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Molecular phylogeny of soft ticks (Ixodida: Argasidae) inferred from mitochondrial genome and nuclear rRNA sequences

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Abstract

The genus-level classification of soft ticks (Argasidae) is controversial. A previous phylogenetic analysis of morphological and developmental characters found that the genus *Ornithodoros* was paraphyletic, and raised a new genus, *Carios*, for species previously in the genera *Antricola, Argas, Ornithodoros*, and *Nothoaspis* (Klompen and Oliver, 1993). Genetic analyses of soft ticks to date have been limited to 16S rRNA, which is not highly phylogenetically informative for this group. We sequenced the entire mitochondrial genomes of seven species of soft ticks, and the partial mitochondrial genomes of a further five species of soft ticks. We used these sequences to test the genus-level classification of soft ticks. Our analyses strongly supported a clade of Neotropical species (mostly bat-associated) within the subfamily Ornithodorinae. This clade, which we call Neotropical Ornithodorinae, has species from two genera, *Antricola* and *Nothoaspis,* and two subgenera, *Ornithodoros* (*Alectorobius*) *and Ornithodoros* (*Subparmatus*). We also addressed the phylogenetic position of *Ornithodoros savignyi,* the type species of the genus *Ornithodoros*. Our analysis had strong support for a clade consisting of *Ornithodoros savignyi* and four other *Ornithodoros* species: *Or. brasiliensis, Or. moubata, Or. porcinus,* and *Or. rostratus*. This clade, *Ornithodoros* sensu stricto, did not contain the *Alectorobius* and *Subparmatus* species, *Or.* (*Alectorobius*) *fonsecai, Or.* (*Alectorobius*) *capensis*, and *Or.* (*Subparmatus*) *marinkellei*, which in traditional classification schemes have been placed in the genus *Ornithodoros*. Our comparison of mitochondrial rRNA, nuclear rRNA, and mitochondrial genome analyses show that only mitochondrial genome sequences have the potential to resolve the controversial phylogenetic relationships within the major soft tick lineages, such as the taxonomic status of *Carios* sensu Klompen and Oliver (1993).

Keywords

Argasidae; phylogeny; mitochondrial genomes; 18S rRNA; *Carios*; *Ornithodoros*

Introduction

Ticks (Ixodida) are haematophagous ectoparasites of vertebrates. There are two main families of ticks: the hard ticks (Ixodidae), with 705 species (Guglielmone et al., 2010; Apanaskevich et al., 2011; Estrada-Peña et al., 2012; Apanaskevich et al., 2013); and the soft ticks (Argasidae), with 198 species (Guglielmone et al., 2010; Nava et al., 2010; Dantas-Torres et al., 2012; Heath, 2012; Venzal et al., 2012, 2013). Soft ticks differ from hard ticks in several fundamental ways: nymphs and adults have a leathery integument, lacking the sclerotised scutum of hard ticks; and have anteroventrally (rather than apically) located mouthparts (Sonenshine, 1991). Additionally, hard ticks take one large blood-meal per life-cycle stage, and engorge over several days, whereas most soft ticks take several smaller blood-meals as nymphs and adults, and engorge rapidly, within minutes to hours (Sonenshine, 1991; Mans and Neitz, 2004). An enigmatic third family of ticks, Nuttalliellidae, shares some morphological and physiological characters with hard ticks, and others with soft ticks, and is represented by a single known species, *Nuttalliella namaqua* (see recent work by Mans et al., 2011, 2012; Latif et al., 2012).

The genus-level classification of hard ticks is no longer controversial, 14 genera are currently recognised (Guglielmone et al., 2010). In contrast, there is no consensus about the genus-level classification of soft ticks. The systematics of soft ticks has been recently reviewed (Nava et al., 2009; Estrada-Peña et al., 2010); there are four competing 'schools' or classification schemes for the genera of soft ticks. These four classification schemes are: the Soviet (or eastern) scheme (Filippova, 1966; Pospelova-Shtrom, 1969); the American (or western) scheme (Clifford et al., 1964; Hoogstraal, 1985); the French scheme (Camicas and Morel, 1977; Camicas et al., 1998); and the cladistic scheme (Klompen and Oliver, 1993) (Table 1). Common to all classification schemes is the division of the Argasidae into two subfamilies: the Argasinae and Ornithodorinae, a division also supported by molecular data (Black and Piesman, 1994; Black et al., 1997; Norris et al., 1999; Klompen et al., 2000, 2007; Mans et al., 2012). There is also agreement among all four schemes on the status of three genera: *Argas* (Argasinae), *Ornithodoros*, and *Otobius* (Ornithodorinae). However, there is disagreement on the species-composition of these three genera, and on the subfamily placement, species-composition, and status of the other genera and subgenera. Thus, 137 of the 198 species of Argasidae (Guglielmone et al., 2010; Nava et al., 2010; Dantas-Torres et al., 2012; Heath, 2012; Venzal et al., 2012, 2013) can be assigned to more than one genus.

All four classification schemes rely on, and are limited to, morphology and physiological or life-cycle characters, as they predate the widespread use of molecular markers. The Soviet and American schemes are based on "overall similarity, with taxonomic rank determined by the degree of phenetic variation" (Estrada-Peña et al., 2010, p. 318); a phenetic approach, rather than the phylogenetic concept of monophyletic clades. The French scheme, especially Camicas et al. (1998), is a simple list of taxa, with no supporting morphological or physiological characters given for any of the groups, so the level of support for these groups cannot be critically examined. In addition, Camicas et al. (1998) contains a *nomen nudum* subgenus, *Reticulibius,* for seven species of *Alectorobius* (Estrada-Peña et al., 2010), and three other unnamed monotypic subgenera of *Alectorobius,* '*Sbg. nov.* 1-3 Morel'.

Klompen and Oliver (1993) were the first to conduct a comprehensive phylogenetic analysis of the Argasidae, from a matrix of 83 morphological and developmental characters. The cladistic scheme proposed by Klompen and Oliver (1993) was a major revision of the genus-level classification of the American scheme: transferring two subgenera (*Alveonasus* and *Proknekalia*) from the subfamily Ornithodorinae to the subfamily Argasinae; and transferring three subgenera (*Carios*, *Chiropterargas,* and *Microargas*) from the Argasinae to the Ornithodorinae. Within the Ornithodorinae, Klompen and Oliver (1993) proposed only three genera: *Otobius*, *Ornithodoros* s.s. (including *Microargas, Alveonasus,* and *Proknekalia*), and *Carios.* The new genus, *Carios*, was raised from the subgenus *Argas* (*Carios*), and contained seven former genera or subgenera (*Alectorobius, Antricola, Carios*, *Chiropterargas, Nothoaspis, Reticulinasus,* and *Subparmatus*). Klompen and Oliver (1993) did not recognise any of these groups as valid subgenera of *Carios,* as their analysis showed that the subgenus *Alectorobius* was paraphyletic. Thus, Klompen and Oliver (1993) argued that recognising subgenera of *Carios* would require elevation of the various paraphyletic *Alectorobius* lineages to subgenera, which "would create a proliferation of poorly supported subgenera or genera" (Klompen and Oliver, 1993, p. 328).

The proposals of Klompen and Oliver (1993) were not universally adopted. Though some later works have used this scheme (Klompen et al., 1996, 1997, 2000, 2007; Black et al., 1997; Ushijima et al., 2003; Labruna and Venzal, 2009; Barros-Battesti et al., 2011; Heath, 2012), eight new species of *Antricola* (Estrada-Peña et al., 2004; Labruna et al., 2008), *Nothoaspis* (Nava et al., 2010), and *Ornithodoros* (*Alectorobius*) (Venzal et al., 2008; 2013; Dantas-Torres et al., 2012) have been described. Indeed, Guglielmone et al. (2005) argued for the retention of *Antricola* as a valid genus, and called for stronger evidence for the genus-

level arrangement of the cladistic scheme. However, some authors have accepted the core proposal of Klompen and Oliver (1993), that the genus *Ornithodoros* in the American scheme (*Ornithodoros* s.l.) is not monophyletic, though with the caveat that support for *Carios* s.l. to date has been weak (Nava et al., 2009; Estrada-Peña et al., 2010; Guglielmone et al., 2010).

In discussing the systematics of soft ticks, other authors (Estrada-Peña et al., 2010; Guglielmone et al., 2010) adopted, but not necessarily endorsed, the genus-level classification of the American scheme, and we follow this convention. As the subgenera are more stable than the genera between the four schemes, we also use subgenus names. However, as with the genus-level classification of the American school, we do not necessarily endorse the validity of all soft tick subgenera, since not all subgenera are likely to be monophyletic.

Phylogenetic analyses of soft ticks to date have been limited by a lack of suitable markers. Mitochondrial 16S rRNA, the most widely sequenced molecular marker in soft ticks, suffers from a lack of phylogenetic resolution at the genus level within the Argasidae (Nava et al., 2009; Estrada-Peña et al., 2010). 16S rRNA has the potential for resolving phylogenetic relationships among closely related species or within species, but not higher-order relationships among groups of species or genera.

Nuclear 18S rRNA, a common genetic marker in molecular phylogenetic studies of hard ticks, has not been widely used in soft ticks. Only six near-complete (ca. 1.7 kb) soft tick 18S rRNA sequences are currently available on GenBank. Black et al. (1997) analysed the 18S rRNA of five soft ticks (in addition to 13 hard ticks), and found strong support for the transfer of the subgenus *Ornithodoros* (*Alveonasus*) to the genus *Argas* (Klompen and Oliver, 1993). A later study also supported this transfer, and had stronger support for the placement of *Otobius megnini* as the sister group to *Or.* (*Ornithodoros*) *moubata* and *Or.* (*Alectorobius*) *puertoricensis* (Dobson and Barker, 1999). Klompen et al. (2000) used 18S rRNA, in combination with partial 28S rRNA, 16S rRNA, and morphology in a total evidence analysis of hard ticks with the same five soft tick taxa, and supported the transfer of *Ornithodoros* (*Alveonasus*) to *Argas*. More recent studies of 18S rRNA in hard ticks have strong support for monophyly of most hard tick genera, with the exception of closely-related genera like *Rhipicephalus* and *Hyalomma* and a few enigmatic species such as *Amblyomma sphenodonti* and *Am. elaphense* (Burger et al., 2012; 2013). However, 18S rRNA alone does not have sufficient phylogenetic information to resolve the relationships among hard tick genera.

Previous studies of arthropods found that 18S rRNA is most useful for resolving relationships among higher taxa (phyla and superphyla) and suggested that 28S rRNA is more useful at lower taxonomic levels (Mallatt et al., 2004; Mallatt and Giribet, 2006), though a study of the parasitiform mites found that partial 28S sequences were not highly informative for relationships among mesostigmatid mite lineages (Klompen et al., 2007).The addition of partial 28S rRNA sequences to the analysis of 18S rRNA improves the support for some relationships among hard tick genera, but does not strongly resolve all the relationships among hard tick genera (Burger et al., 2012; 2013).

We have had some success using mitochondrial (mt) genomes to address phylogenetic relationships among hard tick lineages (Burger et al., 2012; 2013; Burger et al., Submitted). In this study, we sequenced entire mt genomes of *Antricola* (*Antricola*) *mexicanus, Argas* sp. SpringbokSA-QMS95171, *Argas* (*Persicargas*) *miniatus, Argas* (*Argas*) *lagenoplastis, Otobius megnini* (the type species of the genus *Otobius*), *Ornithodoros* (*Pavlovskyella*) *rostratus*, and *Ornithodoros* (*Pavlovskyella*) *brasiliensis*. We also sequenced partial mt genomes of *Ornithodoros* (*Subparmatus*) *marinkellei, Ornithodoros* (*Alectorobius*) *fonsecai, Nothoaspis amazoniensis, Antricola* (*Parantricola*) *marginatus* (the type species of the subgenus *Parantricola*), and *Ornithodoros* (*Ornithodoros*) *savignyi* (the type species of the genus *Ornithodoros*). Additionally, we sequenced the entire mt genome of *Allothyrus* sp. LamingtonNP-QMS95173 (Holothyrida). The Holothyrida, free-living mites which feed on the body fluids of dead arthropods (Walter and Proctor, 1998), have long been thought to be the sister group to the Ixodida. This sister group relationship has been supported by phylogenetic analysis of morphological and developmental characters (Lindquist, 1984; Lehtinen, 1991) and nuclear rRNA sequences (Murrell et al., 2005; Klompen et al., 2007). We used these mt genome sequences to investigate the phylogeny of the soft ticks, particularly with reference to the competing schemes of soft tick classification.

