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Phylogenetic analysis of the mitochondrial genomes and nuclear rRNA genes of ticks reveals a deep phylogenetic structure within the genus *Haemaphysalis* and further elucidates the polyphyly of the genus *Amblyomma* with respect to *Amblyomma sphenodonti* and *Amblyomma elaphense*

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ABSTRACT

We sequenced the entire mitochondrial genomes of 3 species of metastriate ticks: *Haemaphysalis formosensis*, *H. parva*, and *Amblyomma cajennense*. We also sequenced two thirds (ca. 9500 bp) of the mitochondrial genomes of *H. humerosa* and *H. hystricis*. We used these 5 mitochondrial genome sequences together with the 13 tick mitochondrial genomes we sequenced previously and the 2 tick mitochondrial genomes sequenced by Black and Roehrdanz (1998), as well as the nuclear rRNA genes from 84 ticks and mites, in phylogenetic analyses. Our analyses reveal deep phylogenetic structure within the genus *Haemaphysalis*, with at least 2 species, *H. parva* and *H. inermis* that are highly divergent from the rest of the genus *Haemaphysalis*. We identify a region of the 18S rRNA gene which correlates with this division of the genus *Haemaphysalis* as well as a novel insertion in the mitochondrial genome of *H. parva*. We reject the hypotheses of Hoogstraal and Aeschlimann (1982) and Barker and Murrell (2004) on the relationships among metastriate genera. Instead, our analysis provides further evidence for the division of the Metastriata into 2 major lineages: (i) *Amblyomma* s.s. plus Rhipicephalinae (i.e. *Rhipicephalus*, *Hyalomma*, *Rhipicentor*, and *Dermacentor*); and (ii) *Haemaphysalis* plus *Bothriocroton* plus *Amblyomma sphenodonti*. We also provide further evidence for the polyphyly of the genus *Amblyomma* with respect to *A. sphenodonti* and *A. elaphense*. The most likely position of *A. elaphense* is sister to the rest of the Metastriata; the most likely position of *A. sphenodonti* is sister to the genus *Bothriocroton*. These 2 species do not belong in the genus *Amblyomma*, and we propose that new genera are required for *A. sphenodonti* and *A. elaphense*.

Introduction

Ticks (Chelicerata: Anactinotrichida: Ixodida) are blood-feeding ectoparasites of terrestrial vertebrates. Hard ticks (Ixodidae) comprise 702 of the 896 valid tick species (Guglielmone et al., 2010) and are organised into 2 groups: Prostriata, containing only the genus *Ixodes*, and Metastriata, containing the 13 other currently recognised hard tick genera (Hoogstraal and Aeschlimann, 1982; Guglielmone et al., 2010). The metastriate genus *Haemaphysalis* is the second most specious tick genus, with 166 currently valid species. The genus is distributed globally, though the greatest diversity is found in southeastern Asia (Kolonin, 2009).

One of the defining morphological features of the genus *Haemaphysalis* is the presence of a prominent "blade-like dorsal retrograde process" (Nuttall and Warburton, 1915) on trochanter I (i.e. a large backwards-facing hump or spur on the first segment of the first leg). Also characteristic of the genus are short, wide palps, with the palp femur projecting laterally over the rectangular basis capitulum. The genus *Haemaphysalis* was studied by Hoogstraal and Kim (1985), who proposed a trend in the evolution of morphology within and among the subgenera of *Haemaphysalis* from atypical and 'primitive' *Amblyomma*-like forms to typical *Haemaphysalis*-like forms. Hoogstraal and Kim (1985) 'graded' the subgenera of *Haemaphysalis* into 3 categories: (i) Structurally Primitive, (ii) Structurally Intermediate, and (iii) Structurally Advanced. Hoogstraal and Kim (1985) also placed much emphasis on tick–host specificity and apparent tick–host coevolution; more recent work suggests that the degree to which ticks are host-specific is overestimated and that ecological specificity is more important in tick evolution (Klompen et al., 1996).

