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Seventy-eight entire mitochondrial genomes and nuclear rRNA genes provide insight into the phylogeny of the hard ticks, particularly the *Haemaphysalis* species, *Africaniella transversale* and *Robertsicus elaphensis*

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ABSTRACT

Hoogstraal and Kim (1985) proposed from morphology, three groups of *Haemaphysalis* subgenera: (i) the "structurally advanced"; (ii) the "structurally intermediate"; and (iii) the "structurally primitive" subgenera. Nuclear gene phylogenies, however, did not indicate monophyly of these morphological groups but alas, only two mitochondrial (mt) genomes from the "structurally intermediate" subgenera had been sequenced. The phylogeny of *Haemaphysalis* has not yet been resolved. We aimed to resolve the phylogeny of the genus *Haemaphysalis*, with respect to the subgenus *Alloceraea*. We presented 15 newly sequenced and annotated mt genomes from 15 species of ticks, five species of which have not been sequenced before, and four new *18S* rRNA and *28S* rRNA nuclear gene sequences. Our datasets were constructed from 10 mt protein-coding genes, *cox1*, and the *18S* and *28S* nuclear rRNA genes. We found a 132-bp insertion between tRNA-Glu (E) gene and the *nad1* gene in the mt genome of *Haemaphysalis* (*Alloceraea*) *inermis* that resembles insertions in *H.* (*Alloceraea*) *kitaokai* and *Rhipicephalus* (*Boophilus*) *geigyi*. Our mt phylogenies had the three species of *Amblyomma* (*Aponomma*) we sequenced embedded in the main clade of *Amblyomma: Am.* (*Aponomma*) *fimbriatum, Am.* (*Aponomma*) *gervaisi* and *Am.* (*Aponomma*) *latum*. This is further support for the hypothesis that the evolution of eyes appears to have occurred in the most-recent-common-ancestor of Amblyocephalus (i.e. Amblyomminae plus Rhipicephalinae) and that eyes were subsequently lost in the most-recent-common-ancestor of the subgenus *Am.* (*Aponomma*). Either *Africaniella transversale* or *Robertsicus elaphensis*, or perhaps *Af. transversale* plus *Ro. elaphensis*, appear to be the sister-group to the rest of the metastriate Ixodida. Our *cox1* phylogenies did not indicate monophyly of the "structurally primitive", "structurally intermediate" nor the "structurally advanced" groups of *Haemaphysalis* subgenera. Indeed, the subgenus *Alloceraea* may be the only monophyletic subgenus of the genus *Haemaphysalis* sequenced thus far. All of our mt genome and *cox1* phylogenies had the subgenus *Alloceraea* in a clade that was separate from the rest of the *Haemaphysalis* ticks. If *Alloceraea* is indeed the sister to the rest of the *Haemaphysalis* subgenera this would resonate with the argument of Hoogstraal and Kim (1985), that *Alloceraea* was a subgenus of "primitive" *Haemaphysalis. Alectorobius capensis* from Japan had a higher genetic-identity to *A. sawaii*, which was also from Japan, than to the *A. capensis* from South Africa. This indicates that *A. capensis* from Japan may be a cryptic species with respect to the *A. capensis* from South Africa.

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1. Introduction

The hard ticks, Ixodidae, comprise the Metastriata and the Prostriata. The Metastriata has been divided into: (1) Haematobothrion which has *Archaeocroton, Bothriocroton* and *Haemaphysalis* (Burger et al., 2013); and (2) Amblyocephalus which has *Amblyomma, Anomalohimalaya, Cosmiomma, Dermacentor, Hyalomma, Margaropus, Rhipicentor*, and *Rhipicephalus* (Burger et al., 2013, 2012; Kelava et al., 2021) [We note, that it is not yet known where *Africaniella transversale* (Kelava et al., 2021) and *Robertsicus elaphensis* (Barker and Burger, 2018) fit into this scheme]. The genus *Haemaphysalis* (Koch, 1844), has 166 species and is the largest genus of the Metastriata and the second largest genus of ticks (Barker and Murrell, 2004; Guglielmone et al., 2010; Guglielmone and Robbins, 2018; Guglielmone et al., 2020) [the largest genus of ticks is the genus *Ixodes* with 243 species]. The genus *Haemaphysalis* has 16 subgenera: *Aboimisalis, Aborphysalis, Alloceraea, Allophysalis, Dermaphysalis, Elongiphysalis, Garnhamphysalis, Gonixodes, Haemaphysalis, Herpetobia, Kaiseriana, Ornithophysalis, Rhipistoma, Segalia, Sharifiella* and *Subkaiseriana* (Camicas et al., 1998).

Hoogstraal and Kim (1985) characterised the subgenera of *Haemaphysalis* by their morphology into either "structurally primitive", "structurally intermediate" or "structurally advanced" groups (Fig. 1). Four subgenera, *Alloceraea, Allophysalis, Aboimisalis,* and *Sharifiella,* were classified as "structurally primitive" (Hoogstraal and Kim, 1985). The "structurally primitive" subgenera had projections (lateral convexity) on each side of the basis capitula and narrow elongated palps like *Amblyomma, Bothriocroton, Hyalomma* and some species of *Ixodes* (Hoogstraal and Aeschlimann, 1982; Hoogstraal and Kim, 1985) (Fig. 1). The "structurally advanced" subgenera of *Haemaphysalis* (i.e. *Aborphysalis, Dermaphysalis, Elongiphysalis, Garnhamphysalis, Gonixodes, Haemaphysalis, Kaiseriana, Ornithophysalis, Rhipistoma, Segalia* and *Subkaiseriana*) on the other hand had broader palps than both the "structurally primitive" and the "structurally intermediate" genera (Hoogstraal and Kim, 1985) (Fig. 1). The basis capitula of the "structurally advanced" *Haemaphysalis*, in all life-stages, were rectangular with posterior cornua present in most species (we note the absence of cornua in adult stages of *H.* (*Rhipistoma*) *lemuris*) (Fig. 1). The single subgenus of the "structurally intermediate" *Haemaphysalis, Herpetobia*, had a more "structurally advanced" rectangular basis capitula than those of "structurally primitive" ticks, but also had compact and partially salient palps which Hoogstraal and Kim (1985) thought might be the precursors to the broad palps of the "structurally advanced" subgenera (Hoogstraal and Kim, 1985) (Fig. 1). The subgenus *Alloceraea* had the most "primitive" morphological characters of the *Haemaphysalis* ticks (Hoogstraal and Kim, 1985). Hoogstraal and Kim (1985) proposed that the evolution of the different *Haemaphysalis* morphological characters was were adaptations to life on reptilian hosts and then later, to mammalian hosts. Recent phylogenies of nuclear *18S* and *28S* rRNA genes (Burger et al., 2013; Beati and Klompen, 2019), however, did not have the "structurally primitive", "structurally intermediate" and

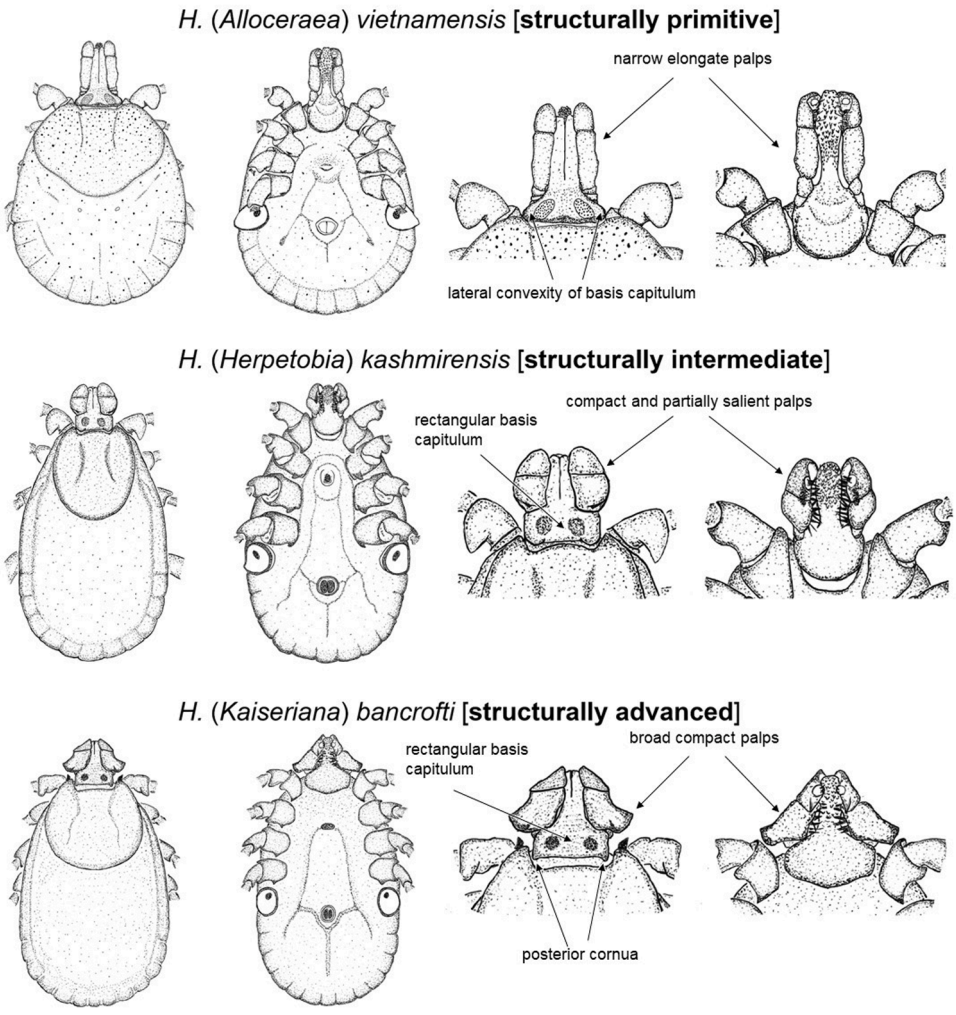

Fig. 1. Examples of the "structurally primitive", "structurally intermediate" and "structurally advanced" subgenera of *Haemaphysalis* of Hoogstraal and Kim (1985) redrawn with minor modifications from Hoogstraal and Wilson (1966, *H.* (*Alloceraea*) *vietnamensis*), Hoogstraal and Kim (1985, *H.* (*Herpetobia*) *kashmirensis*) and Barker and Walker (2014, *H.* (*Kaiseriana*) *bancrofti*). Note that *H.* (*Alloceraea*) *vietnamensis*), is also known as *H.* (*Alloceraea*) *colasbelcouri* (Guglielmone et al., 2020).