Materials and methods

Specimens and DNA extraction

Specimens used for mt genome sequencing in this study are listed in Table 2, along with accession numbers for sequences deposited in GenBank. DNA was extracted from tick specimens using the DNeasy Tissue Extraction Kit (QIAGEN). Individual tick specimens

were cut in half longitudinally, using a scalpel under a dissecting microscope. Half of each tick 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 a micropestle prior to DNA extraction. Voucher specimens were deposited in the Queensland Museum, South Brisbane, Queensland 4010, under registration numbers QMS95171-95182. No voucher specimens were deposited for *Argas lagenoplastis* as the entire specimen was used in DNA extraction. Additionally, we also sequenced the 18S and partial 28S rRNA genes of *Ornithodoros* (*Alectorobius*) *mimon*, *Ornithodoros rondoniensis* (described as *Carios rondoniensis* Labruna et al., 2008), as well as the 18S rRNA sequence of *Argas* (*Argas*) *monachus*. Additionally, we sequenced the 18S and 28S rRNA of eight hard ticks for which mt genome sequences had been sequenced previously (Burger et al., Submitted). These sequences are available on GenBank with accession numbers KC769599, KC769627, KC769602, KC769623, KC769609, KC769614-KC769621, and KC769635-KC769642.

We were unable to identify two of our specimens to species. *Allothyrus* sp. LamingtonNP-QMS95173 was collected from Lamington National Park, Queensland, Australia, and is an undescribed species of holothyrid mite; only 27 species of Holothyrida have been formally described to date (Beaulieu et al., 2011). *Argas* sp. SpringbokSA-QMS95171 was collected from an abandoned mud nest of a bird near Springbok, South Africa by SCB.

PCR amplification and sequencing

Short (ca. 400-700 bp) regions of the *cox1* and *cytb* 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 three regions. Entire mt genomes were then amplified in two overlapping fragments on the forward (majority or J) strand: (i) a ca. 6 kb fragment from *cytb* to *cox1*; and (ii) a ca. 9 kb fragments from *cox1* to *cytb* (Figure 1). Partial mt genomes were sequenced from only one of the two fragments: the 9 kb fragment in *Or. marinkellei*, and the 6 kb fragment in *No. amazoniensis*, *An. marginatus*, *Or. fonsecai*, and *Or. savignyi*. 18S rRNA and 28S rRNA genes were amplified and sequenced as per Burger et al. (2012), using previously reported primers (Hillis and Dixon, 1991; Black et al., 1997; Whiting et al., 1997; Dobson and Barker, 1999).

Expand Long Range dNTPack kits (Roche) were used to amplify all long mt PCR products (> 1 kb). TaKaRa Ex Taq DNA polymerase kits (Takara Biotechnology) were used to

amplify short mt gene fragments (< 1 kb) as well as nuclear rRNA genes. 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 for Ex Taq were: 94°C for 60 sec, followed by 40 cycles of 98°C for 10 sec, 60°C for 30 sec, 72°C for 2 min and a final extension of 72°C for 5min. General PCR conditions for Expand dNTPack were: 92°C for 120 sec, 10 cycles of 92°C for 10 sec**,** 55°C for 15 sec, 60°C for 8 min, followed by 25 cycles of 92°C for 10 sec 55°C for 15 sec, 60°C for 8 min (increasing by 20 sec per cycle), and a final extension of 68°C for 7 min. PCR products were examined on 1% agarose gels stained with ethidium bromide. DNA Molecular Weight Marker VII (Roche Diagnostics) and Low DNA Mass Ladder (Invitrogen) were used to estimate the length and concentration of PCR products, respectively. Wizard SV Gel and PCR Clean-up System (Promega) was used to purify PCR products for use in sequencing reactions. Sequencing reactions used the ABI Prism BigDye v3.1 Terminator kit (Applied Biosystems) and an Applied Biosystems 3730*xl* DNA Analyzer at the Australian Genome Research Facility (AGRF). The mt genome of *Ar. lagenoplastis* was sequenced by primer walking at the AGRF. All other mt genomes were sequenced using an Illumina HiSeq run at BGI-Hong Kong.

Contig assembly and mitochondrial genome annotation

Geneious Pro 5.6 (Drummond et al., 2012) was used to assemble Sanger and Illumina sequence reads. Protein coding genes were identified by BLAST searches of open reading frames, and tRNA genes were identified with tRNAscan-SE (Lowe and Eddy, 1997) and ARWEN (Laslett and Canback, 2008). Non-coding regions and rRNA genes were identified by BLAST search and alignment with other tick mt genomes.

Sequence alignment

The entire mt genomes of 27 tick species, as well as two mesostigmatid mites and two horseshoe crabs, and the partial mt genomes of five ticks were retrieved from GenBank (Table 3). TranslatorX (Abascal et al., 2010) was used to align the nucleotide sequences of mt protein coding genes based on their amino acid sequences. MAFFT (Katoh et al., 2002) was chosen as the alignment algorithm in TranslatorX and Gblocks (Castresana, 2000) was used to trim the alignments of ambiguously aligned sites. Individual gene alignments were concatenated into an alignment of all 13 mt protein coding genes for phylogenetic analysis.

The final mtDNA alignment was 10,119 nucleotides long, and is available on Dryad (doi:10.5061/dryad.XXXXX).

18S rRNA sequences of 73 ticks, 10 holothyrid and opilioacarid mites, and two horseshoe crabs were retrieved from GenBank, along with 48 partial 28S rRNA sequences from the same species (Table S2). To these we added the 24 18S rRNA and 22 28S rRNA sequences which we obtained from the same species as the mt genomes in this study, as well as eight mt genomes of hard ticks from a previous study (Burger et al., Submitted). In total 109, 18S rRNA and 70 28S rRNA sequences were aligned using MAFFT (Katoh et al., 2002), and adjusted and trimmed manually. The final 18S and 28S alignments were 1,862 and 2,535 nucleotides long, respectively. For phylogenetic analysis, the two rRNA alignments were concatenated into a single alignment, which is available on Dryad (doi:10.5061/dryad.XXXXX).

We also retrieved 48 partial 16S rRNA sequences and seven partial 12S rRNA sequences from GenBank, and aligned these sequences with the rRNA genes from the mt genomes. Both mt rRNA genes were aligned with MAFFT using the Q-INS-i setting, which uses RNA secondary structure to aid the alignment. The alignments were then trimmed manually, resulting in a 16S rRNA alignment of 529 nucleotides, and a 12S rRNA alignment of 896 nucleotides (doi:10.5061/dryad.XXXXX).

Phylogenetic analysis

Phylogenetic analyses of mtDNA, mt amino acid, mt rRNA, and nuclear rRNA sequences were executed with GARLI (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006). We partitioned the mtDNA alignments by codon position and used jModeltest 2 (Darriba et al., 2012) to select optimal substitution models for each partition (HKY+I+G for 12S rRNA, and 16S rRNA alignments; GTR+I+G for 18S rRNA, 28S rRNA and all mtDNA partitions).

A recent study of tick mt genomes (Mans et al., 2012) used the putative amino acid sequence of five of the 13 mt genes for phylogenetic interference (cox1, cytb, nad1, nad2, and nad4), which were found to be the most phylogenetically informative in an analysis of insect mt genomes (Talavera and Vila, 2011). We set out to determine the optimal subset of the 13 mt protein-coding genes to use for phylogenetic analysis of ticks. We used GARLI to determine the Maximum Likelihood (ML) tree for each gene alignment, and used Ktreedist (Soria-

Carrasco et al., 2007) to compare each gene tree with the ML tree for the concatenated mt genome alignment. We analysed both the DNA and amino acid data for each gene, and kept the taxa set the same for each gene by excluding all partial tick mt genomes. We then ranked each gene tree by the number of symmetric differences to the concatenated data tree, and chose two different subsets. The first cutoff includes only the three to four genes with the lowest number of symmetric differences (<20, indicated by the dashed line in Table S3), and the second cutoff excludes three genes with the highest number of symmetric differences (>28, indicated by the dotted line in Table S3). In total, we tested 12 concatenated mt gene alignments: mtAA4 (amino acid sequences of cox1, nad1, nad4, and nad5); mtAA10 (amino acid sequences of cox1, cox2, cox3, cytb, nad1, nad2, nad3, nad4, nad5, and nad6); mtAA13 (amino acid sequences of all 13 mt genes); mtAA5 (amino acid sequences of the five genes from analysis of insect mt genomes: cox1, cytb, nad1, nad2, and nad4); mtDNA3 (DNA sequences of cox1, cox3, and nad1); mtDNA10 (DNA sequences of atp6, cox1, cox2, cox3, cytb, nad1, nad2, nad3, nad4L, and nad5); mtDNA13 (DNA sequences of all 13 mt genes); mtDNA5 (DNA sequences of the five genes from analysis of insect mt genomes, as above); and the four mtDNA datasets with third codon positions excluded. These alignments are available on Dryad (doi:10.5061/dryad.XXXXX).

For each alignment, GARLI was instructed to estimate the free parameters of each chosen model, to treat all partitions as unlinked and to perform 1000 bootstrap replicates, which were summarised with SumTrees, part of the DendroPy package (Sukumaran and Holder, 2010). Our analyses ran on the GARLI web service (Bazinet and Cummings, 2011), which uses a special programming library and associated tools (Bazinet et al., 2007) and grid computing (Cummings and Huskamp, 2005) through The Lattice Project (Bazinet and Cummings, 2008), and includes clusters and desktops in one encompassing system (Myers et al., 2008). The GARLI web service distributes files among hundreds of computers where the analyses were conducted asynchronously in parallel, following the general computational model of a previous phylogenetic study (Cummings et al., 2003), using an earlier grid computing system (Myers and Cummings, 2003).

Results

Mitochondrial genome organisation

Allothyrus sp. LamingtonNP-QMS95173 (Holothyrida) and all the soft ticks sequenced in this study have the ancestral arthropod mt gene arrangement (Figure 1) previously reported in

soft ticks (Shao et al., 2004; 2005a), *Nu. namaqua* (Mans et al., 2012), prostriate hard ticks (Black and Roehrdanz, 1998; Campbell and Barker, 1998; Shao et al., 2005b; Montagna et al., 2012), and horseshoe crabs (Lavrov et al., 2000). The mt genome of *Allothyrus* sp. (Holothyrida) differs slightly from the mt genomes of soft ticks; it is closer in length to non-Australasian *Ixodes* mt genomes (*Allothyrus* sp. is 13-40 bp longer) than to soft tick mt genomes (*Allothyrus* sp. is 90-201 bp longer). Additionally, the anticodon of tRNA-Ser_1 (AGN) is TCT in *Allothyrus* sp. and hard ticks, but in all soft ticks and *Nu. namaqua*, the tRNA-Ser_1 (AGN) anticodon is GCT.

The Tick-Box motif, recently reported by Montagna et al. (2012), is a degenerate 17 bp motif (ttgyrtchwwwtwwgda) which punctuates the 3' ends of 16S rRNA and *nad1* in chelicerate mt genomes. We identified both sites of the Tick-Box motif (Montagna et al., 2012) in all of the soft tick mt genomes in this study (Figure S1), except for the 9 kb partial mt genome of *Or. marinkellei*, which does not include 16S rRNA or *nad1*. We also identified both sites of the Tick-Box in *Allothyrus* sp. (Holothyrida), however we noted that the *nad1* Tick-Box of *Allothyrus* sp. differs from the *nad1* Tick-Box found in ticks as it does not appear to punctuate the 3' end of *nad1*. The *nad1* gene of *Allothyrus* sp. has a complete stop codon before the start of the Tick-Box motif. The other site of the Tick-Box motif is the same in *Allothyrus* sp. as in soft ticks and Australasian *Ixodes* ticks; within tRNA-Leu_2 (CUN) after the 3' end of 16S rRNA (Figure S1).

Phylogenetic analysis of mitochondrial rRNA genes

The ML tree inferred from 16S rRNA (529 bp) has moderate support (70%) for monophyly of Argasinae, and weak support (58%) for monophyly of Ornithodorinae, but relationships within the two soft tick subfamilies are not resolved (Figure S2). The phylogenetic position of *Ar.* (*Carios*) *vespertilionis* (the type species of the subgenus *Carios*) is not strongly supported, but it clusters within the Ornithodorinae (47%). Where conspecific sequences are available, sequences from the mt genomes in this study cluster with them, with the exception of *Ar. miniatus* (Brazil), which clusters with *Ar. robertsi* (Australia) (99.7% identical). *Argas* sp. SpringbokSA-QMS95171 clusters with *Ar. africolumbae* (91.5% identical).