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Hoogstraal and Kim's (1985) hypothesis on the relationships among species of *Haemaphysalis* have not yet been fully tested by phylogenetic analysis. Klompen et al. (1997) addressed some of the relationships among *Haemaphysalis* subgenera in a phylogenetic analysis of the Metastriata, based on morphology. Klompen et al. (1997) supported monophyly of *Haemaphysalis*, but could not resolve the phylogenetic position of the Structurally Primitive subgenera with respect to the rest of the genus *Haemaphysalis*.

Sampling of the genus *Haemaphysalis* in molecular analysis has been limited; most sequences of *Haemaphysalis* spp. in GenBank are from Structurally Advanced species. One Structurally Primitive species, *H. inermis* did not cluster with the rest of the genus *Haemaphysalis* in analyses of mitochondrial 12S and 16S rRNA (Norris et al., 1999) and nuclear 18S rRNA (Dobson and Barker, 1999; Miller et al., 2007). However, a total evidence analysis of morphology, 18S, partial 16S, and partial 28S rRNA data supported monophyly of *Haemaphysalis* with respect to *H. inermis*, though only 2 other *Haemaphysalis* species (Structurally Primitive *H. punctata* and Structurally Advanced *H. leporispalustris*) were included in the analysis (Klompen et al., 2000). Recent analysis of both 18S and partial 28S nuclear rRNA sequences including 6 species of *Haemaphysalis* had some support for the position of *H. inermis* as sister to the rest of the *Haemaphysalis* (Burger et al., 2012). This study also included another Structurally Primitive species, *H. punctata*, which did not cluster with *H. inermis*, but was within the main *Haemaphysalis* clade.

A recent phylogenetic analysis of complete mt genomes and nuclear rRNA genes was informative for the genus *Amblyomma*, suggesting that the genus *Amblyomma* was paraphyletic with respect to *A. sphenodonti* and *A. elaphense* (Burger et al., 2012). However, this analysis could not conclusively resolve the phylogenetic position of *A. sphenodonti* or *A. elaphense*, though there was moderate support for *A. sphenodonti* as sister to the genus *Bothriocroton*. The most likely position of *A. elaphense* was either as sister to the rest of the Metastriata or sister to the genus *Haemaphysalis* (Burger et al., 2012). In addition, though there was no strong evidence for a sister group relationship between *A. elaphense* and *A. sphenodonti*, this possibility could not be excluded.

Burger et al. (2012) also proposed that the Metastriata comprised 2 clades: (i) *Amblyomma* s.s. (excluding *A. sphenodonti* and *A. elaphense*) plus Rhipicephalinae (*Rhipicephalus*, *Hyalomma*, and *Dermacentor*); and (ii) *Haemaphysalis* plus *Bothriocroton* and *A. sphenodonti*. This division of the Metastriata into 2 clades contrasts with the proposal of Hoogstraal and Aeschlimann (1982) and the working hypothesis of Barker and Murrell (2004), though a previous analysis of nuclear 18S rRNA had moderate support for *Amblyomma* s.s. plus Rhipicephalinae (Dobson and Barker, 1999), and a total evidence analysis of morphology and rRNA had moderate support for both clades (Klompen et al., 2000). For convenience, in this paper, we call these 2 clades Haematobothrion, for *Haemaphysalis* plus *Bothriocroton* plus *A. sphenodonti*; and Amblyocephalus for *Amblyomma* s.s. plus Rhipicephalinae. These names are a combination of the names of the 2 major genera of each clade. We do not intend for these clade names to become formal taxonomic ranks, rather we use them to assist in the discussion of the phylogenetic relationships among metastriate lineages.