"structurally advanced" groups of Hoogstraal and Kim (1985) as monophyletic.

Burger et al. (2013) inferred phylogenies from *18S* and *28S* rRNA where *Haemaphysalis* (*Aboimisalis*) *punctata*, a "structurally primitive" species, and *H.* (*Herpetobia*) *sulcata*, a "structurally intermediate" species, were in a clade with nine other "structurally advanced" *Haemaphysalis* species with 100% ML support, to the exclusion of *H.* (*Alloceraea*) *inermis*, a "structurally primitive" species. An *18S* rRNA phylogeny, moreover, had *H.* (*Al.*) *punctata*, a "structurally primitive" species, in a clade with eight "structurally advanced" species to the exclusion of two "structurally primitive" species, *H.* (*Al.*) *inermis* and *H.* (*Al.*) *aponommoides* (Beati and Klompen, 2019). The monophyly of the THREE morphological groups of Hoogstraal and Kim (1985), has, thus, not been found in gene-trees so far, but too few species of *Haemaphysalis* have been studied to reject the hypotheses of Hoogstraal and Kim (1985). *H.* (*Al.*) *inermis* and *H.* (*Al.*) *kitaokai* formed a clade well removed from the rest of the *Haemaphysalis* species in the trees of Kelava et al. (2021) (See Figs. 6, 8 and 10 but note that *H.* (*Al.*) *inermis* was incorrectly called *H.* (*Segalia*) *parva* in these trees; that error is discussed below in the present paper). For convenience, we will refer to the *H.* (*Al.*) *inermis* and *H.* (*Al.*) *kitaokai* as the *Alloceraea* clade. *18S* and *28S* rRNA gene-trees also indicated that *H.* (*Al.*) *aponommoides* and *H.* (*Al.*) *inermis* form a clade with high support (98% MP, 1.0 BI) (Beati and Klompen, 2019; Burger et al., 2013). Despite the genus *Haemaphysalis* being the second largest genus of ticks, the phylogeny of the genus is poorly understood. At the time of writing, 11 entire mt genomes of *Haemaphysalis* species had been published: *H.* (*Haemaphysalis*) *concinna, H.* (*H.*) *flava, H.* (*H.*) *japonica, H.* (*H.*) *megaspinosa, H.* (*H.*) *pentalagi, H.* (*Kaiseriana*) *cornigera, H.* (*K.*) *hystricis, H.* (*K.*) *longicornis, H.* (*Al.*) *inermis, H.* (*Al.*) *kitaokai* and *H.* (*Aborphysalis*) *formosensis*. Resolution of the phylogeny of the genus *Haemaphysalis*, and its subgenera, may provide insight into the phylogenetic positions of the putative sister-genus/genera of the genus *Haemaphysalis*, such as *Bothriocroton* and *Archaeocroton*. Indeed, the positions of *Bothriocroton, Archaeocroton* and *Haemaphysalis* in the clade Haematobothrion are not known.

Here, we present 15 new mt genome sequences of ticks; five of these are from species published for the first time [*Amblyomma* (*Aponomma*) *gervaisi, Am.* (*Ap.*) *latum, Am.* (*Xiphiastor*) *nuttalli, H.* (*K.*) *yeni* and *I.* (no subgenus) *columnae*] whereas the other 10 mt genomes are from species we and others have studied before [*Alectorobius capensis, H.* (*H.*) *concinna, H.* (*H.*) *flava, H.* (*H.*) *japonica, H.* (*K.*) *hystricis, H.* (*K.*) *longicornis, H.* (*Ab.*) *formosensis, H.* (*Al*) *inermis, Ixodes* (*Ixodes*) *pavlovskyi* and *I.* (*I.*) *persulcatus*]. We also sequenced the *18S* rRNA and *28S* rRNA genes of four species: *Africaniella transversale, Am.* (*Ap.*) *gervaisi, Am.* (*X.*) *nuttalli* and *Am.* (*Ap.*) *latum*. Our aim was to contribute to the knowledge of the phylogeny of the genus *Haemaphysalis*, with respect to the subgenus *Alloceraea* and other genera of Metastriata.

2. Materials and methods

2.1. Mitogenome sequencing and assembly

Mt genomes and the *18S* and *28S* rRNA genes were sequenced and assembled according to the methods of Nakao et al. (2011) and Kelava et al. (2021) (Table 1). Briefly, the blackPREP Tick DNA/RNA Kit (Analytik Jena, Germany) was used to extract the DNA from ticks. Long-range and short-range PCRs amplified two overlapping fragments (12-13 kb and 1.5-2.5 kb) of the entire mt genomes. PrimeSTAR® GXL DNA Polymerase (Takara-Bio, Shiga, Japan) was used to amplify all long mt PCR products. Tks Gflex™ DNA Polymerase (Takara-Bio) was used to amplify short mt gene fragments as well as nuclear rRNA genes. The Nextera DNA Library Prep Kit (Illumina, Hayward, CA) and Illumina MiSeq platform with the MiSeq reagent kit v3 was used to construct Illumina sequence libraries from the long and short PCR fragments. Illumina sequence reads were assembled in CLC Genomics Workbench v12.0.3 (Qiagen, Hilden, Germany).

2.2. Mitogenome annotation and alignment

The 15 new tick mt genomes were annotated with Geneious Prime (Kearse et al., 2012). MITOS Web Server (Bernt et al., 2013) was used to identify the protein-coding genes, rRNA genes and tRNA genes. BLAST (Chen et al., 2015) searches of open reading-frames and intergenic regions were done to check the gene annotations. tRNAscan-SE Search Server v1.21 (Lowe and Chan, 2016) was used to identify any tRNA genes that were not found by other methods. The sequences of tRNA genes were confirmed by observation of the putative secondary structure of transcripts as implemented in Geneious Prime (Kearse et al.,

Table 1
The 15 mt genomes that were sequenced by us for the present paper.

Genus	Genus (subgenus) Species	Sex	Location	Host	Collector	bp
Haemaphysalis	*H.* (*Haemaphysalis*) *concinna*	M	Hokkaido, Japan	vegetation	R. Nakao	14,683
	H. (*Haemaphysalis*) *flava*	M	Wakayama, Japan	vegetation	R. Nakao	14,682
	H. (*Aborphysalis*) *formosensis*	F	Kumamoto, Japan	vegetation	R. Nakao	14,674
	H. (*Kaiseriana*) *hystricis*	F	Kumamoto, Japan	vegetation	R. Nakao	14,745
	H. (*Alloceraea*) *inermis**	M	Lunca Banului, Romania	vegetation	L. Chitima	14,852
	H. (*Haemaphysalis*) *japonica*	F	Yamagata, Japan	vegetation	R. Nakao	14,682
	H. (*Kaiseriana*) *longicornis*	F	Wakayama, Japan	vegetation	R. Nakao	14,696
	H. (*Kaiseriana*) *yeni*	F	Nagasaki, Japan	vegetation	R. Nakao	14,714
Amblyomma	*Am.* (*Aponomma*) *gervaisi*	M	Imported to Japan from Sri Lanka	*Boiga forsteni* (Forsten's cat snake)	K. Okamoto	14,709
	Am. (*Aponomma*) *latum*	M	Imported to Japan from Ghana	*Python regius* (ball python)	Unknown	14,658
	Am. (*Xiphiastor*) *nuttalli***	N	Imported to Japan from Zambia	*Geochelone pardalis* (leopard tortoise)	Unknown	14,681
Ixodes	*I.* (no subgenus) *columnae*	N	Hokkaido, Japan	*Parus minor* (Japanese tit)	K. Tamada & T. Ito	14,524
	I. (*Ixodes*) *pavlovskyi*	M	Hokkaido, Japan	vegetation	R. Nakao	14,559
	I. (*Ixodes*) *persulcatus*	F	Hokkaido, Japan	vegetation	R. Nakao	14,542
Alectorobius	*A. capensis*	N	Kagoshima, Japan	from bird nest	T. Honda, F. Sato, H. Torikai & H. Kawabata	14,420

* NC020335 was taken to be *H.* (*Segalia*) *parva* by Burger et al. (2013). One of us (Samuel Kelava), however, realised from this sequence and the morphology of the voucher specimen in the Queensland Museum (QMS93628) that NC020335 was *H.* (*Alloceraea*) *inermis* not *H.* (*Segalia*) *parva*.
** We only provisionally identify this tick as *Amblyomma nuttalli* Dönitz, 1909 since the morphological identification of species in the *Amblyomma marmoreum* complex of species *sensu* Guglielmone et al. (2020) is fraught with difficulties. Eventually, these difficulties will be resolved and the exoskeleton that we hold can be assigned to a species of tick with confidence.