The ML tree inferred from 12S rRNA sequences (896 bp; Figure 2) has moderate support for monophyly of the Argasidae (62%), as well as the Argasinae (72%) and the Ornithodorinae (78%). However, the relationships among the lineages within the Ornithodorinae are not strongly supported. In contrast to the 16S rRNA tree, there is strong support for monophyly

of the subgenus *Ornithodoros* (94%), and weak support for a cluster of *Antricola*, *Nothoaspis*, and *Ornithodoros* (*Alectorobius*) species (Neotropical Ornithodorinae; 56%). The phylogenetic position of *Ar.* (*Carios*) *vespertilionis* (the type species of the subgenus *Carios*) is not strongly supported, but its placement within the Ornithodorinae rather than the Argasidae is supported (78%). There is no support in the 12S rRNA analysis for inclusion of *Ar. vespertilionis* within the Neotropical Ornithodorinae clade.

Phylogenetic analysis of nuclear rRNA genes

The ML tree inferred from nuclear 18S and partial 28S rRNA sequences (4397 bp; Figure 3) has strong support (100%) for monophyly of the Argasidae and Ornithodorinae, and for two clades within the Ornithodorinae: *Ornithodoros* s.s. (85%) and Neotropical Ornithodorinae (100%). There is little phylogenetic resolution within the Neotropical Ornithodorinae clade, other than support for monophyly of the genus *Antricola* (92%). In contrast, relationships within the *Argas* and *Ornithodoros* s.s. clades are strongly resolved (71-100%). There is also strong support for placement of *Or.* (*Alveonasus*) *lahorensis* within the genus *Argas*, as in previous nuclear rRNA phylogenies (Black et al., 1997; Klompen et al., 2007; Mans et al., 2012). The nuclear rRNA tree is unable to resolve the phylogenetic positions of *Ot. megnini* or *Or. coriaceus* within the Ornithodorinae. Some higher-order relationships are strongly supported in the nuclear rRNA tree: monophyly of the Ixodida, Ixodidae, Metastriata, Opilioacarida, and the holothyrid families Holothyridae and Neothyridae (95-100%). However the placement of *Nu. namaqua* in relation to the Ixodidae and the Argasidae is not strongly supported (< 50%) in the nuclear rRNA tree, and paraphyly of Prostriata and Holothyrida is weakly supported (52-54%).

Phylogenetic analysis of mitochondrial genome sequences

We tested 12 different subsets of the mt genome data: mtAA4, mtAA10, mtAA5, mtAA13, mtDNA3, mtDNA10, mtDNA5, mtDNA13, and the four mtDNA datasets with third codons excluded. The most strongly resolved tree was inferred from the mtDNA 10 genes dataset (mtDNA10): *atp6*, *cox1*, *cox2*, *cox3*, *cytb*, *nad1*, *nad2*, *nad3*, *nad4L*, and *nad5* (7,914 bp; Figure 4 and Table 4). Though the mtDNA10 tree was the most strongly resolved overall, the mtAA all genes (mtAA13) dataset had the strongest support for relationships within the Neotropical Ornithodorinae, and differed from the topology of the mtDNA10 tree (inset, Figure 4). Excluding third codons from the mtDNA analyses had little effect on the level of bootstrap support (Table S4).

There is strong support (93-100%) in the mtDNA10 tree (Figure 4) for monophyly of the *Argas*, *Ornithodoros* s.s., and Neotropical Ornithodorinae clades, and for monophyly of the Ornithodorinae and the placement of *Ot. megnini* as the sister group to rest of the Ornithodorinae. There is also strong support for the placement of *Nu. namaqua* as the sister group to Ixodidae (95%). This placement of *Nu. namaqua* is incompatible with the Bayesian inference analysis of Mans et al. (2012), which had *Nu. namaqua* as the sister group to the Ixodidae plus Argasidae. Our mtAA4 and mtDNA5 datasets also had weak support for *Nu. namaqua* as the sister group to the Ixodidae plus Argasidae (Table 4). Our mtDNA and mtAA analyses differed on the placement of *Allothyrus* sp.; mtDNA analyses supported placement as the sister group to Ixodida plus Mesostigmata (41-86%) but mtAA analyses supported *Allothyrus* sp. as the sister group to Ixodida (68-80%). Common to all mtAA and mtDNA analyses was the lack of strong support for relationships within the Neotropical Ornithodorinae, other than monophyly of the genus *Antricola*. However, the mtAA13 tree (Figure 4 inset) has moderate support for *Or.* (*Subparmatus*) *marinkellei* as the sister group to the rest of Neotropical Ornithodorinae (60%), and for *Or.* (*Al.*) *fonsecai* plus *Or.* (*Al.*) *capensis* (74%).

The mtDNA10 tree conflicts with the nuclear rRNA in the placement of *Or.* (*Pavlovskyella*) *rostratus* as the sister group to *Or.* (*Pavlovskyella*) *brasiliensis* (100%), rather than sister to the other *Ornithodoros* s.s. (85% in nuclear rRNA analysis, Figure 3). Other than this conflict, the mtDNA10 tree is compatible with the nuclear rRNA tree for most comparable nodes with in the Ixodida, but the mtDNA10 tree has stronger support for the monophyly of Prostriata, the placement of *Nu. namaqua* and *Ot. megnini*, and for the relationships within *Ornithodoros* s.s. and *Argas*.

The results of the Approximately Unbiased (AU) test of confidence in tree selection (Table 5) show that there is uncertainty in the mtDNA data over the phylogenetic position of *Nu. namaqua*; alternate placements as the sister group to the Ixodidae (supported in our mtDNA10 analysis; Figure 4) and sister to the Ixodidae plus Argasidae (supported by the analyses of Mans et al. 2012) are not rejected by the AU test. However, placement of *Nu. namaqua* as the sister group to Argasidae is rejected. There is similar uncertainty in the placement of *Allothyrus* sp.; the unconstrained mtDNA10 tree has Allothyrus as the sister group to the Ixodida + Mesostigmata, but the alternate placement of *Allothyrus* sp. as the sister group to the Ixodida is not rejected. Within the Neotropical Ornithodorinae, we can reject two alternate clades: *Or.* (*Al.*) *capensis* plus *Or.* (*Al.*) *fonsecai* plus *No. amazoniensis*,

and *Or.* (*Al.*) *capensis* plus *Or.* (*Al.*) *fonsecai* plus *Or.* (*S.*) *marinkellei*. All the other alternate arrangements within this clade we tested were not rejected by the AU test (see Tables 6, S5). We also tested an alternate placement of *Ot. megnini* (sister to *Carios* sensu Klompen and Oliver, 1993) as well as constraining the clade of *Amblyomma* s.s. plus Rhipicephalinae clade (Amblyocephalus sensu Burger et al., 2013) to be paraphyletic. Both of these constraints were rejected by the AU test. The alternative arrangements for these taxa in the unconstrained analysis (monophyly of *Amblyomma* s.s. plus Rhipicephalinae and *Ot. megnini* sister to *Ornithodoros* s.s. plus Neotropical Ornithodorinae) were the only strongly supported clades when the AU test was broken down by clade (Table S5).

Discussion

Our phylogenetic analysis of mtDNA and nuclear rRNA strongly supports division of the Ornithodorinae into three lineages: *Ornithodoros* s.s. (including *Or. savignyi*, the type species of *Ornithodoros*), *Otobius*, and a clade of Neotropical species which we call the Neotropical Ornithodorinae. However, we were unable to unequivocally resolve the relationships within the Neotropical Ornithodorinae species, other than monophyly of the two species of *Antricola* in our analysis. The clustering of *Or.* (*Alectorobius*) *fonsecai* with *Or.* (*Alectorobius*) *capensis*, representing monophyly of the *Alectorobius* species in this analysis, was moderately supported in the mtAA data (Figure 4 inset). The monophyly of *Alectorobius* has been supported in previous studies of 16S rRNA (Labruna et al., 2008; Nava et al., 2009), and was not rejected in the AU test (Table 5). However, given the suggestion of paraphyly of this subgenus by Klompen and Oliver (1993) on the basis of morphology, we would require sampling of more species, particularly the type species, *Or.* (*Alectorobius*) *talaje*, to confirm or disprove monophyly of the subgenus *Alectorobius*.

Our analysis was inconclusive as to the status of *Argas* sp. SpringbokSA-QMS95171 with respect to *Argas africolumbae*. A study of the *Or. sonrai* group using 16S rRNA reported up to 16.4% divergence between specimens of *Or. sonrai*, though the divergence within the *Or. moubata* complex between *Or. moubata* and *Or. porcinus* was only 6.5% (Vial et al., 2006). The divergence between *Ar. africolumbae* and *Argas* sp. SpringbokSA-QMS95171 is intermediate between those two figures (8.5%) for the 16S rRNA marker region. Thus it is unclear based on our molecular analysis whether *Argas* sp. SpringbokSA-QMS95171 may be a new species of *Argas* or a genetically divergent type of *Ar. africolumbae*. Before making a taxonomic decision, *Argas* sp. SpringbokSA-QMS95171 should be morphologically and

genetically compared to other *Argas* species, especially those occurring in South Africa, not sampled in the present study.

The 16S rRNA marker region also revealed a very close phylogenetic relationship between our *Ar.* (*Persicargas*) *miniatus* from Brazil and a specimen of *Ar.* (*Persicargas*) *robertsi* from Australia. These two sequences only differed by 1 bp over the ca. 400 bp 16S rRNA marker region (99.7% identical), suggesting that these two sequences are from the same species. However, *Ar. miniatus* and *Ar. robertsi* are distinct species morphologically and geographically. *Ar. miniatus* is only known to parasitise domestic chickens in South America (Kohls et al., 1970; Guglielmone et al., 2003; Gonzalez-Acuna and Guglielmone, 2005; Nava et al., 2007), whereas *Ar. robertsi* parasitizes domestic chickens as well as numerous wild birds (some migratory) in Australia, Indonesia, Thailand, India, Sri Lanka and Taiwan (Hoogstraal et al., 1968; 1974). *Ar. miniatus* and *Ar. robertsi* are distinguishable from *Ar. persicus*, a globally distributed parasite of domestic chickens, both morphologically (Hoogstraal et al., 1968; 1974; Kohls et al., 1970) and in our 16S rRNA analysis (Figure S2). In our view the two most likely explanations for the near-identical 16S rRNA marker region of the two sequences are either: (i) *Ar. miniatus* and *Ar. robertsi* are regional variants of the same species, i.e. that *Ar. miniatus* is a South American morphological variant of the more widespread *Ar. robertsi*; or (ii) the 16S sequence of *Ar. robertsi* AY436768 on GenBank is actually *Ar. miniatus* and may have been misidentified as the only *Persicargas* species reported from Australia are *Ar. persicus* and *Ar. robertsi*. Further study of *Ar. robertsi* (Australia and Asia) and *Ar. miniatus* (South America) is required to discern between these two possibilities. We note that the GenBank *Ar. robertsi* were collected from cattle egrets (Petney et al., 2004) and *Ar. miniatus* has only been reported in the literature from domestic chickens (Kohls et al., 1970; Guglielmone et al., 2003). Additionally, only three *Persicargas* species are currently known from Australia: *Ar. persicus*, *Ar. robertsi* and an undescribed species, represented by *Argas* sp. J7224 AY436771 on Genbank (Petney et al., 2004).

Our analysis of nuclear rRNA had strong support for most of the same relationships within the Argasidae as the mtDNA analysis. However, the phylogenetic position of two species of Ornithodorinae, *Or. coriaceus* and *Ot. megnini*, were not strongly supported in our nuclear rRNA analysis. Only 18S rRNA is available for *Or. coriaceus*; the 18S rRNA gene evolves slowly, and there are few sites supporting monophyly of the *Ornithodoros* s.s. clade. It is possible that addition of 28S rRNA sequence from *Or. coriaceus* may be able to resolve its phylogenetic position, as 28S rRNA sequences increase support for several nodes in the

Metastriata. Thus, future phylogenetic studies of soft ticks should sequence 28S rRNA in addition to 18S rRNA, especially for phylogenetically controversial species. The lack of support for the phylogenetic position of *Ot. megnini,* for which both 18S rRNA and 28S rRNA are available, could be addressed by addition of the other species of *Otobius, Ot. lagophilus*, as well as further representatives of *Ornithodoros* s.s. and the Neotropical Ornithodorinae; however, the phylogenetic position of *Ot. megnini* is strongly supported in the mtDNA data.

