloratus*, *R.* (*Boophilus*) *geigyi*, *R.* (*Boophilus*) *kohlsi* and *R.* (*Boophilus*) *microplus*. *Rhipicephalus* (*Boophilus*) *kohlsi* was not included in this study, as it is only present in the Middle East and found exclusively on sheep and goat.

The remaining *Boophilus* species all colonize Africa and *R.* (*Boophilus*) *microplus*, being the most important cattle tick, has only just recently been introduced in West Africa. The recognition of this species amongst the three other closely related Boophilids, endemic in the area, is extremely difficult and could therefore easily be overlooked. The first indication of the introduction of this species was the failure of acaricide treatment as a result of the known high degree of resistance which characterizes this species (George et al., 2004).

In all of the tropical and subtropical areas where *R.* (*Boophilus*) *microplus* had been introduced, the tick became a serious hindrance to livestock production. Parasitism by *R.* (*Boophilus*) *microplus* results in poor condition, weight loss, reduced meat and milk production, and potential transmission of *Babesia bovis*, *B. bigemina* and *Anaplasma marginale* (Estrada-Peña and Venzal, 2006).

Recent publications indicate an extension of the distribution of *R.* (*Boophilus*) *microplus* and the displacement of *R.* (*Boophilus*) *decoloratus* in different countries like in Tanzania (Lynen et al., 2008) and in South Africa (Tonnesen et al., 2004).

The conventional method to identify *R.* (*Boophilus*) spp. relies on comparison of morphological characteristics of the different species, which is extremely cumbersome as size and differences between species are limited and sometimes even variable.

To be able to identify *R.* (*Boophilus*) *microplus* with certainty and so differentiate the four cattle related *R.* (*Boophilus*) spp., the development of a PCR–RFLP, based on sequence differences in the second internal transcribed spacer (ITS2), was the main objective of this study.

To validate the test, tick samples from West Africa were collected and identified both morphologically and genetically.

## 2. Material and methods

### 2.1. Sources of ticks

A preliminary cross-sectional tick survey was carried out in West Africa between May 2006 and February 2008 which yielded tick samples from nine countries: Senegal, Togo, Burkina Faso, Guinea-Conakry, Cameroon, Mauritania, Niger, The Gambia and Ivory Coast. In each country between three and 10 cattle were sampled. These ticks were stored in 70% alcohol, labeled and sent to the Institute of Tropical Medicine in Antwerp (Belgium) for analysis.

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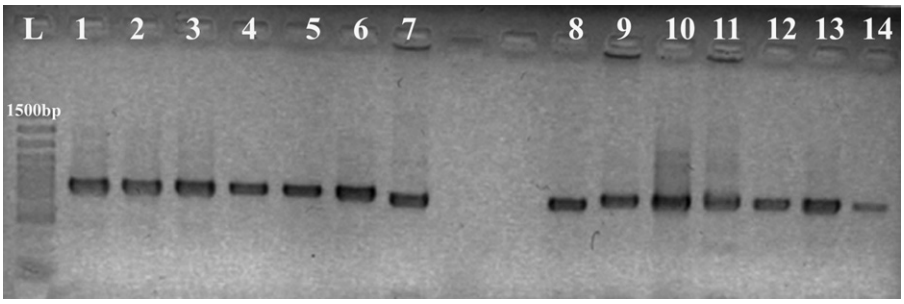


**Fig. 1.** PCR products. Ladder at the left (L), *R.* (*Boophilus*) *microplus* reference ticks in the columns 1–6, *R.* (*Boophilus*) *geigyi* reference ticks in column 7, *R.* (*Boophilus*) *decoloratus* reference ticks in columns 8–10 and *R.* (*Boophilus*) *annulatus* reference ticks in columns 11–13, positive control in column 14.

### 2.2. Morphological identification

The first step of the morphological identification of the ticks up the genus level was done using a stereomicroscope (Zeiss Stemi 2000) at 60× magnification. In a next step a microscope (Olympus) at the 100× magnification was used to identify *R.* (*Boophilus*) ticks at species level. For the latter, the hypostome dentition, presence of a protuberance bearing setae on palpal segment 1, external spur on coxae II and III, caudal process on male engorged ticks and shape of the internal and external spurs on adanal plates were used as discriminating characteristics (Walker et al., 2003).

