

A revision of two distinct species of *Rhipicephalus*: *R. microplus* and *R. australis*

Uma revisão sobre duas espécies distintas de *Rhipicephalus*: *R. microplus* e *R. australis*

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— REVIEW —

ABSTRACT

Rhipicephalus (Boophilus) species are monoxenous ticks with seasonal distribution in tropical and subtropical regions. For many years, *Rhipicephalus microplus* was considered as a single species; however, further analysis split these ticks into two distinct species. Because *R. microplus* and *R. australis* share similar attributes, it is hard to discriminate these two species and explain the changes in the classification of these parasites over the past decades. The reappearance of *R. australis* is an outcome of new research, which has afforded to better characterize these probably cryptic species. Evidence based on morphological features, the lack of conspecificity, microsatellite markers, mitochondrial 12S and 16S ribosomal DNA, and mitochondrial genome supports the re-classification of *R. microplus* as different species. Therefore, populations of *R. microplus* from Australia, Cambodia, Philippines, Indonesia, New Caledonia, Borneo, New Guinea, Tahiti and parts of Southeast Asia were recently reinstated as *R. australis*. Moreover, a better knowledge on the speciation between these two species could pave the way to important advances in tick control strategies.

Key words: *Rhipicephalus*, *Boophilus*, *microplus*, *australis*, taxonomy.

RESUMO

As espécies pertencentes ao gênero *Rhipicephalus (Boophilus)* são carrapatos monoxenos de distribuição sazonal em regiões tropicais e subtropicais. Por muito anos, duas espécies de carrapatos foram consideradas como *Rhipicephalus microplus*. Contudo, estudos recentes reclassificaram esse

carrapato em duas espécies: *R. microplus* e *R. australis*. Em razão de diversas semelhanças entre *R. microplus* e *R. australis*, distinguir essas duas espécies torna-se uma tarefa árdua, o que explica as mudanças de classificação dessas espécies nas últimas décadas. O reaparecimento da espécie *R. australis* surge com novas pesquisas, resultado de uma melhor caracterização dessas prováveis espécies crípticas. Evidências baseadas em análises das características morfológicas, na ausência de co-especificidade, em marcadores de microssatélites, no DNA ribossomal mitocondrial 12S e 16S, assim como no genoma mitocondrial, suportam a re-classificação de *R. microplus* como duas espécies distintas. Nesse sentido, populações de *R. microplus* da Austrália, Camboja, Nova Caledônia, Bornéu, Filipinas, Nova Guiné, Indonésia e Taiti foram recentemente renomeadas como *R. australis*. Além disso, um melhor entendimento sobre a especiação e localização dessas duas espécies pode trazer avanços importantes para melhorar as estratégias de controle desses carrapatos.

Palavras-chave: *Rhipicephalus*, *Boophilus*, *microplus*, *australis*, taxonomia.

INTRODUCTION

Records of ticks date back to approximately 2,000 years, when they were considered anus-less animals by Pliny, the Elder (A.D. 23-79) (HILLYARD, 1996). Later, reverend Dr. Thomas Moufet (A.D. 1553-1604) noted that *Ricinus* means “filled with

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food abundantly and yet there is no passage for any excrement” (BOWMAN & SAUER, 2004). To answer the question from “where the first ticks were evolved?” DOBSON & BARKER (1999) suggested that ticks evolved in the part of the supercontinent Gondwana that became Australia, in the early Devonian era, around 390 Mya ago. Contrarily, KLOMPEN et al. (2000) suggested that the hard ticks evolved 120 Mya later, after Australia became relatively isolated.

Ticks are hematophagous acarines mainly distributed in two major families, *Ixodidae* (hard tick) and *Argasidae* (soft tick) (BLACK & PIESMAN, 1994), while only one tick species is present in the *Nuttalliellidae* family (GUGLIELMONE et al., 2010). Argasid ticks feed fast, but take in small volumes of blood in each meal, contrasting with Ixodid ticks, which have a longer-feeding process and ingest large amounts of blood. Ixodid ticks are unique among ectoparasites due to their long host attachment period (SAUER et al., 2000) and are divided into metastriate and prostriate ticks. Metastriate ticks include *Rhipicephalus* genera, characterized by relatively short mouthparts and secretion of copious amounts of cement or glue responsible for the firm attachment to its host. Prostriate ticks include the *Ixodes* genera, with longer and barbed mouthparts that are responsible for attachment to the host (FRANCISCHETTI et al., 2009).