The failure of our mtDNA phylogeny to resolve the relationships among species within the Neotropical Ornithodorinae clade may be due to one or both of two factors: (i) lack of data in the 6 kb region of the mt genome we sequenced in four species (Figure 1); and (ii) a rapid divergence at the base of the Neotropical Ornithodorinae clade. The 6 kb region of the mt genome we sequenced from four species in this study contains only two complete protein-coding genes (*nad1* and *nad2*) and two partial protein-coding genes (*cox1* and *cytb*) as well as the two rRNA genes. While these four mt protein-coding genes are sufficient to resolve the phylogenetic position of *Or. savignyi* within *Ornithodoros* s.s., they are not sufficient to resolve the placement of Neotropical Ornithodorinae species (Figure 4). In our mtDNA tree, and in the trees inferred from other markers, the Neotropical Ornithodorinae clade has long terminal and short internal branches, which suggests rapid divergence in this group. Our work on hard ticks suggested partial mt genomes were sufficient for placement of species of *Haemaphysalis* and *Rhipicephalus* (Burger et al., 2013; Burger et al., Submitted). However, these hard tick species were closely related to other species for which the entire mt genome was available, and the partial genomes were 10 kb long and contained a greater number of phylogenetically informative genes (*cox1, cox2, cox3, atp8, atp6, nad1, nad2, nad3*, and partial *cytb*). Thus, we suggest future studies aim to sequence entire mt genomes for soft ticks. We sequenced only the 6 kb region due to difficulty in amplifying the 9 kb region in four species (see Figure 1). Thus, we suggest the 9 kb region should be amplified in two sections with primers in the *nad5* or *cox3* genes; the entire mt genomes we sequenced of *An. mexicanus*, *Or. brasiliensis*, *Or. rostratus*, *Ot. megnini* and the 9 kb region of *Or. marinkellei* should make designing primers for these less-conserved genes easier.

Our analyses differ from the mt genome phylogeny of Mans et al. (2012) on the phylogenetic position of *Nu. namaqua,* and relationships within the Metastriata. Their analysis has *Nu. namaqua* as the sister group the Ixodidae plus Argasidae, strongly supported in Bayesian analysis of both 18S rRNA and mtAA. Our ML analysis of 18S rRNA and 28S rRNA cannot

resolve the phylogenetic placement of *Nu. namaqua*. However, most of our mt datasets have support for *Nu. namaqua* as the sister group to the Ixodidae (Figure 4 and Table 4). Only two of our 12 datasets, mtAA4 (63%) and mtDNA5 (71%) had support for the alternate placement of *Nu. namaqua* as the sister group to the Ixodidae plus Argasidae (Table 4). The AU test on our mtDNA10 dataset did not reject either placement of *Nu. namaqua*, though it did reject placement as the sister group to the Argasidae.

Our mtDNA and nuclear rRNA phylogenies have strong support for a clade of Neotropical Ornithodorinae, including representatives of the genera or subgenera *Antricola*, *Nothoaspis*, *Alectorobius*, and *Subparmatus*. Klompen and Oliver (1993) also suggested the species in our Neotropical Ornithodorinae clade were closely related, and placed them in the genus *Carios* s.l., along with three other non-Neotropical subgenera: *Carios*, *Chiropterargas* and *Reticulinasus*. However, the phylogenetic placement of these non-Neotropical subgenera is yet to be tested by molecular analysis. Thus, our Neotropical Ornithodorinae clade may be compatible with the genus *Carios* sensu Klompen and Oliver (1993), but to test this will require more data, particularly from the type species of *Carios*, *Argas* (*Carios*) *vespertilionis*. Our analysis of partial 12S and 16S rRNA sequences could not resolve the placement of *Argas* (*Carios*) *vespertilionis* with respect to the rest of the Ornithodorinae. 12S and 16S rRNA sequences are generally unsuited to resolving the higher level phylogenetic relationships among ticks; resolving the phylogenetic placement of *Ar. vespertilionis* will likely require the entire mt genome and nuclear rRNA sequences.

Two aspects of the cladistic scheme of soft tick classification (Table 1; Klompen and Oliver, 1993) have been supported by molecular analyses to date: the transfer of *Ornithodoros* (*Alveonasus*) to the Argasinae, and the transfer of *Carios* to the Ornithodorinae. *Or.* (*Alveonasus*) *lahorensis* is strongly supported as a species of *Argas* in analysis of nuclear rRNA sequences (Figure 3; Black et al., 1997; Dobson and Barker, 1999; Klompen et al., 2000; 2007). Our analysis of partial 12S and 16S rRNA sequences (Figures 2 and S2), had support for monophyly of the genus *Argas* excluding *Argas* (*Carios*) *vespertilionis* (the type species of *Carios*), and for the placement of *Ar.* (*Carios*) *vespertilionis* within Ornithodorinae. However, the placement of *Ar. vespertilionis* with respect to the rest of the Ornithodorinae cannot be resolved with 12S and 16S rRNA as these markers are most useful for resolving phylogenetic relationships among closely related species or within species.

Several other key aspects of the proposals of Klompen and Oliver (1993) have yet to be tested with molecular phylogenetics, such as the paraphyly of the subgenera *Alectorobius* and *Pavlovskyella*; as argasid subgenera are defined on the basis of morphology, it is also possible that other subgenera are paraphyletic. Additionally, the transfer of *Microargas transversus* to the genus *Ornithodoros*, and the transfer of *Or. sparnus* to the genus *Otobius* have yet to be tested in molecular analysis. Although previous phylogenetic analyses of morphology (Klompen and Oliver, 1993) and 18S rRNA (Black et al., 1997) had only weak support for the phylogenetic placement of the genus *Otobius*, our mtDNA analysis has strong support for the placement of *Ot. megnini* as the sister group to the rest of the Ornithodorinae in both bootstrap and AU tests (Figure 4 and Table S5).

The most controversial aspect of the proposals of Klompen and Oliver (1993) was the erection of the genus *Carios* without any subgenera, due to the paraphyly of the subgenus *Alectorobius*, though monophyly for at least four former genera or subgenera was supported: *Reticulinasus*, *Antricola*, *Nothoaspis* and *Carios* (Klompen and Oliver, 1993). We suggest that future taxonomic revisions of argasid genera should keep any monophyletic genera as subgenera; we consider that subgenera should be used only for monophyletic groups, but subgenera are not required for all species in a genus. Future studies should also focus on the type species of genera and subgenera, as suggested by other authors (Estrada-Peña et al., 2010; Guglielmone et al., 2010).

In conclusion, our analysis of mt genome and nuclear rRNA has strong support for a group of Neotropical Ornithodorinae, including species of the genera *Antricola* and *Nothoaspis*, and the subgenera *Alectorobius, Parantricola,* and *Subparmatus*, and demonstrates the potential for mt genome sequences to resolve the controversial phylogenetic relationships among soft tick lineages. The genera and subgenera in our Neotropical Ornithodorinae clade were placed in the genus *Carios* s.l. by Klompen and Oliver (1993). However, our analyses of mt 16S and 12S rRNA were unable to resolve the precise phylogenetic position of *Argas* (*Carios*) *vespertilionis* (the type species of *Carios*) though it was within the Ornithodorinae. Our analysis of 18S and 28S nuclear rRNA also had strong support for the three clades in our mtDNA analysis (*Argas, Ornithodoros* s.s., and Neotropical Ornithodorinae), but could not strongly resolve phylogenetic relationships within these clades, or the placement of two species (*Or. coriaceus* and *Ot. megnini*). In contrast to our analyses of nuclear rRNA, our analysis of mt genome sequences had strong support for most relationships within the Argasidae, including monophyly of *Argas* and *Ornithodoros* s.s., and the placement of *Ot.*

megnini as the sister group to the rest of the Ornithodorinae, but relationships within the Neotropical Ornithodorinae clade are not strongly supported, in part due to our use of partial mt genomes. However, to resolve relationships within the major soft tick lineages, especially the phylogenetic position of *Argas* (*Carios*) *vespertilionis* and the paraphyly of *Alectorobius*, and thus the validity of the genus *Carios* sensu Klompen and Oliver (1993), will likely require entire mt genome sequences.

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Figure Legends

Figure 1. The arrangement of genes in the mitochondrial genomes sequenced in this study. *Allothyrus* sp. (Holothyrida) and all soft ticks (Argasidae) sequenced to date have the ancestral arthropod gene arrangement, also shared by *Nuttalliella namaqua* (Nuttalliellidae), prostriate hard ticks (*Ixodes* spp.) and horseshoe crabs (Xiphosura). Genes on the outside of the circle are on the forward (majority or J) strand of the mt genome, genes on the inside of the circle are on the complementary (minority or N) strand of the mt genome. The inner circles show the two PCR products amplified to sequence the mt genomes in this study, labelled by approximate size. Diagram constructed with GenomeVX (Conant and Wolfe, 2008) and Inkscape. Abbreviations: *An, Antricola*; *Ar, Argas*; *Or, Ornithodoros*; *No, Nothoaspis*.

* These sequences are partial mitochondrial genomes, from one of the two PCR products.

Figure 2. ML tree inferred from 12S rRNA sequences (896 bp). Percentage bootstrap support is given at each node, and nodes with <25% bootstrap support are collapsed. Clades and branches within the Argasidae are colour-coded: *Argas*, green; *Otobius*, teal; *Ornithodoros* s.s., olive; Neotropical Ornithodorinae, *Ornithodoros turicata,* and *Carios vespertilionis*, red. GenBank accession numbers are after taxa labels. The Prostriata and Metastriata clades have been condensed, and outgroup taxa omitted, see the mtDNA tree (Figure 4) for the composition of these clades. Species sequenced in this study are in bold.

Figure 3. ML tree inferred from nuclear rRNA sequences (18S and 28S rRNA; 4,397 bp). Percentage bootstrap support is given at each node, and nodes with <50% bootstrap support are collapsed. Genera of hard ticks (Ixodidae) have been condensed, and the number of species is in parentheses. Clades and branches within the Argasidae are colour-coded: *Argas*, green; *Otobius*, teal; *Ornithodoros* s.s., olive; Neotropical Ornithodorinae; and *Ornithodoros coriaceus,* red. The labels for Prostriata and Holothyrida are dashed lines as monophyly for these clades is not supported. Species sequenced in this study are in bold, see Table S2 for GenBank accession numbers.

*Bootstrap support for these nodes is increased with addition of 28S rRNA sequences

Figure 4. ML tree inferred from the concatenated DNA sequences of the 10 mitochondrial genes (7,914 bp): *atp6, cox1, cox2, cox3, cytb, nad1, nad2, nad3, nad4L*, and *nad5* (see Methods, Table S3). Percentage bootstrap support is given at each node, and nodes with

<25% bootstrap support are collapsed. Clades and branches within the Argasidae are colour-coded: *Argas*, green; *Otobius*, teal; *Ornithodoros* s.s., olive; Neotropical Ornithodorinae, red. Species sequenced in this study are in bold, and GenBank accession numbers are after the taxa labels. Inset: Neotropical Ornithodorinae clade in the ML tree inferred from the putative amino acid sequences of all thirteen mt protein-coding genes (mtAAall).

Figure S1. The Tick-Box motif in soft ticks (Argasidae), *Nuttalliella namaqua* (Nuttalliellidae) and *Allothyrus* sp. (Holothyrida). These species have two Tick-Box motifs, which punctuate the 3' ends of *nad1* and 16S rRNA transcripts (Montagna et al., 2012). The *nad1* Tick-Box motif overlaps the 3' end of *nad1* by 2 bp and provides a complete stop codon after 3' polyadenylation of the *nad1* mRNA. The 16S rRNA Tick-Box motif is within the tRNA-Leu_1 (CUN) gene. Species sequenced in this study are in bold. Diagram constructed with Geneious (Drummond et al., 2012) and Inkscape.

* The *nad1* gene in *Allothyrus* sp. has a complete stop codon before the start of the Tick-Box motif.

Figure S2. ML tree inferred from 16S rRNA sequences (529 bp). Percentage bootstrap support is given at each node, and nodes with <25% bootstrap support are collapsed. Clades and branches within the Argasidae are colour-coded: Argasinae, green; Ornithodorinae, red; *Otobius*, teal. The GenBank accession numbers are after taxa labels. Species sequenced in this study are in bold.

*This specimen identified on GenBank as *Ornithodoros mimon*, is apparently *Or.* (*Alectorobius*) *hasei* (Labruna et al., 2011).

Table 1. Alternate classification schemes for the Argasidae adapted from Klompen and Oliver (1993). Genera recognised in each scheme are in bold, followed by the subgenera included in each genus. Subgenera in parentheses are included in the above genus or subgenus, but not recognised as valid by the authors of the scheme. The two subfamilies, Argasinae and Ornithodorinae, are separated by the dotted line.