The morphological identification of a subset of the ticks was confirmed by Prof. Ivan Horak (Faculty of Veterinary Tropical Diseases, University of Pretoria, South Africa).

### 2.3. Development of PCR and RFLP technique

To develop the PCR and RFLP technique, 27 reference ticks were included: nine *Rhipicephalus* (*Boophilus*) *microplus* adults from Ivory Coast, eight *R.* (*Boophilus*) *decoloratus* of which three came from Cameroon, two from Ivory Coast and three from The Gambia. Were also included five *R.* (*Boophilus*) *geigyi*: two from Ivory Coast and three from Guinea as well as five *R.* (*Boophilus*) *annulatus* from Cameroon. The ITS2 region of some morphologically identified specimens was sequenced to confirm the initial identification.

#### 2.3.1. Primers design

ITS2 sequences for *R.* (*Boophilus*) *microplus*, *geigyi*, *annulatus* and *decoloratus* (GenBank accession nos. U97715, AF271273, AF271272 and U97716) were downloaded and aligned using Clustal W software (Thompson et al., 1994). Primers were designed using a DNA computer software program Web primer (http://seq.yeastgenome.org/cgi-bin/web-primer). Amplification was done on the ITS2 gene using the forward primer Boophits2 F 5′-GCC-GTC-GAC-TCG-TTT-TGA-3′ and Boophits2 R 5′-TCC-GAA-CAG-TTG-CGT-GAT-AAA-3′ as reverse primer. GC content and self-annealing was checked on the Oligo Calc web site (http://www.basic.northwestern.edu/biotools/oligocalc.html). These primers were tested in the AmpliX program (http://ifrjr.nord.univ-mrs.fr/AmplifX) and the expected amplicon lengths were estimated. For *R.* (*Boophilus*) *microplus* the expected length was 829 bp, for *R.* (*Boophilus*) *geigyi* 765 bp, for *R.* (*Boophilus*) *annulatus* 832 bp and for *R.* (*Boophilus*) *decoloratus* 821 bp.

#### 2.3.2. DNA extraction

DNA was extracted using the method of Boom (Boom et al., 1990). This method is based on lytic activity and nuclease inactivating properties of proteinase K together with the nucleic acid-binding properties of silica particles.

#### 2.3.3. DNA amplification

Standard PCR amplifications were carried out in 25 μl reaction mixtures containing 5 μl of the extracted DNA, 1.65 mM $MgCl_2$, 0.2 mM of the four dNTPs, 10 pM of each primer, 1 U *Taq* polymerase enzyme (Promega) and 1 μl Yellow Sub™ (GENEO Bioproducts, Hamburg, Germany). The reaction mixture was overlaid by a drop of fine neutral mineral oil (ICN) and placed on a heating block of a programmable thermocycler (Biometra, Westburg). After a denaturation step of 4 min at 94 °C each of the 40 cycles consisted of 30 s at 92 °C, 45 s at 58 °C and 60 s at 72 °C and ended with an extension step of 8 min at 72 °C.

The mixtures were examined for the presence of DNA fragments by loading 5 μl of each reaction mixed with 2 μl of loading buffer onto 2% agarose gels (Sigma). A 1.5 kb DNA ladder (MBI Fermentas, Lithuania) was loaded on every gel. The samples were run for 20 min at 100 V, stained in ethidium bromide for 30 min, washed under running tap water and photographed under UV illumination. For further typing of the fragments, RFLP based methods were used.

#### 2.3.4. Restriction fragment length polymorphism (RFLP)

Suitable restriction enzymes were identified with the aid of the online tools of The Restriction Enzyme Database (Rebase® http://rebase.neb.com/rebase/rebase.html). Restriction site positions and fragment lengths were determined with DNALC Sequence utilities (http://www.dnalc.org/bioinformatics/dnalc_nucleotide_analyzer.htm). PCR products were digested by *Msp1* enzyme (6 U) with 4 μl of amplified DNA in 15 μl total volume. The reaction was left overnight at 37 °C. Four microliters of all restricted sample mixed with 2 μl of loading buffer was transferred onto a polyacrylamide gel and a 1 kb DNA superladder (Eurogentec) was added to all gels for fragment size determination. DNA fragments were separated by vertical electrophoresis in TBE buffer at 100 V for 2.5 h. The gel was stained using Sybr Green I (Cambrex Bio Science, Rockland, Inc.) during 40 min.