2012). We assembled eight datasets for phylogenetic analyses: (i) nucleotide sequences of 10 concatenated mt protein-coding genes (10PCGDNA) (as per Kelava et al. (2021)); (ii) nucleotide sequences of 10 concatenated mt protein-coding genes without *Ro. elaphensis* (10PCGDNA_No_Robertsicus); (iii) nucleotide sequences of 10 concatenated mt protein-coding genes without *Af. transversale* (10PCGDNA_No_Africaniella); (iv) amino-acid sequences of 10 concatenated mt protein-coding genes (10PCGAA) (as per Kelava et al. (2021)); (v) nucleotide sequences of the *18S* and *28S* rRNA nuclear genes (*18S*+*28S*); (vi) nucleotide sequences of 10 concatenated mt protein-coding genes plus the nucleotide sequences of the *18S* and *28S* rRNA nuclear genes (10PCGDNA+18S+28S) (like Burger et al. (2013)); (vii) nucleotide sequences of the *cox1* gene (CO1DNA); and nucleotide sequences of the entire mt genomes (not partitioned by gene) of seven *Alectorobius* tick sequences and two *Nothoaspis* sequences. The 10PCGDNA dataset had 78 species: 15 newly sequenced genomes plus 63 genomes gleaned from NCBI GenBank (Ncbi, 2016). The CO1DNA dataset had 66 *cox1* sequences. The 18S+28S dataset was comprised of 58 *18S* rRNA and *28S* rRNA sequences, whereas the 10PCG+28S+28S dataset was comprised of 46 mt genome, *18S* rRNA and *28S* rRNA sequences. The MAFFT G-INS-I (Katoh and Standley, 2013) algorithm as implemented in Geneious Prime was used to align the 10 protein-coding genes: *atp6, cox1, cox2, cox3, cytb, nad1, nad2, nad3, nad4, nad5* and the *18S* and *28S* rRNA nuclear genes. The protein-coding genes were aligned on the basis of their putative amino-acid sequences. The *18S* and *28S* rRNA genes were aligned on the basis of their nucleotide sequences. Gblocks (Castresana, 2000) was used to remove regions with gaps in more than 50% of sequences and highly variable regions in all gene alignments before concatenation.

2.3. Pairwise (%) genetic-identity

For comparisons of pairwise (%) genetic-identity within and among the genera *Haemaphysalis* and *Bothriocroton*, whole mt genomes and the *cox1* gene of *Haemaphysalis* and *Bothriocroton* species were studied. Similarly, the entire mt genomes were used for comparisons of pairwise (%) genetic-identity within and among the genera *Alectorobius* and *Nothoaspis*. Pairwise (%) genetic-identity among species was calculated from the genome and gene pairwise alignments in Geneious Prime, presented as percentage genetic-identity.

2.4. Phylogenetic inference

Phylogenies were inferred by Maximum Likelihood (ML) and Bayesian Inference (BI), implemented in RAXML-HPC2 v 8.2.12 (Stamatakis, 2014) and MrBayes v 3.2.2 (Ronquist et al., 2012), respectively. In all ML and BI runs, concatenated datasets were partitioned by gene. JmodelTest2 v 2.1.6 software (Darriba et al., 2012) was used to identify the optimal nucleotide substitution model for the nucleotide datasets for each gene. The GTR + G + I model was found to be the best fit for the nucleotide gene datasets. ModelTest-NG v 0.1.5 software (Darriba et al., 2020) was used to find the best fit amino-acid substitution model. Phylogenies were inferred in the CIPRES Science Gateway v.3.3 (Miller et al., 2010) as outlined in Kelava et al. (2021). RAXML-HPC2 v 8.2.12 (Stamatakis, 2014) was used to execute Rapid bootstrapping of 1000 replicates on our datasets. Two simultaneous BI runs were executed: 10 million generations sampled every 1000 MCMC steps. Four MCMC chains (three heated and one cold) were run for every BI analysis. A burn-in of the first 25% of steps were discarded. Tracer v 1.5 (Rambaut, 2009) was used to observe the effective sample size (ESS) and convergence of independent runs. The trees were displayed in FigTree v 1.4.4 (Rambaut, 2012). Branch support was assessed by RAXML-HPC2 v 8.2.12 (Stamatakis, 2014) and MrBayes v 3.2.2 (Ronquist et al., 2012). *Allothyrus* sp. from Lamington (KC769586) was set as the outgroup in all trees except for the *cox1* and *Alectorobius* trees which had *Nuttalliella namaqua* (KR907248) and *Nothoaspis amazoniensis* (KC769595, NC033900) as the outgroups respectively.

2.5. Tick morphology and classification

Ticks were identified by their morphology. We discovered that mt genome, NC_020335, of Burger et al. (2013) was not from *H.* (*Segalia*) *parva* but rather from *H.* (*Al.*) *inermis*. We concluded this after comparing the morphology of NC_020335 with that of the voucher specimen of *H.* (*Al.*) *inermis* (OL741739) in the Queensland Museum, the position of NC_020335 in our phylogenies and the % genetic-identity of NC_020335 in our pairwise matrices (Appendix Figs. 6–8, Table 2 and Appendix Table 2). We used the revised classification scheme of Mans et al. (2021) for the Argasidae; therefore, *Ornithodoros* (*Alectorobius*) *capensis* and *Carios* (*Alectorobius*) *capensis* are referred to as *Alectorobius capensis*.

3. Results

3.1. Mitochondrial genomes

The mt genomes of five species of ticks were sequenced for the first time: *Amblyomma* (*Aponomma*) *gervaisi, Am.* (*Ap.*) *latum, Am.* (*Xiphiastor*) *nuttalli, Ixodes* (no subgenus) *columnae* and *Haemaphysalis* (*Kaiseriana*) *yeni* (Fig. 2). Four new *18S* rRNA and partial *28S* rRNA sequences for *Am.* (*Africaniella*) *transversale, Am.* (*Ap.*) *gervaisi, Am.* (*Ap.*) *latum* and *Am.* (*X.*) *nuttalli* were also obtained. All five of these mt genomes had the gene-content typical of animals: 13 protein-coding genes, two ribosomal RNAs, 22 tRNAs and either one or two control regions (Fig. 2). The mt gene arrangements were the same as previously reported for the Argasidae, prostriate Ixodidae and metastriate Ixodidae (Fig. 2) (Black and Roehrdanz, 1998; Campbell and Barker, 1998; Shao et al., 2004). The *H.* (*Al.*) *inermis*, sequenced in the present study (OL741739), had a 132-bp insertion in its mt genome between the tRNA-Glu (E) gene and the *nad1* gene, similar (but not identical) to the insertion in *H.* (*Al.*) *inermis* (NC_020335) and *H.* (*Al.*) *kitaokai* (Burger et al., 2013; Kelava et al., 2021) (Fig. 3). A 118-bp portion of the 132-bp insertion was near identical to the 3' end of the *16S* rRNA gene whereas a 37-bp fragment was near-identical to the 3' end of the *nad1* gene in this 132-bp insert in *H.* (*Al.*) *inermis* (Fig. 3).

Our matrices of pairwise (%) genetic-identity showed that most of the gene and genome sequences of the same species had >99% pairwise identity (Table 2, Appendix Tables 2 and 3). Intriguingly, *H.* (*H.*) *megaspinosa* had a pairwise (%) genetic-identity of >98.9% to the two *H.* (*H.*) *japonica* sequences in both the entire mt genome and *cox1* matrices (Table 2, Appendix Table 2). The voucher specimens of *H.* (*H.*) *megaspinosa* (MT371804) and *H.* (*H.*) *japonica* (OL741742) were archived in the collection of tick bioresource with the specimen IDs of HM762 and HJ1240 under the project ID of HT at the Faculty of Veterinary Medicine, Hokkaido University.

Alectorobius capensis had a pairwise (%) genetic-identity of ~98.8% to the NC_005291 *A. capensis* sequence from Japan, but interestingly, had ~93.9% identity to the three *A. capensis* genomes from South Africa (KJ133586, KJ133587 and KR907245) (Appendix Table 3). *Alectorobius sawaii*, intriguingly, had ~95.4%-95.6% identity to the two *A. capensis* sequences from Japan (OL741737 and NC_005291) (Appendix Table 3).