Soviet scheme Filippova (1966) Pospelova-Shtrom (1969)	American scheme* Clifford et al. (1964) Hoogstraal (1985)	French scheme Camicas and Morel (1977) Camicas et al. (1998)	Cladistic scheme Klompen & Oliver (1993)
Argasinae	Argasinae		Argasinae
Argasini			
Argas	***Argas***	***Argas***	***Argas***
Argas	*Argas*	*Argas*	*Argas*
Persicargas	*Persicargas*	*Persicargas*	(incl. *Persicargas*)
		Carios	
Carios	*Carios*	*Carios*	
Chiropterargas	*Chiropterargas*	*Chiropterargas*	
		Ogadenus	
		Ogadenus	*Ogadenus*
Secretargas	*Secretargas*	*Secretargas*	*Secretargas*
		Proknekalia	*Proknekalia*
			Alveonasus
	Microargas		
Ornithodorinae	Ornithodorinae	Ornithodorinae	Ornithodorinae
Otobiini			
Otobius	***Otobius***	***Otobius***	***Otobius***
Alveonasus	***Ornithodoros* (s.l.)**	***Alveonasus***	
Alveonasus	*Alveonasus*	*Alveonasus*	
Proknekalia	*Proknekalia*		
Ogadenus			
Ornithodorini			
Ornithodoros		***Ornithodoros***	***Ornithodoros* (s.s.)**
Ornithodoros	*Ornithodoros*	*Ornithodoros*	(incl. *Ornithodoros*,
Ornamentum	*Ornamentum*	*Ornamentum*	*Ornamentum, Microargas,*
		Alectorobius	*Pavlovskyella, Theriodoros*)
Pavlovskyella	*Pavlovskyella*	*Theriodoros*	
Theriodoros	(incl. *Theriodoros*)	(incl. *Pavlovskyella*)	
			***Carios* (s.l.)**
Alectorobius	*Alectorobius*	*Alectorobius*	(incl. *Carios, Chiropterargas,*
Reticulinasus	*Reticulinasus*	*Reticulinasus*	*Alectorobius, Reticulinasus,*
Subparmatus	*Subparmatus*	*Subparmatus*	*Subparmatus, Antricola,*
Antricola	***Antricola***	***Antricola***	*Parantricola, Nothoaspis*)
Antricola	*Antricola*		
Parantricola	*Parantricola*	***Parantricola***	
	Nothoaspis	***Nothoaspis***	
		Microargas	

* Note that Guglielmone et al (2010) adopted but 'did not necessarily endorse' the American scheme of soft tick genera.

Table 2. Collection data for mitochondrial genome voucher specimens used in this study, along with collection data and accession numbers for nucleotide sequences deposited in GenBank.

Species	**Collection locality**	**Collector/Donor**	**Mt genome**	**18S and 28S rRNA**
Allothyrus sp. LamingtonNP-QMS95173	Lamington National Park, QLD, Australia	Matthew Shaw	KC769586	KC769613 KC769634
Argas (*Argas*) *lagenoplastis*	Gatton, QLD, Australia	Stephen C Barker	KC769587	-
Argas (*Persicargas*) *miniatus*	Campo Grande, MS, Brazil	Marcos Garcia & Renato Andreotti	KC769590	KC769610 KC769631
Argas sp. SpringbokSA-QMS95171	Springbok, South Africa	Stephen C Barker	KC769588	KC769611 KC769632
Antricola (*Parantricola*) *marginatus*	Calcehtok Caves, Yucatán, Mexico	Marcelo Labruna	KC769598	KC769606 KC769622
Antricola (*Antricola*) *mexicanus*	Yucatán, Mexico	Santiago Nava & Alberto Guglielmone	KC769591	KC769603 KC769626
Ornithodoros (*Alectorobius*) *fonsecai*	Bonito, MS, Brazil	Marcelo Labruna	KC769597	KC769608 KC769633
Ornithodoros (*Subparmatus*) *marinkellei*	Porto Velho, RO, Brazil	Marcelo Labruna	KC769594	KC769601 KC769625
Ornithodoros (*Pavlovskyella*) *brasiliensis*	São Francisco de Paula, RS, Brazil	Darci Barros-Battesti	KC769593	KC769604 KC769629
Ornithodoros (*Pavlovskyella*) *rostratus*	Corumbá, MS, Brazil	Marcelo Labruna	KC769592	KC769605 KC769628
Ornithodoros (*Ornithodoros*) *savignyi*	Aden, Yemen	Stephen C Barker	KC769596	KC769612
Otobius megnini	Antananarivo, Madagascar	Frédéric Stachurski	KC769589	KC769607 KC769630
Nothoaspis amazoniensis	Porto Velho, RO, Brazil	Marcelo Labruna	KC769595	KC769600 KC769624

Table 3. Mitochondrial genome sequences retrieved from GenBank.

Higher Taxon	Species	GenBank ID	Reference
Metastriata	*Amblyomma cajennense*	JX573118	Burger et al. (2013)
	Amblyomma elaphense	JN863729	Burger et al. (2012)
	Amblyomma fimbriatum	JN863730	Burger et al. (2012)
	Amblyomma sphenodonti	JN863731	Burger et al. (2012)
	Amblyomma triguttatum	AB113317	Shao et al. (2005a)
	Bothriocroton concolor	JN863727	Burger et al. (2012)
	Bothriocroton undatum	JN863728	Burger et al. (2012)
	Dermacentor nitens	KC503258	Burger et al. (Submitted)
	Haemaphysalis flava	AB075954	Black & Roehrdanz (1998)
	Haemaphysalis formosensis	JX573135	Burger et al. (2013)
	Haemaphysalis humerosa	JX573138	Burger et al. (2013)
	Haemaphysalis hystricis	JX573137	Burger et al. (2013)
	Haemaphysalis parva	JX573136	Burger et al. (2013)
	Rhipicephalus annulatus	KC503256	Burger et al. (Submitted)
	Rhipicephalus appendiculatus	KC503257	Burger et al. (Submitted)
	Rhipicephalus australis	KC503255	Burger et al. (Submitted)
	Rhipicephalus geigyi	KC503263	Burger et al. (Submitted)
	Rhipicephalus kohlsi	KC503262	Burger et al. (Submitted)
	Rhipicephalus microplus	KC503259	Burger et al. (Submitted)
	Rhipicephalus microplus	KC503260	Burger et al. (Submitted)
	Rhipicephalus microplus	KC503261	Burger et al. (Submitted)
	Rhipicephalus sanguineus	AF081829	Shao et al. (2004)
Prostriata	*Ixodes hexagonus*	AF081828	Black & Roehrdanz (1998)
	Ixodes persulcatus	AB073725	Shao et al. (2005b)
	Ixodes uriae	AB087746	Shao et al. (2005b)
	Ixodes holocyclus	AB075955	Shao et al. (2005b)
	Ixodes ricinus	JN248424	Montagna et al (2012)
Argasidae	*Argas africolumbae*	JQ665720	Mans et al. (2012)
	Ornithodoros moubata	AB073679	Shao et al. (2004)
	Ornithodoros porcinus	AB105451	Shao et al. (2005a)
	Carios capensis	AB075953	Shao et al. (2004)
Nuttalliellidae	*Nuttalliella namaqua*	JQ665719	Mans et al. (2012)
Mesostigmata	*Varroa destructor*	AY163547	Evans & Lopez (2002)
	Stylochyrus rarior	GQ927176	Swafford & Bond (2009)
Xiphosura	*Tachypleus tridentatus*	FJ860267	Unpublished data
	Limulus polyphemus	AF216203	Lavrov et al. (2000)

Table 4. Bootstrap support values for nodes within Argasidae and other controversial nodes in the different mitochondrial gene datasets. Nodes are labelled as per Figure 4; nodes with <50% bootstrap support are indicated with --.

	Node	mtAA 4	mtAA 10	mtAA 13	mtAA 5	mtDNA 3	mtDNA 10	mtDNA 13	mtDNA 5
A	*Allothyrus* sp.*	-- (80)	-- (76)	-- (68)	-- (80)	77	**86**	83	--
B	*Nuttalliella namaqua**	-- (63)	66	74	63	93	**95**	72	-- (71)
C	*Ixodes*	94	86	94	46	**100**	**100**	94	72
D	*Amblyomma* s.s. + Rhipicephalinae	62	95	95	78	50	**100**	97	91
E	Argasidae	99	**100**	**100**	99	**100**	**100**	**100**	**100**
F	Argasinae	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
G	*Ar. lagenoplastis* + *Ar. miniatus**	--	**92**	**94**	**92**	-- (50)	69	68	73
H	*Argas* sp. + *Ar. africolumbae*	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
I	Ornithodorinae	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
J	*Otobius*	94	89	90	75	89	**96**	94	72
K	*Ornithodoros* s.s.*	-- (61)	83	63	82	58	**93**	67	59
L	*Or. rostratus* + *Or. brasiliensis*	83	96	99	71	98	**100**	68	87
M	Neotropical Ornithodorinae	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
N	*Antricola*	**100**	**100**	**100**	**100**	96	**100**	**100**	**100**
O	*Or. fonsecai* + *Or. capensis*	**74**	71	**74**	53	--	--	--	--

* Some datasets had stronger bootstrap support for the alternate placements taxa compared to the mtDNA10 tree (Figure 4). These alternate placements were: *Allothyrus* sp. as the sister group to Ixodida, *Nuttalliella namaqua* as the sister group to Ixodidae + Argasidae, *Ar. lagenoplastis* sister to all other *Argas*, and *Or. rostratus* + *Or. brasiliensis* sister to Neotropical Ornithodorinae. Bootstrap support values for this alternate placement are in parenthesis.

Table 5. Results of the Approximately Unbiased (AU) test of confidence in tree selection on 13 topological constraints.

	Topology constraint*	-lnL	p-value**
1	unconstrained (Figure 4)	183,145	0.918
2	*Or.* (*S.*) *marinkellei* + *No. amazoniensis*	183,146	0.697
3	*Or.* (*Al.*) *capensis* + *Or.* (*Al.*) *fonsecai* (*Alectorobius* monophyly)	183,148	0.513
4	*Or.* (*S.*) *marinkellei* + *Or.* (*Al.*) *fonsecai*	183,151	0.175
5	*Allothyrus* sp. sister to Ixodida	183,153	0.239
6	*Haemaphysalis* + *Am. sphenodonti* + *Bothriocroton* (Haematobothrion sensu Burger et al. 2013)	183,156	0.203
7	*Nuttalliella namaqua* sister to Ixodidae + Argasidae	183,156	0.114
8	***Nuttalliella namaqua* sister to Argasidae**	**183,158**	**0.035**
9	*Or.* (*S.*) *marinkellei* + *Or.* (*Al.*) *capensis*	183,161	0.059
10	***Otobius megnini* sister to Neotropical Ornithodorinae**	183,174	**0.002**
11	**Rhipicephalinae + *Haemaphysalis* + *Am. sphenodonti* + *Bothriocroton***	**183,207**	**< 0.001**
12	***Or.* (*Al.*) *capensis* + *Or.* (*Al.*) *fonsecai* + *No. amazoniensis***	**183,238**	**< 0.001**
13	***Or.* (*Al.*) *capensis* + *Or.* (*Al.*) *fonsecai* + *Or.* (*S.*) *marinkellei***	**183,379**	**< 0.001**

* Full topological constraints are available in Newick tree format on Dryad (doi:10.5061/dryad.XXXXX).