Restriction sites are located for *R.* (*Boophilus*) *microplus* at 241 bp, 311 bp and 408 bp, for *R.* (*Boophilus*) *geigyi* at 108 bp, 323 bp, 333 bp, 385 bp, 709 bp, for *R.* (*Boophilus*) *annulatus* at 108 bp, 241 bp, 311 bp, 408 bp and for *R.* (*Boophilus*) *decoloratus* at 107 bp, 241 bp, 311 bp, 391 bp, 407 bp, 442 bp, 765 bp. Thus, in agarose gel, theoretically for *R.* (*Boophilus*) *microplus* four bands of 421 bp, 241 bp, 97 bp and 70 bp had to be found. For *R.* (*Boophilus*) *geigyi*, six bands of 324 bp, 215 bp, 108 bp, 56 bp, 52 bp and 10 bp. For *R.* (*Boophilus*) *annulatus* five bands of 424 bp, 133 bp, 108 bp, 97 bp and 70 bp. For *R.* (*Boophilus*) *decoloratus* eight bands of 323 bp, 134 bp, 107 bp, 80 bp, 70 bp, 56 bp, 35 bp, 16 bp.

## 3. Results

The morphological identification of 1070 ticks revealed that the *R.* (*Boophilus*) specimens represented 39% of the collected ticks, predominantly *R.* (*Boophilus*) *geigyi* except in Ivory Coast where *R.*

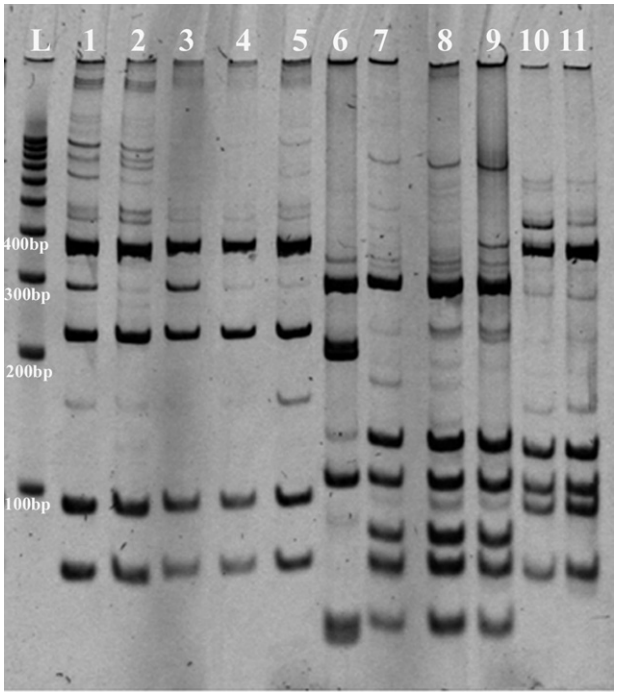


**Fig. 2.** RFLP profiles. Ladder at the left (L), *Rhipicephalus* (*Boophilus*) *microplus* (bands around 400 bp, 250 bp, 100 bp, 70 bp) in columns 1–5; *R.* (*Boophilus*) *geigyi* (bands around 300 bp, 200 bp, 100 bp, and 2 bands around 50 bp) in the column 6; *R.* (*Boophilus*) *decoloratus* (bands around 300 bp, 150 bp, 100 bp, 80 bp, 70 bp, 50 bp, 20 bp) in columns 7–9; *R.* (*Boophilus*) *annulatus* (bands around 400 bp, 150 bp, 100 bp, 90 bp, 75 bp) in column 10 and 11.

(*Boophilus*) *microplus* probably overtop all other *R.* (*Boophilus*) spp. One third of these *Boophilus* ticks remained unidentified at species level because of damaged mouthparts.

The 27 reference ticks allowed the development of a PCR–RFLP. This PCR–RFLP allowed then the identification of the damaged *Rhipicephalus* (*Boophilus*) ticks.

The PCR amplification gave for 55 tested samples a major product between 765 bp and 832 bp (see Fig. 1) as predicted by AmpliX program. The RFLP using *Msp* I digestion gave very distinct profiles differentiating the four *Rhipicephalus* (*Boophilus*) species as theoretically expected (see Fig. 2).

