Molecular Phylogenetics and Evolution 64 (2012) 45–55

Contents lists available at SciVerse ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Phylogenetic analysis of ticks (Acari: Ixodida) using mitochondrial genomes and nuclear rRNA genes indicates that the genus *Amblyomma* is polyphyletic

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ARTICLE INFO

Article history:
Received 2 November 2011
Revised 9 February 2012
Accepted 6 March 2012
Available online 17 March 2012

Keywords:
Ixodida
Phylogeny
Mitochondrial genomes
18S rRNA
28S rRNA
Amblyomma
Polyphyly

ABSTRACT

Our understanding of the phylogenetic relationships among tick lineages has been limited by the lack of resolution provided by the most commonly used phylogenetic markers. Mitochondrial genomes are increasingly used to address controversial phylogenetic relationships. To date, the complete mitochondrial genomes of eleven tick species have been sequenced; however, only three of these species are metastriate ticks, the most speciose lineage of ticks. In this study, we present the nucleotide sequences of the complete mitochondrial genomes of five more species of metastriate ticks: *Amblyomma elaphense*, *Amblyomma fimbriatum*, *Amblyomma sphenodonti*, *Bothriocroton concolor* and *Bothriocroton undatum*. We use complete mitochondrial genome sequences to address the phylogenetic placement of two morphologically 'primitive' species – *Am. elaphense* and *Am. sphenodonti* – with respect to the genus *Amblyomma*. Our analysis of these five mitochondrial genomes with the other eleven tick mitochondrial genomes, as well as analysis of nuclear rRNA genes, provides strong evidence that the genus *Amblyomma* is polyphyletic with the inclusion of *Am. sphenodonti* and *Am. elaphense*. A new genus or two new genera may be required to describe *Am. sphenodonti* and *Am. elaphense*. It is also possible that these two species are sisters to two established genera, *Bothriocroton* in the case of *Am. sphenodonti*, and *Haemaphysalis* in the case of *Am. elaphense*. However, other arrangements of these taxa cannot be excluded with the current data. Thus, while *Am. sphenodonti* and *Am. elaphense* do not belong in the genus *Amblyomma*, the phylogenetic placement of these two species cannot be resolved without more data from metastriate ticks, either greater sampling of mitochondrial genomes, or a large data set of nuclear genes.

1. Introduction

Ticks (Ixodida) are blood-feeding ectoparasites of vertebrates, globally important as vectors of disease and parasites of livestock. There are 896 tick species currently recognised (Guglielmone et al., 2010) in three families: the Ixodidae (hard ticks), the Argasidae (soft ticks), and the monotypic Nuttalliellidae. The Ixodidae are further divided into two lineages: the Prostriata, which contains only the genus *Ixodes*, and the Metastriata, which contains all other hard tick genera (Keirans, 2009).

Traditional views of the relationships among the genera of ticks are derived from Hoogstraal's work on the evolution of ticks, which included a 'family tree' of the relationships between tick lineages (Hoogstraal and Aeschlimann, 1982). Although several aspects of Hoogstraal's 'family tree' have withstood challenges by new data (i.e. morphological and DNA analysis), there have been several revisions proposed to the systematics of the ticks. Within the Metastriata, phylogenetic analysis has shown that the five species in the genus *Boophilus* are embedded in the genus *Rhipicephalus*, making *Rhipicephalus* paraphyletic (Beati and Keirans, 2001; Murrell et al., 2000, 2001), hence *Boophilus* was made a junior synonym of *Rhipicephalus* (Murrell and Barker, 2003). In addition, the genus *Aponomma* was revealed to be polyphyletic, consisting of several species that cluster within the genus *Amblyomma*, and another group of species which appeared to form a new genus, *Bothriocroton* (Dobson and Barker, 1999; Klompen et al., 2002, 2000).