** Topological constraints rejected by the AU test ($p < 0.05$) are in bold type.

Figure 1

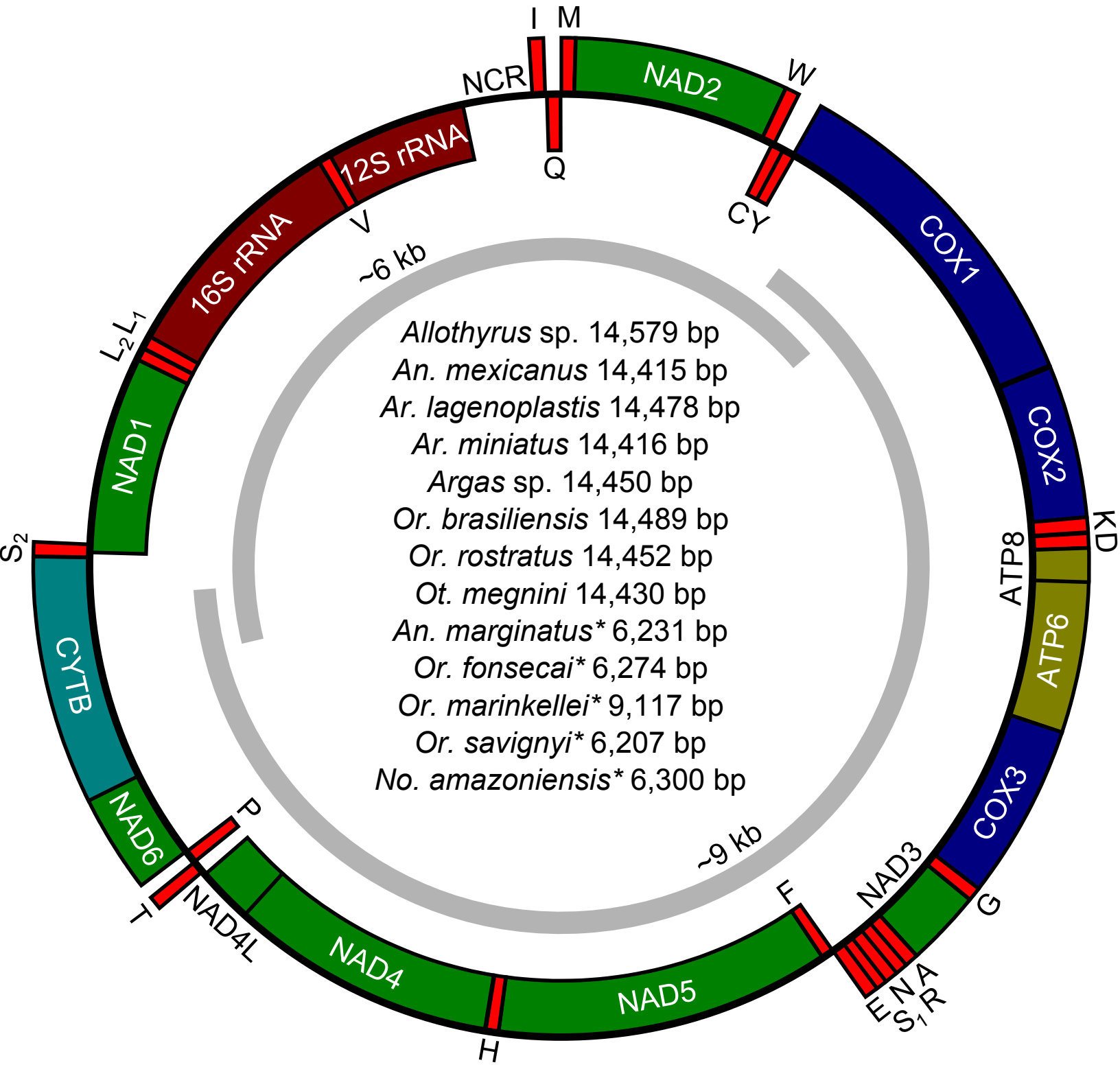

I
M
NCR
NAD2
W
Q
12S rRNA
CY
V
16S rRNA
~6 kb
COX1
L2 L1
COX2
NAD1
KD
S2
ATP8
Allothyrus sp. 14,579 bp
An. mexicanus 14,415 bp
Ar. lagenoplastis 14,478 bp
Ar. miniatus 14,416 bp
Argas sp. 14,450 bp
Or. brasiliensis 14,489 bp
Or. rostratus 14,452 bp
Ot. megnini 14,430 bp
An. marginatus* 6,231 bp
Or. fonsecai* 6,274 bp
Or. marinkellei* 9,117 bp
Or. savignyi* 6,207 bp
No. amazoniensis* 6,300 bp
ATP6
CYTB
COX3
NAD6
P
~9 kb
NAD3
T
NAD4L
F
G
NAD4
NAD5
E
N
A
S1
R
H

Figure 2

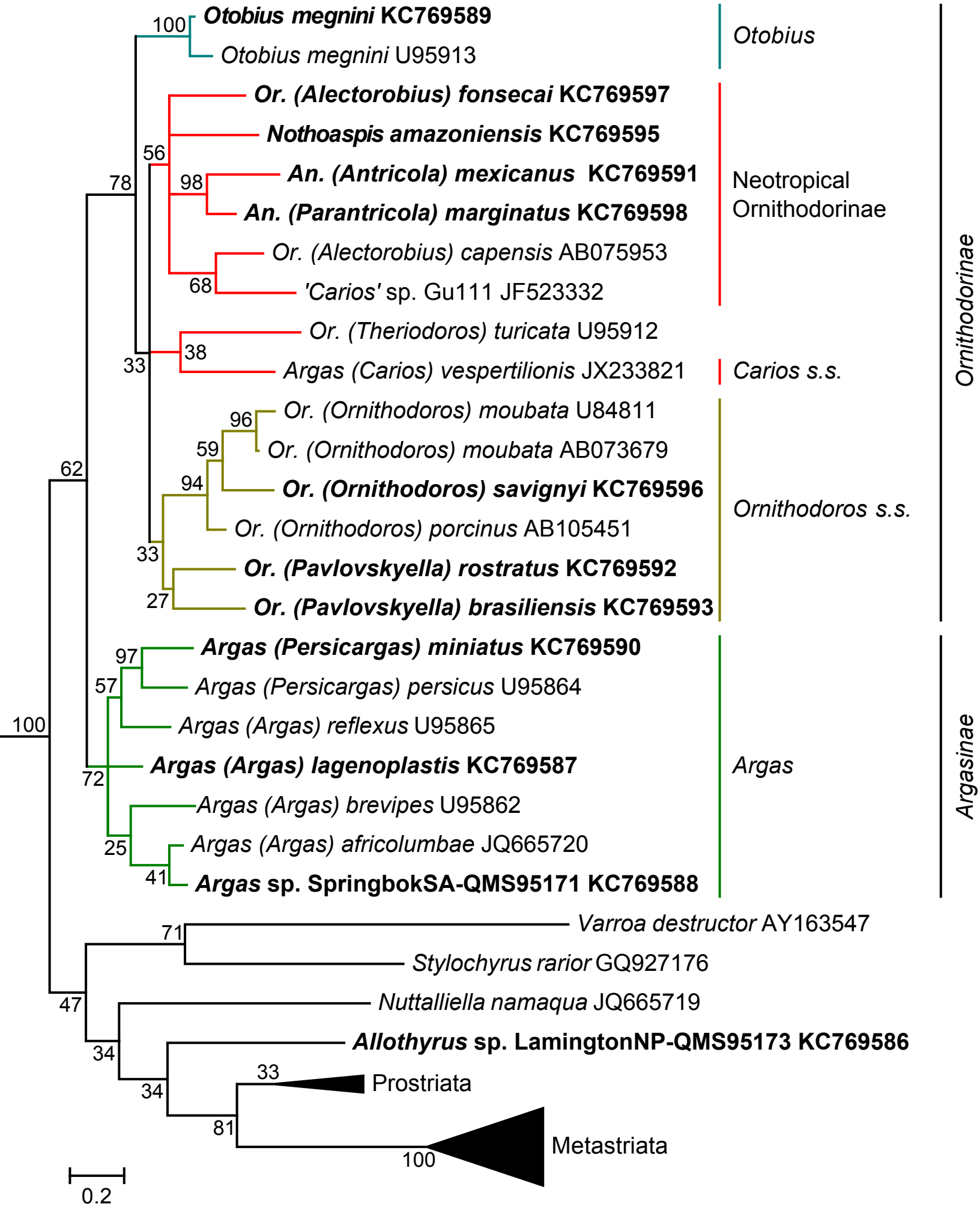

Otobius megnini KC769589
Otobius megnini U95913
Or. (Alectorobius) fonsecai KC769597
Nothoaspis amazoniensis KC769595
An. (Antricola) mexicanus KC769591
An. (Parantricola) marginatus KC769598
Or. (Alectorobius) capensis AB075953
'Carios' sp. Gu111 JF523332
Or. (Theriodoros) turicata U95912
Argas (Carios) vespertilionis JX233821
Or. (Ornithodoros) moubata U84811
Or. (Ornithodoros) moubata AB073679
Or. (Ornithodoros) savignyi KC769596
Or. (Ornithodoros) porcinus AB105451
Or. (Pavlovskyella) rostratus KC769592
Or. (Pavlovskyella) brasiliensis KC769593
Argas (Persicargas) miniatus KC769590
Argas (Persicargas) persicus U95864
Argas (Argas) reflexus U95865
Argas (Argas) lagenoplastis KC769587
Argas (Argas) brevipes U95862
Argas (Argas) africolumbae JQ665720
Argas sp. SpringbokSA-QMS95171 KC769588
Varroa destructor AY163547
Stylochyrus rarior GQ927176
Nuttalliella namaqua JQ665719
Allothyrus sp. LamingtonNP-QMS95173 KC769586
Prostriata
Metastriata
Otobius
Neotropical Ornithodorinae
Carios s.s.
Ornithodoros s.s.
Argas
Ornithodorinae
Argasinae
100
56
78
98
68
33
38
96
59
62
94
33
27
100
97
57
72
25
41
71
47
34
34
33
81
100
0.2

Figure 3

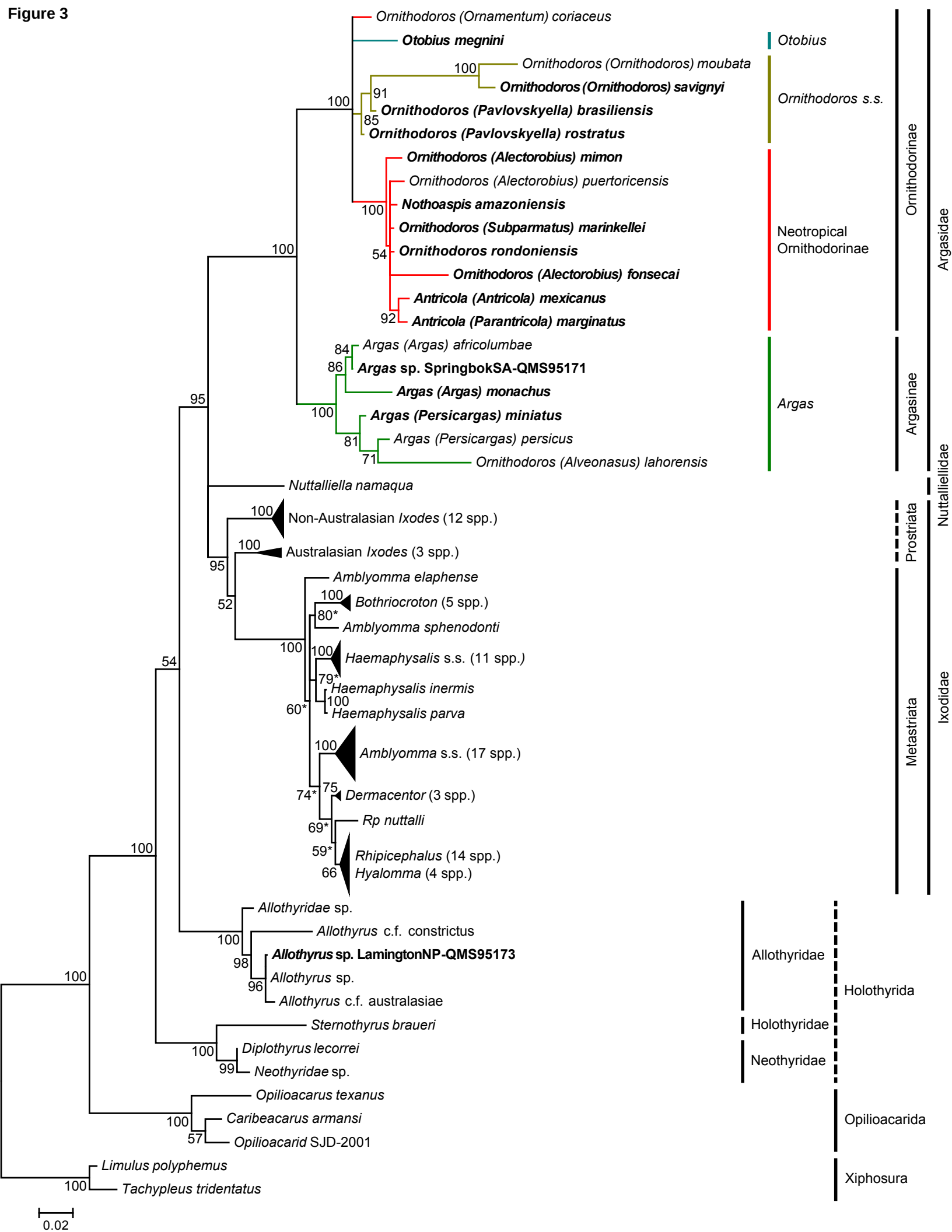
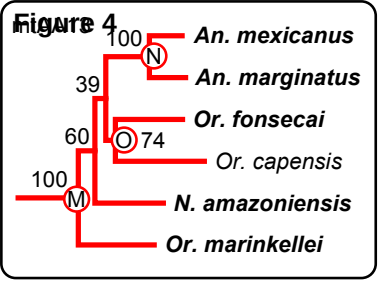