3.2. Phylogenetic analyses

The genus *Amblyomma* was monophyletic in all of our phylogenetic trees (Figs. 4–6, Appendix Figs. 1, 3 and 4). Our 10PCGDNA trees had high support for a clade that had *Am.* (*Xiphiastor*), *Am.* (*Aponomma*), and *Am.* (*Cernyomma*) *geoemydae* (100 ML, 1.0 BI) (Fig. 4, Appendix Figs. 1 and 2). This clade was paraphyletic concerning *Am.* (*C.*) *triguttatum* and *Am.* (*C.*) *geoemydae* (Fig. 4, Appendix Figs. 1–3). *Am.* (*X.*) *hebraeum* was the sister to *Am.* (*X.*) *nuttalli* plus *Am.* (*X.*) *marmoreum* to the exclusion of the rest of the other *Amblyomma* ticks (100 ML, 1.0 BI) (Fig. 4, Appendix Figs. 1 and 2). The positions of *Am.* (*Ap.*) *gervaisi, Am.* (*Ap.*) *latum* and

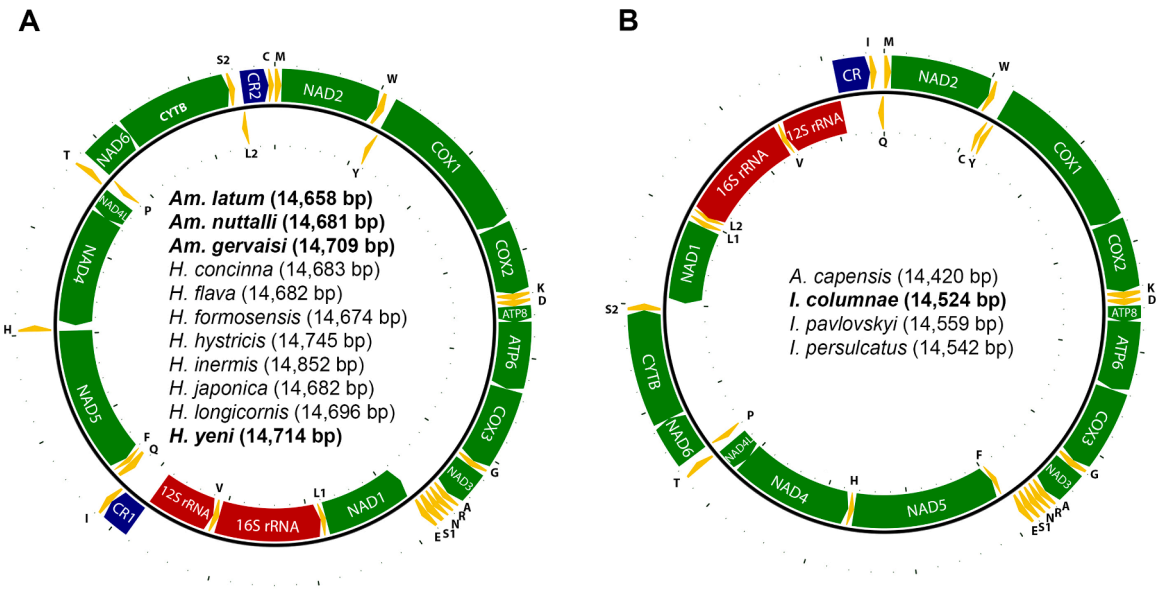

Fig. 2. The fifteen mt genomes that were sequenced by us for the present paper: (A) metastriate Ixodidae; and (B) prostriate Ixodidae and Argasidae. Protein-coding genes are shown in green, tRNAs are in yellow, rRNAs are in red whereas control regions are in blue. Protein-coding genes are labelled by their four-character abbreviations, tRNAs are labelled by their one-letter amino-acid abbreviations whereas control regions are labelled as CR, CR1 and CR2. Other abbreviations: *Am.*, *Amblyomma; H.*, *Haemaphysalis; A.*, *Alectorobius; I.*, *Ixodes*. The sizes of the mt genomes are indicated in brackets. The five species that were sequenced for the first time are in bold font.

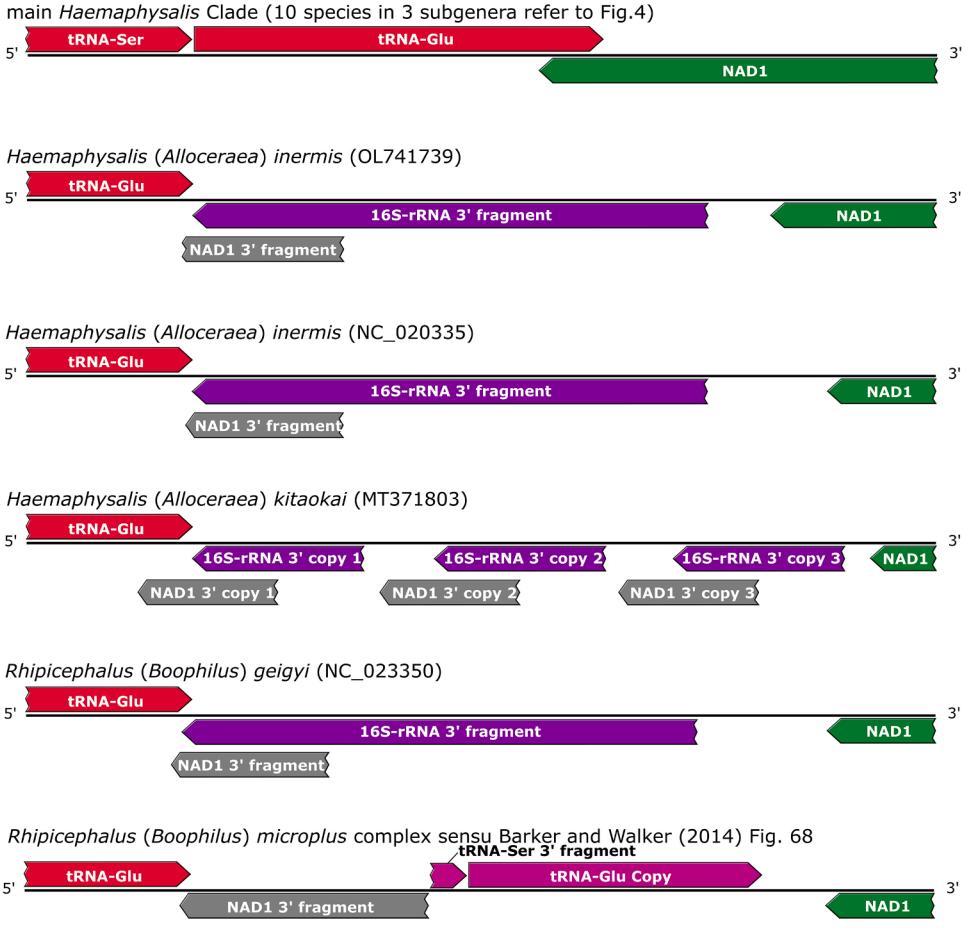

Fig. 3. The six types of insertions and tandem repeats that have been found between the tRNA-Glu (E) and *nad1* genes in the mt genomes of ticks. The protein-coding genes *nad1* are shown in green, the tRNA-Glu (E) and tRNA-Ser (S) genes are shown in red, the partial 3' end *16S* rRNA fragments are shown in purple, the partial 3' end *nad1*gene fragments are shown in grey and the tRNA-Glu (E) complete gene copy is shown in pink. All 10 of the main clade *Haemaphysalis* ticks had the same gene arrangement: *H.* (*Haemaphysalis*) *concinna, H.* (*Kaiseriana*) *cornigera, H.* (*H.*) *flava, H.* (*Ao*) *formosensis, H.* (*K.*) *hystricis, H.* (*H.*) *japonica, H.* (*H.*) *megaspinosa, H.* (*K.*) *longicornis, H.* (*H.*) *pentalagi* and *H.* (*K.*) *yeni*. Our so-called "Main *Haemaphysalis* clade" is illustrated in Fig. 4.

Table 2

Pairwise (%) genetic identities of the entire mt genomes of 19 *Haemaphysalis* and two *Bothriocroton* species (14,674-bp to 15,003-bp). Fig. 4 Species sequenced in the present study are in bold. The *H.* (*Alloceraea*) *inermis*, numbered 18, is the so-called *H.* (*Segalia*) *parva* (NC_020335) of Burger et al. (2013): refer to Section 2.5 of the materials and methods. In this table, we show that the seven re-sequenced mt genomes have >98% identity to the sequences of the same species (*H.* (*Haemaphysalis*) *concinna, H.* (*H.*) *flava, H.* (*Aborphysalis*) *formosensis, H.* (*Kaiseriana*) *hystricis, H.* (*H.*) *japonica, H.* (*K.*) *longicornis* and *H.* (*Al.*) *inermis*). *H.* (*H.*) *megaspinosa* and both sequences of *H.* (*H.*) *japonica* had >98% identity. This table also reflects the phylogeny of Fig. 4 in that the average percentage identity is lower between the *Alloceraea* species vs the "main clade of *Haemaphysalis*" (75.92%, 0.7 stdev) than within either the "main clade of *Haemaphysalis*" (83.92%, 4.8 stdev) or the *Alloceraea* (84.42%, 0.003 stdev).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *H. concinna*																				
2. *H. concinna*	98.4315																			
3. *H. cornigera*	80.6341	80.818																		
4. *H. flava*	84.2993	84.3746	80.4503																	
5. *H. flava*	84.3086	84.3829	80.4721	99.8162																
6. *H. formosensis*	87.0057	87.1229	81.2284	84.9304	84.9195															
7. *H. formosensis*	87.0583	87.1891	81.2936	84.9419	84.931	99.8092														
8. *H. hystricis*	80.2042	80.322	84.4021	80.5603	80.5888	81.0902	81.1282													
9. *H. hystricis*	80.1323	80.2497	84.276	80.4672	80.4957	80.9042	80.9353	98.9223												
10. *H. japonica*	84.6096	84.696	80.9942	89.2796	89.2563	85.0261	85.0716	80.5445	80.4461											
11. *H. japonica*	84.8511	84.9511	81.0657	89.4214	89.4254	85.2524	85.2853	80.6292	80.5239	99.1321										
12. *H. longicornis*	80.5682	80.7153	84.7878	80.9934	80.9827	81.4383	81.5036	86.7855	86.6432	81.1057	81.1636									
13. *H. longicornis*	80.625	80.7789	84.7763	80.9675	80.9569	81.4925	81.559	86.8	86.6441	81.1083	81.1785	99.8231								
14. *H. megaspinosa*	84.7075	84.794	80.9831	89.3119	89.3158	85.1786	85.2242	80.6144	80.4955	98.9888	99.2236	81.0946	81.0972							
15. *H. pentalagi*	80.3671	80.5621	84.4191	80.2411	80.2438	81.1967	81.228	85.7191	85.6244	80.6005	80.7463	86.445	86.479	80.6908						
16. *H. yeni*	80.9451	81.0326	84.0014	80.4535	80.4508	81.4787	81.5248	83.8365	83.6801	81.0526	81.214	84.4475	84.4688	81.2005	84.3089					
17. *H. inermis*	76.2748	76.4072	76.6123	76.0586	76.0723	76.8391	76.8628	75.9343	75.8527	76.4889	76.6783	76.15	76.1465	76.6232	76.3378	76.3421				
18. *H. inermis*	76.3641	76.4832	76.6497	76.0104	76.0477	76.8685	76.8788	75.8713	75.7697	76.4444	76.6473	76.1137	76.1102	76.5788	76.4085	76.3527	99.2459			
19. *H. kitaokai*	75.2114	75.2098	74.9389	74.8564	74.8431	75.5569	75.6396	74.4382	74.316	75.4458	75.5368	74.8482	74.8779	75.4955	74.7905	74.8318	84.4193	84.4253		
20. *B. concolor*	73.1736	73.2002	73.1787	72.8827	72.8687	73.3669	73.484	72.6999	72.5797	73.269	73.2752	72.8022	72.8058	73.2484	72.0493	72.9238	74.3368	74.3714	73.6735	
21. *B. undatum*	73.6667	73.7455	73.6421	73.2621	73.2634	73.4934	73.5706	73.1337	73.0676	73.4602	73.4647	72.8397	72.8433	73.4024	73.1796	73.3656	74.6005	74.5371	73.6918	78.468