Kaufman (1972) was the first to suggest the polyphyly of *Aponomma*. He argued that the genus *Aponomma* was a catch-all group of medium to large eyeless ticks of reptiles. Furthermore, Kaufman recognised three groups within *Aponomma* on the basis of morphology: the 'typical' (17 spp.), the 'indigenous Australian' (7 spp.) and the 'primitive' (2 spp.), and suggested that the latter two groups may represent new genera. Two of these groups were recovered in phylogenetic analysis of the 18S rRNA gene; the 'indigenous Australian' *Aponomma* formed a monophyletic group,

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http://dx.doi.org/10.1016/j.ympev.2012.03.004

to the exclusion of all other metastriate ticks, and the 'typical' *Aponomma* clustered within the genus *Amblyomma* (Dobson and Barker, 1999). A 'total evidence' approach that combined 18S rRNA, 28S rRNA, 16S rRNA and morphological data reached the same conclusion (Klompen et al., 2000). Thus the 'indigenous Australian' *Aponomma* were moved to a new genus, *Bothriocroton*, and the genus *Aponomma* was placed under the synonymy of *Amblyomma* (Klompen et al., 2002). However, molecular data from the third group of *Aponomma*, the 'primitive' *Aponomma* were not included in any of these studies. Both 'primitive' species (*Am. sphenodonti* and *Am. elaphense*) were provisionally placed within *Amblyomma* "*until further evidence relating them to other ixodid lineages is generated*" (Klompen et al., 2002). Herein, we use the obsolete term 'primitive' *Aponomma* only as a convenient name for a grouping of the two species *Am. sphenodonti* and *Am. elaphense*.

Few morphological synapomorphies were proposed for the new genus *Bothriocroton*; many of the characters proposed by Kaufman (1972) to define his 'indigenous Australian' *Aponomma* (i.e. *Bothriocroton*) were shared with either the 'typical' or 'primitive' *Aponomma*, or not consistent among *Bothriocroton* species (Klompen et al., 2002). Klompen et al. (2002) suggested that the most promising apomorphy for *Bothriocroton* is the distribution of large wax glands in the larvae. Large wax glands are secretory glands found only in metastriate ticks. *Bothriocroton* larvae have three large wax glands laterally, anterior to the first festoons, whereas other metastriate larvae have at most one large wax gland in that position (Klompen et al., 1996). Klompen et al. (1997) reported the pattern of large wax glands in *Am. elaphense*, but the pattern of large wax glands in *Am. sphenodonti* has not been reported to date.

The first phylogenetic analysis of ticks that included one of the 'primitive' *Aponomma*, *Am. sphenodonti*, had strong support for monophyly of *Bothriocroton* and *Amblyomma* sensu stricto (including the 'typical' *Aponomma*), but *Am. sphenodonti* did not cluster with either (Miller et al., 2007). This tree inferred from 18S rRNA sequences placed *Am. sphenodonti* as sister to a group of all other metastriate ticks excluding *Bothriocroton*, though this was not strongly supported in bootstrap analysis. *Am. elaphense*, the other 'primitive' *Aponomma*, has not to date been included in any molecular phylogenetic analysis. However, a morphological tree of the Metastriata, which included *Am. elaphense* (but not *Am. sphenodonti*) suggested paraphyly of both *Aponomma* and *Amblyomma* (Klompen et al., 1997).

Mitochondrial (mt) genome data are increasingly used for phylogenetic analysis. Eleven tick mt genomes have been sequenced to date: three soft ticks, four prostriate ticks, and three metastriate ticks. Soft ticks and prostriate ticks have the same mitochondrial gene arrangement as the hypothetical ancestor to the arthropods (Shao et al., 2004; Shao and Barker, 2007). The metastriate ticks, however, have a rearrangement of a 5 kb block of genes relative to the ancestral gene order, as well as the rearrangement of two separate tRNA genes and a duplication of the large non-coding region (Black and Roehrdanz, 1998; Campbell and Barker, 1998). These mt gene order rearrangements are a strong synapomorphy for the Metastriata; however, mt gene order is only part of the information contained in the mt genome. In this study, we explore the use of complete mt genome sequences to address the phylogenetic relationships among the lineages of metastriate ticks.