Ornithodoros (Ornamentum) coriaceus
Otobius megnini
Ornithodoros (Ornithodoros) moubata
Ornithodoros (Ornithodoros) savignyi
Ornithodoros (Pavlovskyella) brasiliensis
Ornithodoros (Pavlovskyella) rostratus
Ornithodoros (Alectorobius) mimon
Ornithodoros (Alectorobius) puertoricensis
Nothoaspis amazoniensis
Ornithodoros (Subparmatus) marinkellei
Ornithodoros rondoniensis
Ornithodoros (Alectorobius) fonsecai
Antricola (Antricola) mexicanus
Antricola (Parantricola) marginatus
Argas (Argas) africolumbae
Argas sp. SpringbokSA-QMS95171
Argas (Argas) monachus
Argas (Persicargas) miniatus
Argas (Persicargas) persicus
Ornithodoros (Alveonasus) lahorensis
Nuttalliella namaqua
Non-Australasian Ixodes (12 spp.)
Australasian Ixodes (3 spp.)
Amblyomma elaphense
Bothriocroton (5 spp.)
Amblyomma sphenodonti
Haemaphysalis s.s. (11 spp.)
Haemaphysalis inermis
Haemaphysalis parva
Amblyomma s.s. (17 spp.)
Dermacentor (3 spp.)
Rp nuttalli
Rhipicephalus (14 spp.)
Hyalomma (4 spp.)
Allothyridae sp.
Allothyrus c.f. constrictus
Allothyrus sp. LamingtonNP-QMS95173
Allothyrus sp.
Allothyrus c.f. australasiae
Sternothyrus braueri
Diplothyrus lecorrei
Neothyridae sp.
Opilioacarus texanus
Caribeacarus armansi
Opilioacarid SJD-2001
Limulus polyphemus
Tachypleus tridentatus
0.02
Otobius
Ornithodoros s.s.
Neotropical Ornithodorinae
Argas
Ornithodorinae
Argasinae
Argasidae
Nuttalliellidae
Prostriata
Metastriata
Ixodidae
Allothyridae
Holothyrida
Holothyridae
Neothyridae
Opilioacarida
Xiphosura

Figure 4


100
N
An. mexicanus
An. marginatus
39
Or. fonsecai
60
O
74
Or. capensis
100
M
N. amazoniensis
Or. marinkellei

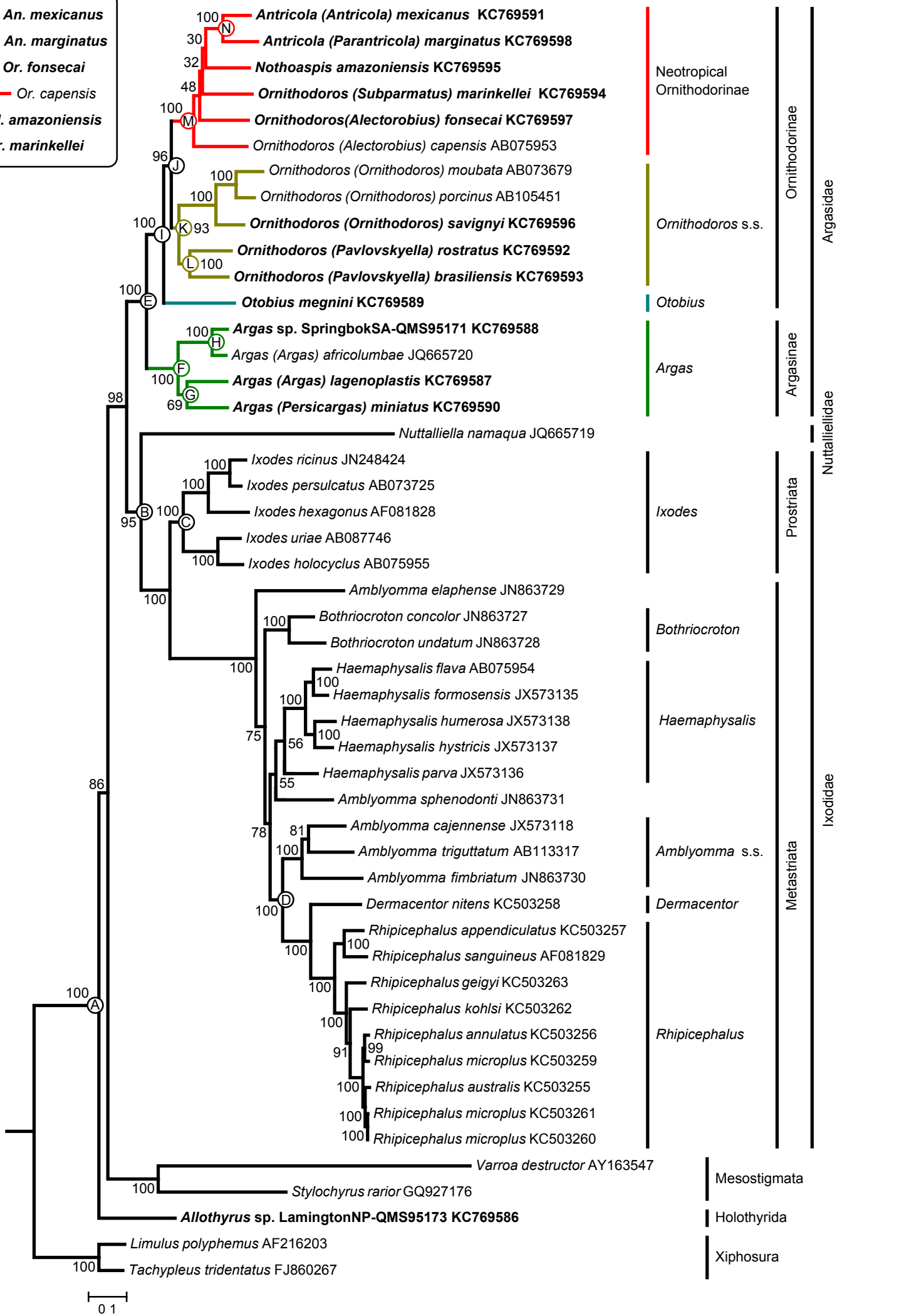

Antricola (Antricola) mexicanus KC769591
Antricola (Parantricola) marginatus KC769598
Nothoaspis amazoniensis KC769595
Ornithodoros (Subparmatus) marinkellei KC769594
Ornithodoros(Alectorobius) fonsecai KC769597
Ornithodoros (Alectorobius) capensis AB075953
Ornithodoros (Ornithodoros) moubata AB073679
Ornithodoros (Ornithodoros) porcinus AB105451
Ornithodoros (Ornithodoros) savignyi KC769596
Ornithodoros (Pavlovskyella) rostratus KC769592
Ornithodoros (Pavlovskyella) brasiliensis KC769593
Otobius megnini KC769589
Argas sp. SpringbokSA-QMS95171 KC769588
Argas (Argas) africolumbae JQ665720
Argas (Argas) lagenoplastis KC769587
Argas (Persicargas) miniatus KC769590
Nuttalliella namaqua JQ665719
Ixodes ricinus JN248424
Ixodes persulcatus AB073725
Ixodes hexagonus AF081828
Ixodes uriae AB087746
Ixodes holocyclus AB075955
Amblyomma elaphense JN863729
Bothriocroton concolor JN863727
Bothriocroton undatum JN863728
Haemaphysalis flava AB075954
Haemaphysalis formosensis JX573135
Haemaphysalis humerosa JX573138
Haemaphysalis hystricis JX573137
Haemaphysalis parva JX573136
Amblyomma sphenodonti JN863731
Amblyomma cajennense JX573118
Amblyomma triguttatum AB113317
Amblyomma fimbriatum JN863730
Dermacentor nitens KC503258
Rhipicephalus appendiculatus KC503257
Rhipicephalus sanguineus AF081829
Rhipicephalus geigyi KC503263
Rhipicephalus kohlsi KC503262
Rhipicephalus annulatus KC503256
Rhipicephalus microplus KC503259
Rhipicephalus australis KC503255
Rhipicephalus microplus KC503261
Rhipicephalus microplus KC503260
Varroa destructor AY163547
Stylochyrus rarior GQ927176
Allothyrus sp. LamingtonNP-QMS95173 KC769586
Limulus polyphemus AF216203
Tachypleus tridentatus FJ860267
Neotropical Ornithodorinae
Ornithodoros s.s.
Otobius
Argas
Ixodes
Bothriocroton
Haemaphysalis
Amblyomma s.s.
Dermacentor
Rhipicephalus
Mesostigmata
Holothyrida
Xiphosura
Ornithodorinae
Argasinae
Argasidae
Nuttalliellidae
Prostriata
Metastriata
Ixodidae
0 1