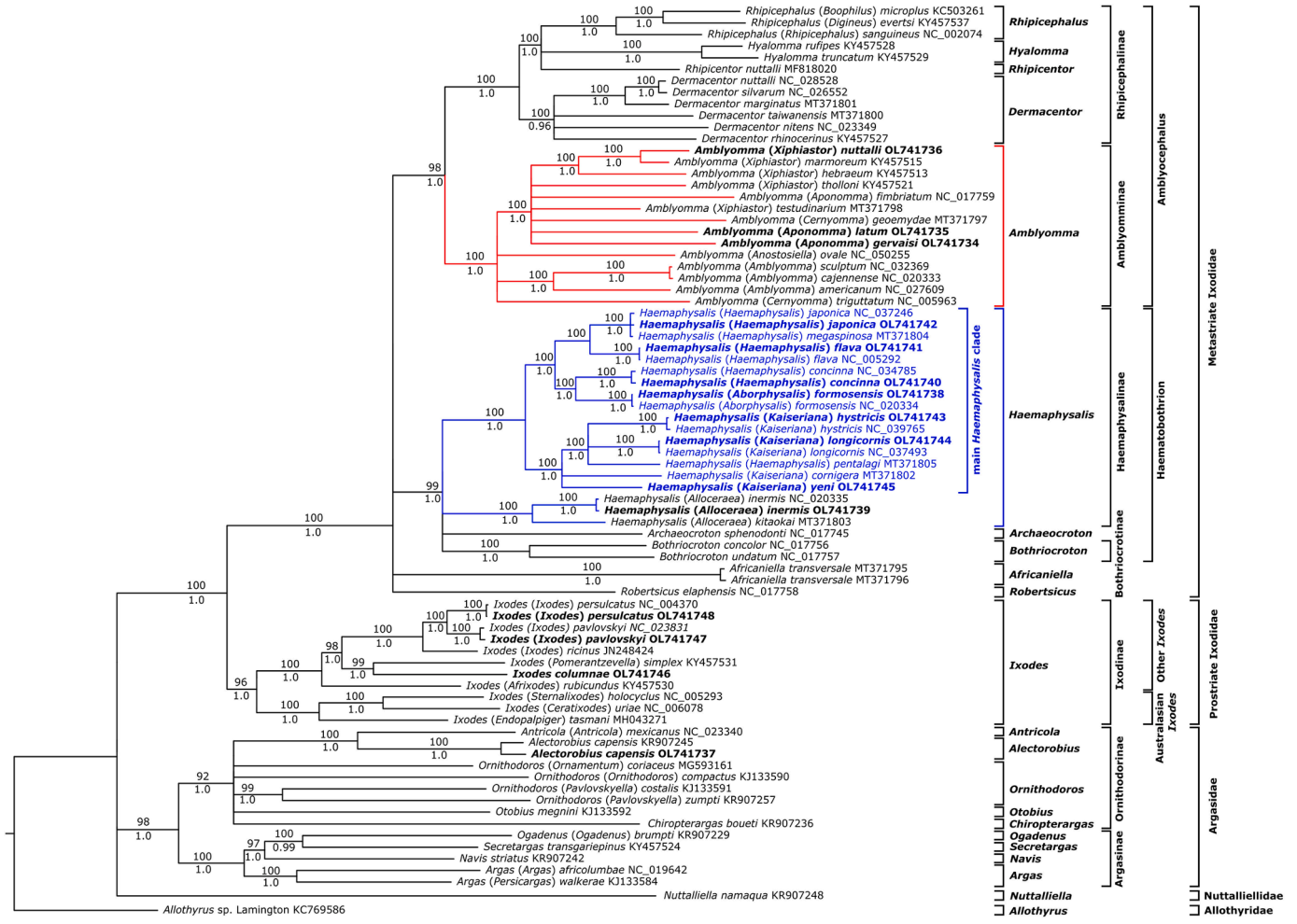

Fig. 4. Maximum Likelihood (ML) phylogenetic trees with Bayesian Inference (BI) from the 10PCGDNA dataset i.e. 10 protein-coding genes, all base positions (9,699-bp). Species sequenced for the present study are in bold. Numbers above branches show Maximum Likelihood support whereas numbers below branches show Bayesian posterior probability. All of the branches with less than 90% Maximum Likelihood or 0.9 Bootstrap and posterior probability support were collapsed. The so-called "main *Haemaphysalis* clade" which we refer to elsewhere in the present paper, is shown in the blue square bracket. *Allothyrus* sp. Lamington (Allothyridae) was set as the outgroup.

Am. (*Ap.*) *fimbriatum*, among the *Am.* (*Xiphiastor*) species and *Am.* (*C.*) *geoemydae* was not resolved (Figs. 4–6, Appendix Figs. 1–4). Amblyocephalus (i.e. Amblyomminae plus Rhipicephalinae) was the sister to Haematobothrion (i.e. *Bothriocroton, Archaeocroton* plus *Haemaphysalis*) to the exclusion of *Af. transversale* and *Ro. elaphensis* in our 10PCGDNA_No_Robertsicus and 10PCGDNA_No_Africaniella trees (Appendix Figs. 1 and 2). The phylogenetic positions of *Af. transversale* and *Ro. elaphensis* were once again not resolved.

The subgenus *Alloceraea* was the only subgenus of *Haemaphysalis* that was monophyletic in all of our phylogenetic trees (Figs. 4–6 and Appendix Figs. 1–4). Our 10PCGAA tree, intriguingly, had *Archaeocroton sphenodonti* as the sister to *H.* (*Al.*) *inermis* and *H.* (*Al.*) *kitaokai* (93 ML, 1.0 BI) (Appendix Fig. 3): the position of *Ac. sphenodonti* in Haematobothrion was not resolved in the other phylogenetic trees. *H.* (*Al.*) *kitaokai* was the sister to *H.* (*Al.*) *inermis* in the 10PCGDNA, 10PCGAA and CO1DNA trees (100 ML, 1.0 BI) (Fig. 4, Appendix Figs. 1, 2 and 4). All of our trees had *Alloceraea* well removed from the other *Haemaphysalis* species (Figs. 4–6, Appendix Figs. 1–4). Indeed, we did not find convincing evidence of the monophyly of the genus *Haemaphysalis* in any of our trees (i.e. >90 ML, >0.9 BI). The CO1DNA tree had *H.* (*He.*) *sulcata* and *H.* (*Ab*) *punctata* plus the subgenera *Haemaphysalis, Kaiseriana* and *Aborphysalis* in a clade to the exclusion of *Alloceraea* (98 ML, 1.0 BI) (Appendix Fig. 4). *Haemaphysalis* (*K.*) *yeni* was embedded among the *Kaiseriana* species and *H.* (*H.*) *pentalagi* with high support (100 ML, 1 BI) (Fig. 4, Appendix Figs. 1–3).

Our phylogenies from nucleotide and amino-acid sequences, had support for *I.* (*Pomerantzevella*) *simplex* as the sister to *I.* (no subgenus) *columnae* (>90 ML, 1.0 BI) (Fig. 4, Appendix Figs. 1–3). The *A. capensis* from Japan and South Africa grouped with *A. sawaii* with high support to the exclusion of *A. fonsecai* (Appendix Fig. 5). The 10PCGDNA+18S+28S tree had *Nuttalliella namaqua* as the sister to the hard ticks plus the soft ticks (Fig. 6) whereas the position of the Nuttalliellidae was unresolved in the rest of our phylogenetic trees. Thus, we have nothing to add to Kelava et al. (2021) regarding the relationships of the three families of ticks, the Ixodidae, Argasidae and Nuttalliellidae.