Studies demonstrated differences of specific *Rhipicephalus* (*Boophilus*) spp. life cycle in different ecological zones, where varying degrees of climatic conditions affect each phase of the tick life cycle (LEGG, 1930; LONDT & ARTHUR, 1975) and changes in these factors can difficult precise establishing of the life cycles of *R. microplus* and *R. australis*.

In recent years, *Boophilus* spp. was reassigned to the genus *Rhipicephalus* (MURRELL & BARKER, 2003). Since, based on molecular and morphological studies, some *Rhipicephalus* species were found to be more closely associated to *R. (Boophilus)* species in comparison to *Rhipicephalus* species (MURRELL et al., 2000). Substantial morphological and molecular data have produced important evidence supporting the genus *Rhipicephalus* as paraphyletic to the genus *Boophilus* (MURRELL & BARKER, 2003; BARKER & MURRELL, 2004). To avoid misunderstanding and disbelief among researchers concerning name changes, *Boophilus* was retained as a subgeneric epithet, and *Boophilus microplus* became *Rhipicephalus (Boophilus) microplus* (GUGLIELMONE et al., 2010) or *Rhipicephalus microplus*.

The controversy about the classification of *R. australis* and *R. microplus* started in 1899, when the morphological description of *Rhipicephalus australis* moved this tick from the group of rhipicephalines species (FULLER, 1899). However, differences in morphological features, reproductive parameters, and genetic constitution between *R. microplus* specimens collected in America and Africa and individuals collected in Australia provide sufficient evidences to reclassify *R. australis* and *R. microplus* as different species (LABRUNA et al., 2009; ESTRADA-PEÑA et al., 2012). Based on such evidence, we reviewed the data supporting the current taxonomic position of *R. microplus* and *R. australis* as distinct species.

Current taxonomy

Morphological differences

R. microplus, earlier named *Haemaphysalis micropla* Canestrini, 1888, was not precisely distinguished from *R. australis* when this species was identified by Fuller (FULLER, 1899). However, SALMON & STILES (1901) classified *R. australis* as a distinct species, while NEUMANN (1901) regarded *R. australis* as a subspecies of *R. microplus*. Several studies initially supported the conspecificity between these two species (BEDFORD, 1932; ROBERTS, 1965). Overall differences in specimens collected in Australia, America, and Africa are allegedly strong enough to support *R. australis* as a distinct species (MINNING, 1934). Contrarily, UILENBERG (1962) concluded that these morphological variations did not validate the notion that *R. australis* is a distinct species; therefore the author synonymized it under the name *B. microplus*. Similarly, for LONDT & ARTHUR (1975) the morphological variation observed between Australian and South African *R. microplus* do not afford to maintain these two tick populations as distinct species. Contrarily, recent studies suggest that both female and male adults of *R. australis*, besides this species' clearly smaller larva, differ from *R. microplus* by a combination of other morphological characters (ESTRADA-PEÑA et al., 2012).

Despite the features closely shared by *R. australis* and *R. microplus*, a clear set of morphological differences persists. Larvae of *R. australis* described as *B. microplus* using specimens collected in Australia are smaller, with a narrow dorsal scutum than *R. microplus* (CLIFFORD et al., 1961; ESTRADA-PEÑA et al., 2012). Adult *R. australis* can be recognized by ventro-medial spurs in male palpal segments, and the copious, plumose, pale white setae on the female dorsum. Variability in other features like

adanal and coxal shields among different populations often lead to incorrect classifications. *R. australis* possess abundant longer and pale dorsal setae, and the median alloscutal setae are arranged in clusters of 4 to 6 rows. However, this feature may be lost in engorged females. *R. microplus* dorsal setae are smaller and slim, and medial alloscutal setae are composed by clusters of 2 to 3 rows (WALKER et al., 2003). As compared to *R. microplus*, the medial scutal setae in *R. australis* female are longer. The setae behind the eyes are visible in *R. australis* female but unapparent *R. microplus* female. The spur in the ventral surface of *R. australis* male is absent in *R. microplus* male. Finally, in *R. microplus* males possess numerous setae on the lateral margins of the ventral surface of the capitulum, which are smaller in *R. australis* (WALKER et al., 2003; ESTRADA-PEÑA et al., 2012).

Genetic differences

Crosses mating trials

Cross mating studies are a useful tool in the differentiation of tick species. For example, *R. annulatus* and *R. microplus* appear to be closely related within the subgenus *Boophilus*, as compared to other *R. (Boophilus)* species (BEATI & KEIRANS, 2001). *R. microplus* and *R. annulatus* cross-breeding has revealed that these two species lack conspecificity (GRAHAM et al., 1972; THOMPSON et al., 1981; DAVEY et al., 1983). A boundary in distributions of *R. microplus* and *R. annulatus* along the Texas-Mexico border suggests that these species reproduced without cross-breeding (LOHMEYER et al., 2011).