Here, we report the complete mt genomes of both of the 'primitive' *Aponomma*: *Amblyomma sphenodonti* and *Amblyomma elaphense*, as well as two members of the 'indigenous Australian' *Aponomma*, *Bothriocroton concolor* and *Bothriocroton undatum*, and a 'typical' *Aponomma*, *Amblyomma fimbriatum*. We use complete mt genome sequences, as well as nuclear rRNA genes, for phylogenetic analysis of the ticks to test the monophyly of the genus *Amblyomma* with respect to *Am. sphenodonti* and *Am. elaphense*. In addition, we report the distribution of large wax glands in the larvae of *Am. sphenodonti*, and ascertain if this morphological character is useful in determining the phylogenetic position of this species.

2. Materials and methods

2.1. Specimens and DNA extraction

DNA was extracted from tick specimens using the DNeasy Tissue Extraction Kit (QIAGEN). A single tick of each species was cut in half 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 were deposited in the Queensland Museum, South Brisbane BC, Queensland 4010, under registration numbers S90969–S90973. Specimens sequenced in this study are listed in Table 1, along with collection data and accession numbers for sequences deposited in GenBank.

2.2. PCR amplification and sequencing

Mitochondrial genomes were amplified in two to five overlapping fragments, using a combination of conserved (Kambhampati and Smith, 1995; Shao et al., 2005; Simon et al., 1994) and species-specific primers (see Tables S1, S2 and S3 for details). The mt genome of *Am. sphenodonti* was sequenced entirely by primer walking. Approximately 7 kb of the mt genomes of *Am. fimbriatum*, *Bt. concolor* and *Bt. undatum* and *Am. elaphense* was also sequenced by primer walking. The remaining 7–8 kb of the mt genomes of these four species was then amplified using species-specific primers (Tables S1 and S2), and all mt genome fragments (including those previously sequenced by primer walking) were sequenced in a single 454 run. Depth and area of coverage for primer walking and 454 sequencing are detailed in Table 2. 18S rRNA genes were amplified and sequenced following Miller et al. (2007) using primers designed by Black et al. (1997) and Dobson and Barker (1999) (Table S3). 28S rRNA genes were amplified and sequenced using primers modified from Hillis and Dixon (1991) and Whiting et al. (1997), to better match known tick 28S rRNA sequences (Table S3).

TaKaRa La Taq DNA polymerase kits (Takara Biotechnology) were used in all PCR reactions. PCR conditions were optimised for each reaction, with the annealing temperature adjusted to suite the primers used, and extension time set to one minute per kb of expected product size. General PCR conditions were: 96 °C for 60 s, followed by 40 cycles of 96 °C for 40 s, 55 °C for 60 s, 72 °C for 8 min, and a final extension of 72 °C for 14 min. PCR products were examined on 1% agarose gel stained with ethidium bromide.

Table 1
Specimens used in this study, along with collection data and GenBank accession numbers for nucleotide sequences deposited in GenBank.

Species	Collection locality	Host	Collector	Mt genome GenBank ID	28S and 18S GenBank ID
Amblyomma sphenodonti	Stephens Island, New Zealand	Sphenodon punctatus	HM	JN863731	JN863726
Amblyomma elaphense	Texas, USA	Bogertophis subocularis	C. Newson	JN863729	JN863722, JN863721
Amblyomma fimbriatum	Lizard Island, QLD, Australia	Varanus gouldii	SCB	JN863730	JN863725
Bothriocroton concolor	Kangaroo Island, SA, Australia	Tachyglossus aculeatus	Ian Beveridge	JN863727	JN863723
Bothriocroton undatum	Fraser Island, QLD, Australia	Varanus gouldii	SCB	JN863728	JN863724