4. Discussion

4.1. The insertion in the mt genomes of Haemaphysalis (Alloceraea) inermis and Haemaphysalis (Al.) kitaokai

H. (*Alloceraea*) *inermis* had an insertion of about 132-bp between the tRNA-Glu (E) gene and the 3' end of the *nad1* gene (Fig. 3). A similar 312-bp insertion was previously reported in *H.* (*Al.*) *kitaokai* in the same location in the mt genome (Kelava et al., 2021). It will be interesting to see if the three other species in the subgenus *Alloceraea* (*H.* (*Al.*) *aponommoides, H.* (*Al.*) *vietnamensis* (also known as *H.* (*Al.*) *colasbelcouri*), *H.* (*Al.*) *primitiva*; Camicas et al. (1998)) also have this insertion. *H.* (*Al.*)

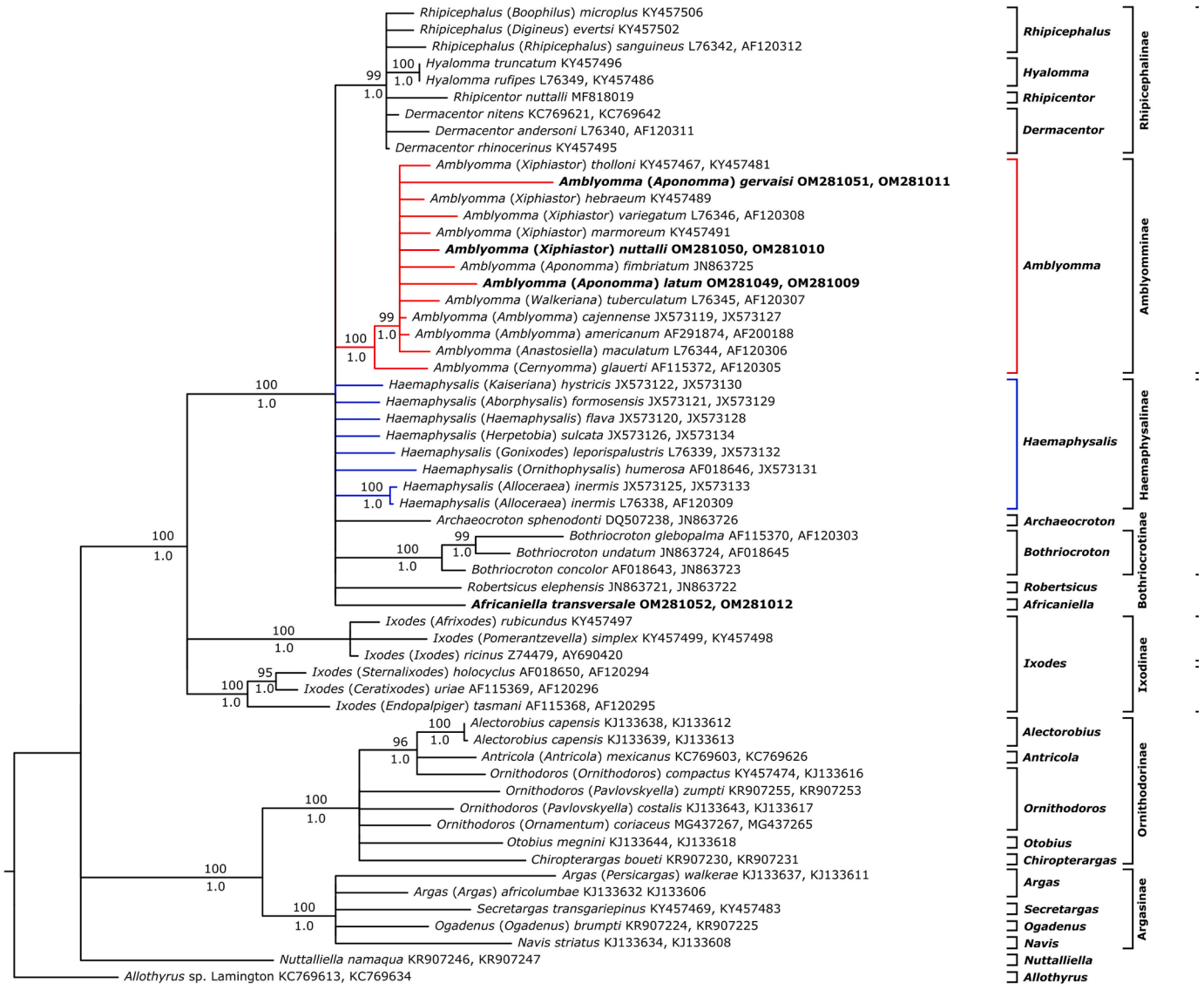

Fig. 5. Maximum Likelihood (ML) phylogenetic trees with Bayesian Inference (BI) from the 18S+28S dataset i.e. *18S* plus *28S* rRNA genes (2,288-bp). Species sequenced for the present study are in bold. Numbers above branches show Maximum Likelihood support whereas numbers below branches show Bayesian posterior probability. All of the branches with less than 90% Maximum Likelihood or 0.9 Bootstrap and posterior probability support were collapsed. *Allothyrus* sp. Lamington (Allothyridae) was set as the outgroup.

inermis had a single partial copy of the 3' end of the *16S* rRNA (118-bp) and *nad1* (37-bp) genes whereas *H.* (*Al.*) *kitaokai* had three copies of these sequences (Fig. 3) (Burger et al., 2013; Kelava et al., 2021). Burger et al. (2014b) reported an insertion in *R. geigyi* that contained part of the 3' end of *16S* rRNA and *nad1* genes (Fig. 3). On the other hand, the *R.* (*Boophilus*) *microplus* species complex and *R.* (*B.*) *decoloratus*, had a tandem repeat of the partial 3' end of *nad1,* tRNA-Ser and partial or whole tRNA-Glu (E) genes in the same location of the mt genome as the insertions in *H.* (*Al.*) *kitaokai, H.* (*Al.*) *inermis* and *R.* (*B.*) *geigyi* (Burger et al., 2014b). Intriguingly, the 132 to 312-bp insertion between the tRNA-Glu (E) gene and the 3' end of the *nad1* gene have been found only in the two species of *Alloceraea* and not in any other subgenus of *Haemaphysalis* sequenced so far.

Montagna et al. (2012) proposed that the "Tick-Box" motif may be involved in the formation of the variable tandem repeats in the *R.* (*B.*) *microplus* species complex and the insertions of 132 to 312-bp discussed above in two *Haemaphysalis* species (*H.* (*Al.*) *inermis* and *H.* (*Al.*) *kitaokai*) and *R.* (*B.*) *geigyi*. The Tick-Box motif, moreover, may be responsible for the rearrangement of the mt genomes in the most-recent-common-ancestor of the metastriate Ixodidae (Montagna et al., 2012). Chen et al. (2020) discovered different mt genome arrangements within a single individual *Dermacentor silvarum* tick: inversions of the block of genes from the *nad1* gene to tRNA-Gln (Q) gene in mt genomes. The *nad1* to tRNA-Gln (Q) gene block, moreover, may be a transposable element where the flanking repeats (Tick-Box motif) function like the inverted repeats of a typical transposable element (Chen et al., 2020). In the light of these proposals, we propose that the inverted repeat Tick-Box found at the 3' end of both *nad1* and *16S* rRNA genes may have caused transposition events and the insertion of partial gene copies in *H.* (*Al.*) *inermis* and other species. It is a mystery why these insertions of partial genes have been found only in species of the subgenus *Alloceraea* and in the subgenus *R.* (*Boophilus*). Mt genome sequences of ticks from other subgenera may reveal other such insertions.

4.2. *The genus Amblyomma and the phylogenetic positions of Africaniella transversale and Robertsicus elaphensis*

Kelava et al. (2021) proposed that the lack of well-developed eyes in the subgenus *Am.* (*Aponomma*), with respect to the rest of the genus *Amblyomma*, may be explained by one of two hypotheses: (i) that eyes