In the same way, genetic differences among *R. microplus* strains found at specific biogeographical and ecological areas led taxonomists to consider boophilid ticks from America/Africa as different species against those from Australia. The first evidence supporting the lack of genetic conspecificity between African and Australian *R. microplus* was provided by SPICKETT & MALAN (1978), regarding these species as 'diverging taxa' under speciation process with recent history as distinct populations. Conversely, GUGLIELMONE et al., (2003) concluded that *R. microplus* from South Africa and Australia should be considered as distinct species. Crossbreeding of Australian and Argentinean or Mozambican population of boophilid ticks has led to infertile animals, differently of Argentinean and Mozambican population crosses, that resulted in fertile offspring. These observations support the conclusion that tick population from Mozambique and Argentina represent a single species, while tick from

Australia is possibly a distinct species (LABRUNA et al., 2009). Furthermore, mating experiments between *R. microplus* from South Africa and *R. australis* from Australia produced infertile offspring (SPICKETT & MALAN, 1978), which suggests reproductive isolation between these two species.

Differences based on mitochondrial genome

In addition to morphological observations, mitochondrial 12S and 16S ribosomal DNA (rDNA) has been considered a promising tool to determine phylogenetic relationships among various tick species (MANGOLD et al., 1998; BEATI & KEIRANS, 2001; LABRUNA et al., 2009; ESTRADA-PEÑA et al., 2012; MCCOOKE et al., 2015). Indeed, phylogenetic analysis based on rDNA also supports the lack of conspecificity among ticks (ESTRADA-PEÑA et al., 2012). In this way, rDNA phylogenies using sequences from a number of Australian populations lend strength to suggestion that two species is classified under the name *R. microplus*. Besides the absence of consistent data on specimens and the few number of populations employed for the molecular and cross-breeding by LABRUNA et al. (2009), the authors believe that the available information is sufficient to support the taxonomic separation of *R. australis* and *R. microplus*.

As discussed above, the phylogenetic relationships among *R. (Boophilus)* species were mostly carried out based on evolutionary relationships, relying on partial rDNA sequences (MURRELL et al., 2000, 2001, 2003; BEATI & KEIRANS, 2001; LABRUNA et al., 2009; ESTRADA-PEÑA et al., 2012). Molecular analysis (BEATI & KEIRANS, 2001) has confirmed the sister-species relationship between *R. annulatus* and *R. microplus*, as previously suggested based on morphology (FELDMAN-MUHSAM & SHECHTER, 1970). Phylogenetic evidence (MURRELL et al., 2001) about the genus *Rhipicephalus* also supports monophyly of the *Boophilus* clade; however, this evidence did not solve the relationships among *R. (Boophilus)* species. Moreover, the '*R. microplus*' specimens used in that work were from Australia (*R. australis*), and did not include *R. microplus* s.s. Recent studies based on a wide geographic sampling range of *R. microplus* also support monophyly of the subgenus *Rhipicephalus (Boophilus)*, thus the reinstatement of *R. australis* (LABRUNA et al., 2009; ESTRADA-PEÑA et al., 2012). Intriguingly, *R. microplus* strains collected in India and Nepal show high divergence from American and African *R. microplus* strains (LABRUNA et al., 2009). However, the phylogenetic placement of

Indian and Nepalese *R. microplus* strains was not resolved, though Indian *R. microplus* clustered with *R. annulatus* in a 16S rRNA analysis (LABRUNA et al., 2009).

Mitochondrial genomes were investigated in tick phylogenetic relationships, and have been proved useful in solving tick lineages (BURGER et al., 2012, 2013, 2014a,b; WILLIAMS-NEWKIRK et al., 2015; MCCOOKE et al., 2015). To date, a few mitochondrial genomes of rhipicephaline ticks have been sequenced, and include *R. sanguineus* (BLACK & ROEHRDANZ, 1998), *R. simus* (Xu et al., 2014) and 13 gene fragments of *R. australis* mitochondrial genome (CAMPBELL & BARKER, 1999). Whole and incomplete mitochondrial genomes of *R. annulatus*, *R. australis*, *R. kohlsi*, *R. geigy*, and *R. microplus* strains from Brazil, Texas (USA) Cambodia and China were recently sequenced (BURGER et al., 2013; MCCOOKE et al., 2015). Mitochondrial, *cox1* and 16S rRNA phylogenetic analyses showed a species complex of *R. annulatus*, *R. australis*, and two clades of *R. microplus*. Morphologically related China and India *R. microplus* population were found closed to *R. annulatus* than other specimens of *R. microplus* from Asia, South America, and Africa. Furthermore, *cox1* and 16S rRNA nucleotide sequences afforded to resolve the phylogenetic relationships within the *R. microplus* complex more successfully, as compared to 12S rRNA or the nuclear marker ITS2 (CAMPBELL & BARKER, 1999; LEMPEREUR et al., 2010; BURGER et al., 2013).