Burger et al. (2012) proposed that analysis of additional mitochondrial genome sequences from the genus *Haemaphysalis* would help to resolve the phylogenetic positions of *A. elaphense* and *A. sphenodonti*. Thus, we sequenced the entire mitochondrial genomes of *Haemaphysalis formosensis* (Structurally Advanced; SA), *Haemaphysalis parva* (SA) and *Amblyomma cajennense*, as well as two thirds of the mitochondrial genome of *Haemaphysalis humerosa* (SA) and *Haemaphysalis hystricis* (SA). We also sequenced nuclear 18S rRNA and partial 28S rRNA genes from these 5 species and from *Haemaphysalis flava* (SA), *Haemaphysalis leporispalustris* (SA), and *Haemaphysalis sulcata* (Structurally Intermediate). We use these sequences (i) to test the monophyly of the genus *Haemaphysalis* and the relationships between *Haemaphysalis* subgenera; and (ii) to improve the phylogeny of the metastriate ticks (and particularly the phylogenetic positions of *A. sphenodonti* and *A. elaphense*) by improving taxon sampling for this group.

Materials and methods

Specimens and DNA extraction

Specimens sequenced in this study are listed in Table 1, along with accession numbers for sequences deposited in GenBank. DNA was extracted from tick specimens using the DNeasy Tissue Extraction Kit (QIAGEN). A single tick of each species was cut in half longitudinally, using a scalpel under a dissecting microscope; half was used for DNA extraction, and the other half kept as a voucher specimen. Tissue for extraction was snap-frozen in liquid nitrogen and ground with micropestle prior to DNA extraction. Voucher specimens for each mt genome we sequenced were deposited in the Queensland Museum, South Brisbane BC, Queensland 4010, under registration numbers QMS93625-QMS93629.

PCR amplification and sequencing

Short (ca. 400–700 bp) regions of the *cox1*, *cytb*, and 12S rRNA genes were first amplified and sequenced using universal arthropod primers (Table S1; Simon et al., 1994; Kambhampati and Smith, 1995; Shao et al., 2005b). Species-specific primers were then designed for each species from these 3 regions and used in conjunction with universal tick primers in the 12S rRNA and *cytb* genes, designed from all known tick 12S rRNA and *cytb* sequences (Table S1). Entire mitochondrial genomes were then amplified in 3 overlapping fragments, from *cytb* to *cox1*, *cox1* to 12S rRNA, and from 12S rRNA to *cytb* (Fig. 1). Partial mitochondrial genomes were sequenced from only the first 2 fragments. 18S rRNA and partial 28S rRNA genes were amplified and sequenced as per Burger et al.

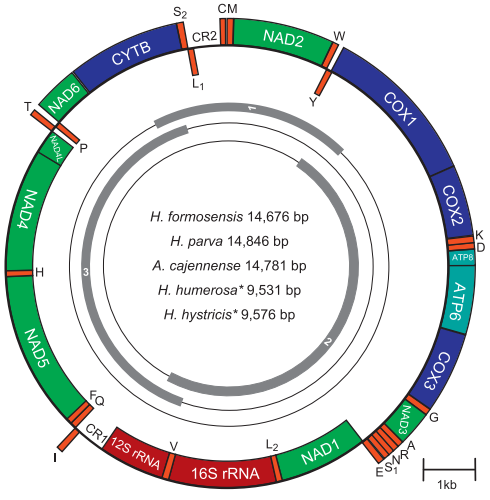

Fig. 1. The relative length and arrangement of genes in the mitochondrial genomes of *Haemaphysalis formosensis*, *H. parva*, and *Amblyomma cajennense* and the partial mt genomes of *H. humerosa* and *H. hystricis*. Genes illustrated on the outside of the main circle are encoded on the forward or majority (J) strand; genes on the inside of the circle are encoded on the reverse or minority (N) strand. The inner 3 circles represent the size and relative position of the PCR fragments amplified in these 5 species. Only fragments 1 and 2, ca. 9500 bp of the mitochondrial genome, were sequenced in *H. humerosa* and *H. hystricis*. Diagram constructed using GenomeVx (Conant and Wolfe, 2008). **H. humerosa* and *H. hystricis* are partial mitochondrial genomes.