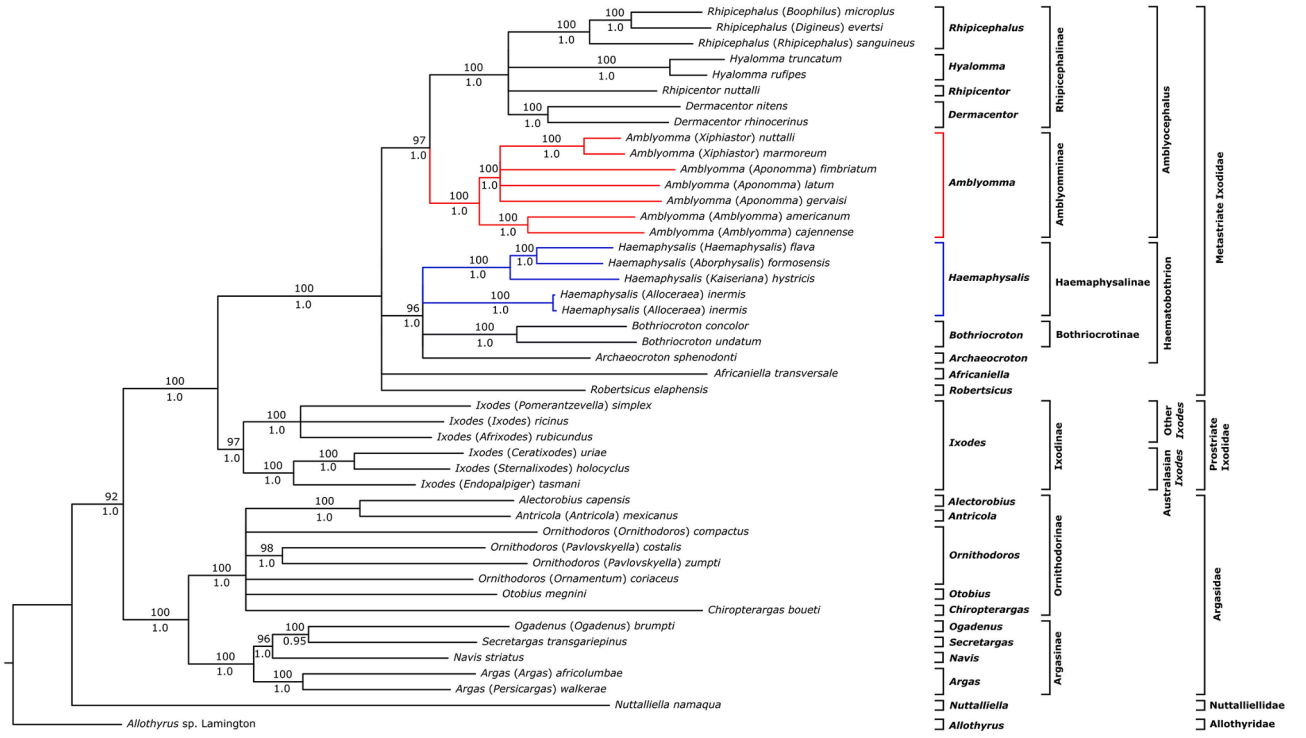

Fig. 6. Maximum Likelihood (ML) phylogenetic trees with Bayesian Inference (BI) from the 10PCGDNA+18S+28S dataset i.e. 10 protein-coding genes, all base positions, plus the *18S* and *28S* rRNA genes (12,117-bp). Species sequenced for the present study are in bold. Numbers above branches show Maximum Likelihood support whereas numbers below branches show Bayesian posterior probability. All of the branches with less than 90% Maximum Likelihood or 0.9 Bootstrap and posterior probability support were collapsed. *Allothyrus* sp. Lamington (Allothyridae) was set as the outgroup.

evolved in the most-recent-common-ancestor of the Amblyocephalus (Amblyomminae plus Rhipicephalinae) but were subsequently lost in the most-recent-common-ancestor of the *Am.* (*Aponomma*) species; or (ii) that eyes evolved independently in the Amblyomminae and the Rhipicephalinae. In our trees, the three species of *Am.* (*Aponomma*) [*Am.* (*Ap.*) *fimbriatum, Am.* (*Ap.*) *gervaisi* and *Am.* (*Ap.*) *latum*] were embedded in the genus *Amblyomma* in a clade with all of the *Am.* (*Xiphiastor*) species and *Am.* (*C.*) *geoemydae* to the exclusion of the *Am.* (*Amblyomma*), *Am.* (*Anastosiella*) subgenera and to *Am.* (*C.*) *triguttatum* (Figs. 4, 6, Appendix Figs. 1–3). The evolution of eyes in the most-recent-common-ancestor of the Amblyomminae and Rhipicephalinae and subsequent loss in the subgenus *Am.* (*Aponomma*) is, therefore, the most parsimonious hypothesis; this was first proposed by Kelava et al. (2021). Whether or not the subgenera of *Amblyomma* are monophyletic is unknown: only the subgenus *Am.* (*Amblyomma*) was monophyletic in our trees (Figs. 4, 6, Appendix Figs. 1–3). Kelava et al. (2021) and Hornok et al. (2020), moreover, proposed that *Africaniella* may not belong to the genus *Amblyomma* and reinstated the genus *Africaniella*.

Ro. elaphensis on the one hand (Appendix Fig. 2) and *Af. transversale* on the other hand (Appendix Fig.1) were sisters to the Amblyocephalus plus the Haematobothrion in our mt genome trees. The mt genome of *Af. transversale* has at least five rearrangements relative to the known mt genomes of the 52 other Metastriata that have been studied (Kelava et al., 2021). Although both *Ro. elaphensis* and *Af. transversale* were put in the "primitive *Amblyomma*" by Kaufman (1972), these two species are not sister-species (Figs. 4–6 and Appendix Figs. 3–4 in the present work and Kelava et al. (2021)). It is our view that *Af. transversale* and *Ro. elaphensis* do not belong in the Haematobothrion nor the Amblyocephalus, rather we propose three alternative phylogenetic arrangements: (i) [(Amblyocephalus, Haematobothrion), (*Af. transversale, Ro. elaphensis*)]; (ii) [(Amblyocephalus, Haematobothrion, *Af. transversale*), *Ro. elaphensis*]; and (iii) [(Amblyocephalus, Haematobothrion, *Ro. elaphensis*), *Af. transversale*] (Fig. 7).

Where and when the most-recent-common-ancestors of the four main lineages, Amblyocephalus, Haematobothrion, *Ro. elaphensis* and *Af. transversale* lived, is of much interest to us; and indeed, potentially instructive about the evolution of the ticks. In a similar way, where and when the most-recent-common-ancestor of the entire Metastriata is also of much interest i.e. Amblyocephalus + Haematobothrion + *Ro. elaphensis* +*Af. transversale*. Which, if any, of the four hypotheses articulated by Barker et al. (2021) is correct: the out-of-Africa, out-of-South-America, out-of-Antarctica or out-of-Australia hypothesis still needs to be determined.

4.3. The phylogeny of the subgenera of Haemaphysalis

Our trees did not indicate monophyly of the subgenus *Haemaphysalis* nor *Kaiseriana* (Fig. 4). Intriguingly, *H.* (*H.*) *pentalagi* grouped with the species *H.* (*K.*) *hystricis* and *H.* (*K.*) *longicornis* from the *Kaiseriana* subgenus, therefore, the subgenera *Kaiseriana* and *Haemaphysalis* were not monophyletic in our phylogenies. Moreover, *H.* (*Aborphysalis*) *formosensis* was embedded in the subgenus *Haemaphysalis* as the sister to *H.* (*H.*) *concinna*. Other recent mt genome phylogenies support the placement of *H.* (*H.*) *pentalagi* among the *Kaiseriana* species as well as *H.* (*Ao.*) *formosensis* as the sister to *H.* (*H.*) *concinna* (Mans et al., 2019; Kelava et al., 2021). Further investigation into the morphology of these species is warranted.

Alloceraea was the only subgenus of *Haemaphysalis* that was monophyletic in our trees. Indeed, our phylogenies indicate that the subgenus *Alloceraea* is monophyletic to the exclusion of the rest of the *Haemaphysalis* ticks. The exact position of *Alloceraea* in the phylogenetic scheme of the Haematobothrion (i.e. *Haemaphysalis, Bothriocroton* plus *Archaeocroton*), however, is unknown. We speculate that *Alloceraea* may be the sister to the rest of the *Haemaphysalis*, even though this arrangement is not well supported in any of our phylogenies because we suspect that the current mt genomes are not sufficiently informative to resolve the deep branches like the most-recent-common-ancestor of *Haemaphysalis* or the position of *Ac. sphenodonti*. Intriguingly, our

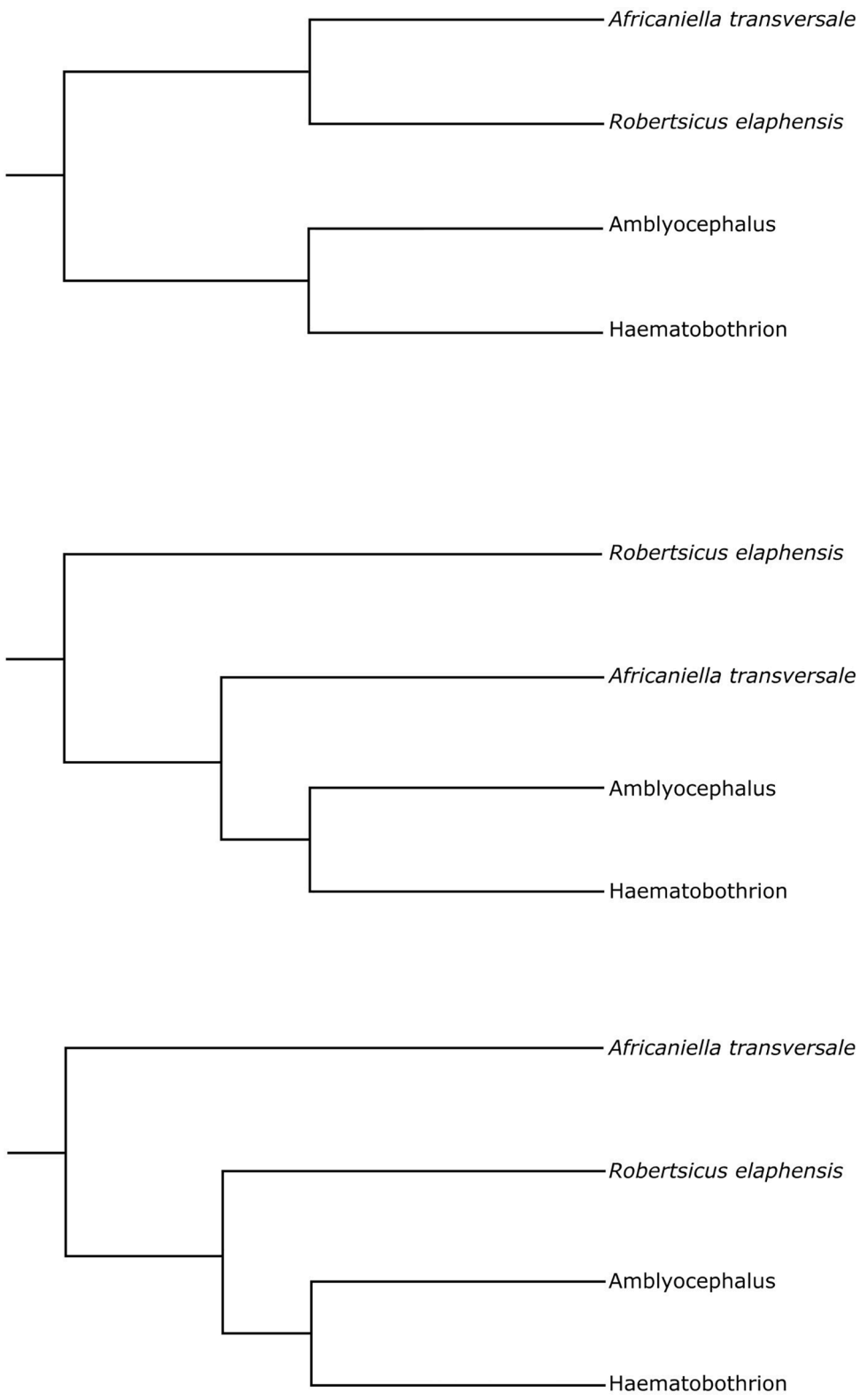

Fig. 7. Three hypotheses for the phylogeny of *Africaniella transversale, Robertsicus elaphensis*, Amblyocephalus (i.e. *Amblyomma* plus the Rhipicephalinae) and Haematobothrion (i.e. *Haemaphysalis* plus *Bothriocroton* and *Archaeocroton sphenodonti*).