Microsatellite markers

The genetic differences between *R. microplus* populations from the TEQA (tick eradication quarantine area) USA, Mexico, and Puerto Rico were first investigated using isozymes over three decades ago (SATTLER et al., 1986). Analysis of fifteen selected polymorphic loci demonstrated heterozygosity to arthropods and high genetic similarity among various strains, suggesting an undifferentiated gene pool in *R. microplus* populations prevalent in North America. Later, microsatellite polymorphism was investigated in tick populations from Argentina, Australia, India, Mozambique, and New Caledonia. Tissues of homologous and heterologous crosses were analyzed, and allele numbers and sizes were determined for each locus. The clustering analysis of microsatellite alleles from *R. microplus* offspring crosses revealed that Australian homologous and heterologous crosses had lower similarity, in comparison to homologous

and heterologous crosses between Mozambique and Argentinian strains (LABRUNA et al., 2009).

In New Caledonia, *R. microplus* has been found in close contact with sympatric hosts, on cattle and on a new host, the rusa deer (*Cervus timorensis*), divided into two differentiated genetic pools. In North America, the white-tailed deer (*Odocoileus virginianus*) and the red deer are also well-studied examples that illustrate this pattern of new sympatric hosts. This phenomenon may have occurred across the globe, in areas where this tick has established some degree of interaction with distinct host species in sympatry with livestock (KOFFI et al., 2006a; DE MEEUS et al., 2010; ARAYA-ANCHETTA et al., 2015). Using eight microsatellite loci, low genetic structure was found in specimens of *R. australis* in New Caledonia (KOFFI et al., 2006b). Analyses using 11 microsatellite markers revealed that the genetic differences between Australian *R. australis* populations endemic in Queensland and New South Wale are not significant; however, the observed diversity among strains within each location proposes limitations to local gene flow (CUTULLÉ et al., 2009). A significant and small genetic structure was detected in ticks that infest rusa deer or domestic cattle (De MEEUS et al., 2010), which suggests that this sympatric adaptation resulted in host-specific populations. Recently, microsatellite markers were successfully used to detect genetic variation among *R. microplus* strains in Texas (BUSCH et al., 2014). Undifferentiated tick collections were analyzed using 11 repeat loci, and it was not possible to identify any genetic divergence between *R. microplus* sampled from white-tailed deer and specimens collected from cattle, even when the ticks were obtained from the same pastures (BUSCH et al., 2014). When parasitized by cattle ticks, these wild ungulates pose a major obstacle to eradication programs, since the difficulty to treat these animals with acaricides (POUND et al., 2010).

Differences in Bm86 gene sequences

Characterization employing an intron and DNA short tandem repeats (STRs) from Bm86 gene of Latin America and Australia *R. microplus* populations revealed significant differences within the Bm86 coding region between ticks from these regions (De La FUENTE et al., 2000). The intron sequence dissipated in the phylogenetic analysis, showing that individuals of the same strain do indeed vary. Moreover, polymorphism analysis at STR suggests differences within and between populations of *R. microplus*, which supports the

molecular level of existence between *R. microplus* populations (De La FUENTE et al., 2000).

Ecological niche

Field data on regional distribution are necessary to clarify the actual distribution and the evolution of *R. microplus* and *R. australis*. It has been hypothesized that *R. microplus* originated in South and Southeast Asia (HOOGSTRAAL, 1985), later spreading to Madagascar and Southern Africa. Data required to shed more light on how and when *R. microplus* spread to Americas is scarce (LABRUNA et al., 2009), although it is reasonable that the tick was brought attached on livestock imported from India or Africa 4-5 centuries ago (BARRÉ & UILENBERG, 2010). Since *R. microplus* is specific to ungulates, the transportation of European cattle (*Bos taurus*) has spread it throughout the tropical and subtropical belt. As compared to tropical *Bovidae*, European bovines are almost incapable of eliciting efficient immune responses to *R. microplus* infestations (FRISCH, 1999). Other factors that may have constrained the geographical distribution of *R. microplus* include climate variables and competition with other tick species (ESTRADA-PENÑA et al., 2006; CHEVILLON et al., 2013). Furthermore, it was suggested that *R. australis* was introduced in Australia and New Caledonia on livestock in 1829 (ANGUS, 1996), and 1942 (VERGES, 1944; DE MEEÛS et al., 2010), respectively. Today, *R. australis* is prevalent in Australia, Cambodia, Philippines, Indonesia, New Caledonia, Borneo, Malaysia, New Guinea and Tahiti (LABRUNA et al., 2009; ESTRADA-PENA et al., 2012; LOW et al., 2015). *R. microplus* is endemic between parallels 32°N and 32°S, where the major cattle breeding countries are, including newly reported ecological preferences in West Africa (MADDER et al., 2007; ESTRADA-PENÑA et al., 2006; LEGER et al., 2013). In addition, *R. microplus* and *R. australis* coexist in southeastern Asian countries (LOW et al., 2015). However, climate changes may further modify the potential geographical distribution of these parasites.