The profile of *R.* (*Boophilus*) *annulatus* showed five bands on agarose gel (around 400 bp, 150 bp, 100 bp, 90 bp, 75 bp) (see Fig. 2 columns 10–11), whereas *R.* (*Boophilus*) *microplus* showed four bands (around 400 bp, 250 bp, 100 bp, 70 bp) (see Fig. 2 columns 1–5). *Rhipicephalus* (*Boophilus*) *geigyi* was characterized by 5 bands (around 300 bp, 200 bp, 100 bp, and 2 bands around 50 bp). One band of 10 bp is missing due to the very short length of this fragment (see Fig. 2 column 6).

*Rhipicephalus* (*Boophilus*) *decoloratus* showed 7 different bands (around 300 bp, 150 bp, 100 bp, 80 bp, 70 bp, 50 bp, 30 bp). One band of 16 bp was missing also due to the very short length of this fragment (see Fig. 2 columns 7–9).

## 4. Discussion

The genus *Boophilus* consists of five species of which three were present in West Africa for many years: *R.* (*Boophilus*) *annulatus*, *R.* (*Boophilus*) *decoloratus* and *R.* (*Boophilus*) *geigyi*. In 2007, *R.* (*Boophilus*) *microplus*, an important vector of *Babesia bovis*, had been identified for the first time in this region where it now seems to cause major problems in dairy farms (Madder et al., 2007) (unpublished data). The introduction most likely occurred during one of the imports of Girolando cattle from Brazil, and this to improve local cattle breeds. The most important questions however, still remains unanswered: when was this tick introduced? It appears that private persons undertook several import actions the last decade. Before 2007 and after the first import of Girolando probably in 2000, no published data are available of the presence or absence of *R.* (*Boophilus*) *microplus*.

As outlined in the introduction, the morphological differences between some of the species of this genus are extremely small and some of the characteristics also seem to be variable. The difference between females of *R.* (*Boophilus*) *microplus* and *R.* (*Boophilus*) *annulatus* is almost exclusively based on the presence or size of the external spurs of the second and third coxae. It was however observed that some specimens originating from Cameroon did show external spurs although the size and shape was somewhat different from those of *R.* (*Boophilus*) *microplus*. The hypostome dentition has been described to be variable as well, *R.* (*Boophilus*) *decoloratus* sometimes presents a dentition of $3.5 \times 3.5$ (Hoogstraal, 1956). In this study we also observed *R.* (*Boophilus*) *microplus* with a dentition of $4.5 \times 4.5$ (three or four extra teeth in between the inner rows). The same applies to the shape of the spurs of the adanal plates of the males, which might be extremely variable in the case of *R.* (*Boophilus*) *microplus*. If the latter species would have been introduced in an area, it could have easily been overlooked, especially when ticks were damaged during collection or not cleaned thoroughly before identification. Especially female ticks are extremely difficult to identify and without males, which are easily been overlooked due to their small size, the presence of *R.* (*Boophilus*) *microplus* is extremely difficult to validate. In fact, morphological identification is far from specific, time consuming and requires sufficient expertise on different populations of the species in question. The molecular tool that was implemented represents a valuable aid for identification and would allow confirmation or disproval of previous or historical identifications.

The first results of the PCR–RFLP tool yielded species-specific profiles and as all the screened populations of the same species originating from different countries showed consistent profiles, the test could therefore be used as a golden standard for tick identification of this genus in the region. The only restriction for the identification of *R.* (*Boophilus*) *microplus* might be the populations from Australia as recent studies have concluded the Australia strain not being able any more of producing fertile crosses with Africa and Latin American strains and being genetically different (Labruna et al., 2009).

The presence of *R.* (*Boophilus*) *microplus* in Ivory Coast was confirmed without any doubt. The samples that were collected in this country only presented *R.* (*Boophilus*) *microplus*, indicating that a recent introduction into this area seems unlikely or extremely successful. From all other countries in this study no *R.* (*Boophilus*) *microplus* were identified so far. It must however be mentioned that the number of samples screened was fairly low and consequently this should be confirmed.

Future investigations are needed to determine the real extent of the presence of *R.* (*Boophilus*) *microplus* in Ivory Coast and neighboring countries and the effect on the transmission dynamics of *Babesia* spp. present in the area. To secure livestock production, improvement of cattle breeds and the import of exotic cattle breeds, the development of this reliable tool could offer opportunities for surveillance of ticks on cattle especially during importation.

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