amino-acid tree indicated that *Ac. sphenodonti* was the sister to *Alloceraea* to the exclusion of the rest of the *Haemaphysalis* (Appendix Fig. 3). The amino-acid tree, therefore, indicated that either *Ac. sphenodonti* may belong to the *Alloceraea*, perhaps as part of the genus *Haemaphysalis*, or the subgenus *Alloceraea* does not belong to the genus *Haemaphysalis* but rather to another genus, perhaps *Archaeocroton*. We, however, do not trust the placement of *Ac. sphenodonti* with *Alloceraea* because this arrangement was not seen in any of our other phylogenetic trees with high support (>90 ML, >0.9 BI). Other recent phylogenies, in contrast, had *Ac. sphenodonti* in a clade with *Bothriocroton* with high phylogenetic

support (92 ML) (Geng et al., 2017). Therefore, due to the conflicting evidence, we argue that indeed *Alloceraea* belongs, for now, in the genus *Haemaphysalis* and is the sister to the rest of the *Haemaphysalis* species, and that *Ac. sphenodonti* is not in the genus *Haemaphysalis*. However, phylogenies with more species from the Haematobothrion (i.e. *Haemaphysalis, Bothriocroton* plus *Archaeocroton*) or using transcriptome data (e.g. Charrier et al., 2019) may be able to resolve the Haematobothrion and accept or reject our hypothesis. Sequences from species closely related to *Ac. sphenodonti* may resolve the deep branches of the Haematobothrion if there is a saturation of substitution sites at synonymous coding positions between the ingroup taxa and the outgroup (Halanych et al., 1999; Weisrock et al., 2005).

The morphological groups of *Haemaphysalis* proposed by Hoogstraal and Kim (1985) were not supported by our *cox1* gene tree (Appendix Fig. 4). Our *cox1* gene tree, rather, had *H.* (*Ab*) *punctata*, "structurally primitive", and *H.* (*He.*) *sulcata*, "structurally intermediate", in a clade with the rest of "structurally advanced" *Haemaphysalis* species to the exclusion of *H.* (*Al.*) *inermis* and *H.* (*Al.*) *kitaokai*, "structurally primitive" (97 ML, 0.98 BI) (Appendix Fig. 4). The morphological phylogenetic trees inferred by Klompen et al. (1997), likewise, indicated that the "structurally primitive" subgenera were paraphyletic: the *Aboimisalis* subgenus ("structurally primitive") and the "structurally intermediate" and "structurally advanced" subgenera of *Haemaphysalis* formed a phylogenetic clade to the exclusion of the subgenera *Alloceraea* and *Allophysalis*. The *18S* plus *28S* rRNA phylogenies of Burger et al. (2013), moreover, had *H.* (*Ab.*) *punctata* and *H.* (*He.*) *sulcata* in a clade with the "structurally advanced" *Haemaphysalis*. We did not include *H.* (*Ab.*) *punctata* in our *18S* plus *28S* rRNA phylogenetic trees because the *28S* rRNA partial gene sequences for this species were too short and incomplete. Our *cox1, 18S* and *28S* rRNA gene phylogenies did not support the monophyly of these three groups of *Haemaphysalis*. We, therefore, agree with the conclusion of Burger et al. (2013) that the "structurally primitive", "structurally intermediate" and "structurally advanced" *Haemaphysalis* may not be monophyletic. Of course, entire mt genome sequences from species that belong to the unrepresented "structurally primitive" and "structurally intermediate" subgenera may resolve the subgenera groups of *Haemaphysalis*.

4.4. *Alectorobius capensis* cryptic species

Mans et al. (2019) proposed that a pairwise (%) genetic-identity of 96% or more of tick mt genomes indicates members of the same species, whereas a pairwise (%) genetic-identity of 94% or less indicate different species. Pairwise (%) genetic-identity of 94%-96% between mt genomes, moreover, may indicate cryptic species (Mans et al., 2019-2021). The mt genomes of *A. capensis* from Japan had a pairwise (%) genetic-identity of ~93.9% to the mt genomes of *A. capensis* ticks from South Africa which indicates that one or more cryptic species may be present within *A. capensis* (Appendix Table 3). Kim et al. (2017), intriguingly, had 94.1%-95.5% pairwise (%) genetic-identity between the *16S* rRNA sequences of *A. capensis* from Korea and Japan, but 90.3%-91.7% pairwise (%) genetic-identity between the *A. capensis* from Korea and *A. capensis* from Peru and the Galapagos Islands. Mans et al. (2019) suggested that the *A. capensis* from Japan and South Africa may represent cryptic species from comparisons of *cox1* and entire mt genome sequences. Our pairwise (%) genetic-identity, moreover, indicated that the mt genomes of *A. capensis* from Japan are closer to that of *A. sawaii* (Japan) than to the *A. capensis* from South Africa. Indeed, *A. capensis* from Japan had a pairwise (%) genetic-identity of 95.4%-95.6% to *A. sawaii* but ~93.9% identity to the South African *A. capensis* (Appendix Table 3). Munoz-Leal et al. (2017) also observed morphological similarity in the larval stages of *A. capensis* from Brazil and *A. sawaii* and their *16S* rRNA sequences of *A. capensis* and *A. sawaii* had a pairwise (%) genetic-identity of >95%. The phylogeny of the *A. capensis* from Japan and South Africa and the relationship to *A. sawaii* is unclear. *A. capensis* (sensu stricto) and *A. sawaii* both belong to the *A. capensis* (formerly *Ornithodoros capensis* see Guglielmone et al. (2010); Mans et al., (2021)) species complex (sensu lato) with nine other species (*A. amblus, A. cheikhi, A. coniceps, A. denmarki, A. maritimus, A. muesbecki, A. spheniscus, A. talaje* and *A. yunkeri*) (Hoogstraal et al., 1985; Dupraz et al., 2016). *Alectorobius capensis* has a worldwide distribution with populations in southern Africa, Madagascar, Iran, Australia, New Zealand, Eastern USA, Brazil, Caribbean Islands, Hawaii, Japan and other smaller Atlantic and Pacific islands (Dupraz et al., 2016; Hoogstraal, 1985; Kim et al., 2017; Munoz-Leal et al., 2017; Munoz-Leal et al., 2020). Phylogenies from the entire mt genomes from the *A. capensis* populations around the world may reveal even more cryptic species and provide insights into the relationships between *A. sawaii* and *A. capensis*.

5. Conclusions

We discovered that *Haemaphysalis* (*Alloceraea*) *inermis* had an insertion between the tRNA-Glu (E) and *nad1* genes. This was similar to insertions in *H.* (*Al.*) *kitaokai*, and *Rhipicephalus* (*Boophilus*) *geigyi* (Burger et al., 2013, 2014b; Kelava et al., 2021). The 132-bp insertion of *H.* (*Al.*) *inermis* had parts of the 3' end *nad1* (37-bp) and *16S* rRNA gene (118-bp) fragments. *Haemaphysalis* (*Al.*) *inermis* and *H.* (*Al.*) *kitaokai* formed a clade separate to the rest of the *Haemaphysalis* ticks. *Africaniella transversale* and *Robertsicus elaphensis* appear to be sisters to the rest of the metastriate Ixodidae although it is not known exactly which of three possible arrangements is correct [(rest of Metastriata), (*Af. transversale, Ro. elaphensis*)] or [(rest of Metastriata, *Af. transversale*), *Ro. elaphensis*)] or [(rest of Metastriata, *Ro. elaphensis*), *Af. transversale*]. The relationships of *Af. transversale* and *Ro. elaphensis* with respect to Haematobothrion and Amblyocephalus and to each other is still not known. Intriguingly, our amino-acid sequence tree had *Archaeocroton sphenodonti* as the sister with high support, to the subgenus *Alloceraea*, therefore, the genus *Haemaphysalis* may not be monophyletic. The *Alectorobius capensis* may contain cryptic species as the *A. capensis* ticks from Japan had a higher pairwise (%) genetic-identity to *A. sawaii* from Japan than the *A. capensis* ticks from South Africa.

CRediT authorship contribution statement

Samuel Kelava: Conceptualization, Methodology, Software, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Ben J. Mans:** Writing – review & editing, Supervision. **Renfu Shao:** Resources, Supervision. **Dayana Barker:** Conceptualization, Resources. **Ernest J.M. Teo:** Conceptualization. **Elisha Chatanga:** Resources, Investigation. **Alexander W. Gofton:** Conceptualization. **Mohamed Abdallah Mohamed Moustafa:** Methodology. **Ryo Nakao:** Methodology, Funding acquisition. **Stephen C. Barker:** Resources, Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.102070.

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