Implications in control methods

Economically, ticks are among the most potentially harmful parasites worldwide (PIESMAN & EISEN, 2008; GRISI et al., 2014). During infestation, ticks may transmit numerous pathogenic bacterial, viral, and protozoan organisms to the host (HAJDUSEK et al., 2013). The combination of the tick vectoring ability and the overall undermining of the host's health caused by blood uptake leads to high losses in livestock and pet industries (WILLADSEN, 2004). *R. microplus* was introduced in the New

World by tick-infested cattle brought by explorers and colonists (HOOGSTRAAL, 1985). Estimated losses caused by these ectoparasites during the first decade of the 19th century reached about US\$63 billion (MOHLER, 1906). Therefore, due to the huge economic impact caused by *R. microplus*, the United States started a national campaign to eradicate *Boophilus* in 1906, and by 1943 this program was declared complete (GRAHAM & HOURRIGAN, 1977). Considering Brazil alone, which has the largest industrial cattle herd in the world; potential losses caused by *R. microplus* are estimated at US\$3.24 billion yearly (GRISI et al., 2014). Tick control is a daunting challenge, because current control methods based on synthetic acaricides are becoming increasingly inefficient (RECK et al., 2014).

Effective new control strategies demand a precisely parasitological epidemiology, because these strategies are affected by the presence of *R. microplus* in a given geographical area. Vaccination experiments demonstrated different degrees of susceptibility for Bm86-based vaccines by *R. microplus* populations, suggesting the existence of genetically distinct strains of *R. microplus* (COBON et al., 1995; GARCIA-GARCIA et al., 2000). The Bm86 vaccine developed from an Australian '*R. microplus*' strain showed very low efficacy when used in Latin America, as compared to Bm95, a Bm86 homologue from the Argentinian *R. microplus* strain, which showed higher vaccinal efficacy in this region (GARCIA-GARCIA et al., 2000). Likewise, a Bm86 formulation from Cuban *R. microplus* showed higher protection levels in America, when compared with Bm86 from Australian '*R. microplus*' (De La FUENTE et al., 2007). Consequently, it is important to clearly describe vaccination data regarding the tick strain analyzed, due to the observed species-specificity of protection levels. Moreover, risk areas for tick-borne disease also need re-analysis, given the potential differences in vector capability by these two tick species.

CONCLUSION

The triumph of taxonomy at species level is an open tool for continuous and exciting discoveries. Better understanding of *R. microplus* and *R. australis* taxonomic structure is pivotal for control measures against these ticks. Further studies on population genetic of *Rhipicephalus* (*Boophilus*) spp. will more thoroughly explain the interactions among these parasites, the pathogens they vector, and their hosts. In addition, such studies will provide more in-depth information

about tick movements, disease dispersal, and designing anti-tick control programs. Genetic variance studies among *Rhipicephalus* (*Boophilus*) spp. will better elucidate the identification of cryptic species. Mixed infestation, when the parasites are in close contact with sympatric alternative hosts (like deer, for instance) is a major hurdle for eradication programs. However, more detailed information about genetic differentiation and genetic population structure could provide tools to improve control strategies. As demonstrated in recent years, the lack of effective protection induced by the Bm86 vaccine can be partly explained considering the presence of different *R. microplus* strains. These failed vaccine experiments can be explained if *R. microplus* is considered not a single, but two distinct species. Although *R. australis* is very similar to *R. microplus*, both the larvae and adults of each species have a clear set of species-specific characters. In conclusion, reported results confirm that two distinct species of ticks were named *R. microplus*.

ACKNOWLEDGMENT

This work was supported by grants from Higher Education Commission, Pakistan Science Foundation (Pakistan), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We would like to thank Dr. Carlos Termignoni for critical reading of this review.

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