Figure S1

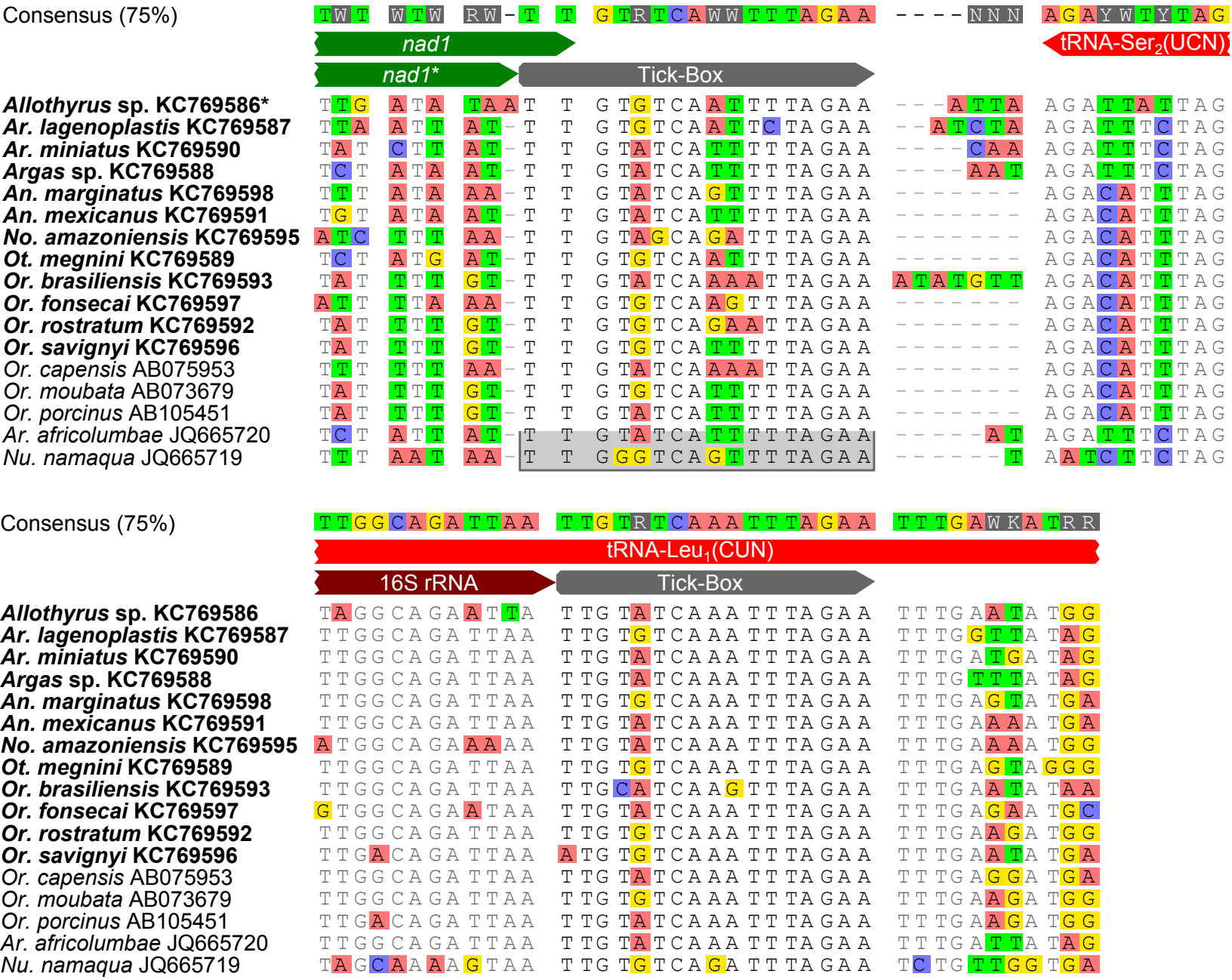
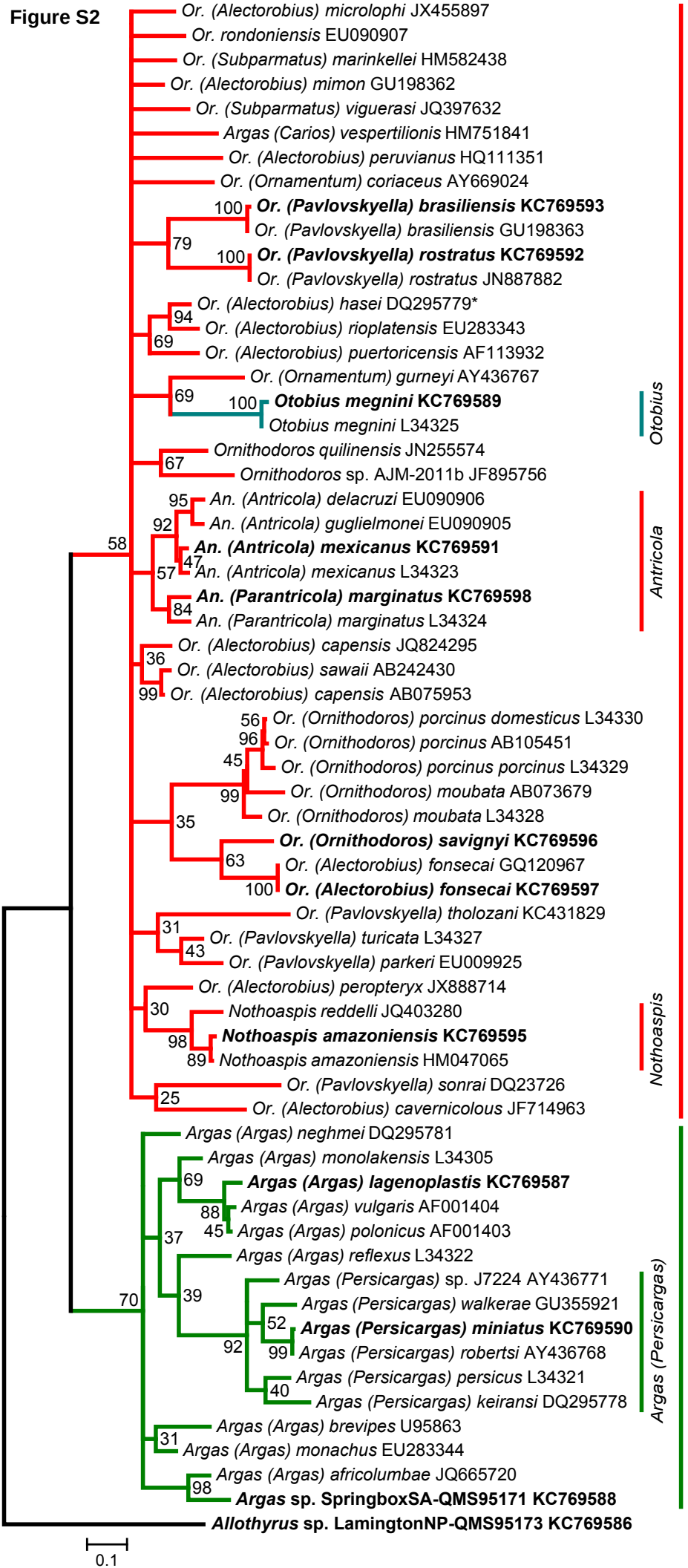

Consensus (75%)
nad1
nad1*
Tick-Box
tRNA-Ser2(UCN)
Allothyrus sp. KC769586*
Ar. lagenoplastis KC769587
Ar. miniatus KC769590
Argas sp. KC769588
An. marginatus KC769598
An. mexicanus KC769591
No. amazoniensis KC769595
Ot. megnini KC769589
Or. brasiliensis KC769593
Or. fonsecai KC769597
Or. rostratum KC769592
Or. savignyi KC769596
Or. capensis AB075953
Or. moubata AB073679
Or. porcinus AB105451
Ar. africolumbae JQ665720
Nu. namaqua JQ665719
Consensus (75%)
tRNA-Leu1(CUN)
16S rRNA
Tick-Box
Allothyrus sp. KC769586
Ar. lagenoplastis KC769587
Ar. miniatus KC769590
Argas sp. KC769588
An. marginatus KC769598
An. mexicanus KC769591
No. amazoniensis KC769595
Ot. megnini KC769589
Or. brasiliensis KC769593
Or. fonsecai KC769597
Or. rostratum KC769592
Or. savignyi KC769596
Or. capensis AB075953
Or. moubata AB073679
Or. porcinus AB105451
Ar. africolumbae JQ665720
Nu. namaqua JQ665719

Figure S2


Or. (Alectorobius) microlophi JX455897
Or. rondoniensis EU090907
Or. (Subparmatus) marinkellei HM582438
Or. (Alectorobius) mimon GU198362
Or. (Subparmatus) viguerasi JQ397632
Argas (Carios) vespertilionis HM751841
Or. (Alectorobius) peruvianus HQ111351
Or. (Ornamentum) coriaceus AY669024
100
Or. (Pavlovskyella) brasiliensis KC769593
Or. (Pavlovskyella) brasiliensis GU198363
79
100
Or. (Pavlovskyella) rostratus KC769592
Or. (Pavlovskyella) rostratus JN887882
Or. (Alectorobius) hasei DQ295779*
94
Or. (Alectorobius) rioplatensis EU283343
69
Or. (Alectorobius) puertoricensis AF113932
Or. (Ornamentum) gurneyi AY436767
69
100
Otobius megnini KC769589
Otobius megnini L34325
Otobius
Ornithodoros quilinensis JN255574
67
Ornithodoros sp. AJM-2011b JF895756
95
An. (Antricola) delacruzi EU090906
92
An. (Antricola) guglielmonei EU090905
58
An. (Antricola) mexicanus KC769591
47
57
An. (Antricola) mexicanus L34323
84
An. (Parantricola) marginatus KC769598
An. (Parantricola) marginatus L34324
Antricola
Ornithodorinae
Or. (Alectorobius) capensis JQ824295
36
Or. (Alectorobius) sawaii AB242430
99
Or. (Alectorobius) capensis AB075953
56
Or. (Ornithodoros) porcinus domesticus L34330
96
Or. (Ornithodoros) porcinus AB105451
45
Or. (Ornithodoros) porcinus porcinus L34329
99
Or. (Ornithodoros) moubata AB073679
35
Or. (Ornithodoros) moubata L34328
Or. (Ornithodoros) savignyi KC769596
63
Or. (Alectorobius) fonsecai GQ120967
100
Or. (Alectorobius) fonsecai KC769597
Or. (Pavlovskyella) tholozani KC431829
31
Or. (Pavlovskyella) turicata L34327
43
Or. (Pavlovskyella) parkeri EU009925
Or. (Alectorobius) peropteryx JX888714
30
Nothoaspis reddelli JQ403280
98
Nothoaspis amazoniensis KC769595
89
Nothoaspis amazoniensis HM047065
Nothoaspis
Or. (Pavlovskyella) sonrai DQ23726
25
Or. (Alectorobius) cavernicolous JF714963
Argas (Argas) neghmei DQ295781
Argas (Argas) monolakensis L34305
69
Argas (Argas) lagenoplastis KC769587
88
Argas (Argas) vulgaris AF001404
37
45
Argas (Argas) polonicus AF001403
Argas (Argas) reflexus L34322
39
Argas (Persicargas) sp. J7224 AY436771
70
Argas (Persicargas) walkerae GU355921
52
Argas (Persicargas) miniatus KC769590
92
99
Argas (Persicargas) robertsi AY436768
Argas (Persicargas) persicus L34321
40
Argas (Persicargas) keiransi DQ295778
Argas (Persicargas)
Argas (Argas) brevipes U95863
31
Argas (Argas) monachus EU283344
Argas (Argas) africolumbae JQ665720
98
Argas sp. SpringboxSA-QMS95171 KC769588
Argasinae
Allothyrus sp. LamingtonNP-QMS95173 KC769586
0.1

Supplementary Tables

Table S1. Mitochondrial gene and nuclear rRNA primers used in this study for PCR and sequencing.

Primer	Sequence 5'-3'	Target	Reference
C1-J-1718	GGAGGATTTGGAAATTGATTAGTTCC	*cox1*	Simon et al. (1994)
C1-N-2329	ACTGTAAATATATGATGAGCTCA	*cox1*	Simon et al. (1994)
SR-J-14199	TACTATGTTACGACTTAT	12S rRNA	Kambhampati and Smith (1995)
SR-N-14594	AAACTAGGATTAGATACCC	12S rRNA	Kambhampati and Smith (1995)
CB-N-11328'	GCATAAGCAAATAAAAAATATCATTC	*cytb*	Adapted from Simon et al. (1994)
CB-J-10933	TATGTACTACCATGAGGACAAATATC	*cytb*	Simon et al. (1994)
AlfonCOBF	TTTACTTTCTGCAATTCCATACATTGG	*cytb*	this study; *Ornithodoros fonsecai*
AlfonCOBR	CTTTGGTTGAGAAATATGGATGAAATGG	*cytb*	this study; *Ornithodoros fonsecai*
AlfonCOX1F	CCATTAGCTTCTAATATTTCTCATTCTGG	*cox1*	this study; *Ornithodoros fonsecai*
AlfonCOX1R	GATTAAAATATAGACTTCTGGATGACC	*cox1*	this study; *Ornithodoros fonsecai*
AnmexCOBF	ATCCCATACATCGGAACAAATATTACAC	*cytb*	this study; *Antricola mexicanus*
AnmexCOBR	GGTTAATCCTATAGGATTAGATGATCCAG	*cytb*	this study; *Antricola mexicanus*
AnmexCOX1F	TCCATTAGCTTCAAACATTTCTCACTCAG	*cox1*	this study; *Antricola mexicanus*
AnmexCOX1R	ATGTGAAATTATTCCAAATCCAGGTAGG	*cox1*	this study; *Antricola mexicanus*
ArgCOB1F	TAATATAAGATTCTGACTCCTACCC	*cytb*	this study; *Argas* sp. SpringbokSA-QMS95171
ArgCOB1R	AATATGGCATAGATTATTCCCAAGG	*cytb*	this study; *Argas* sp. SpringbokSA-QMS95171
ArgCOX1F	TAATATAAGATTCTGACTCCTACCC	*cox1*	this study; *Argas* sp. SpringbokSA-QMS95171
ArgCOX1R	AATATGGCATAGATTATTCCCAAGG	*cox1*	this study; *Argas* sp. SpringbokSA-QMS95171
ArminCOBF	TTTCTGTTGATAATCCTACTTTAACACG	*cytb*	this study; *Argas miniatus*
ArminCOBR	GGAGGAGTTACTAGTGGATTTGCTTC	*cytb*	this study; *Argas miniatus*
ArminCOX1F	TCATTAATTGAAAGAGGAGCTGGAACTG	*cox1*	this study; *Argas miniatus*
ArminCOX1R	TTACATGGGAAATAATTCCAAACCCTG	*cox1*	this study; *Argas miniatus*
NoamaCOBF	GACAATCCTACCTTAACACGATTCTTCAC	*cytb*	this study; *Nothoaspis amazoniensis*
NoamaCOBR	GAGAGGGTTTGAAGAACCTGTTTCGTG	*cytb*	this study; *Nothoaspis amazoniensis*
NoamaCOX1F	AGTCTCCATCTAGCCGGAATCTCATC	*cox1*	this study; *Nothoaspis amazoniensis*
NoamaCOX1R	CGATCTGTTAATAGTATGGTGATTGCTCC	*cox1*	this study; *Nothoaspis amazoniensis*
OrmarCOBF	TTGCTCTCCACTTCTTACTTCCATTCATCC	*cytb*	this study; *Ornithodoros marinkellei*
OrmarCOBR	CTGTTGGTTAGCCCTAATGGATTAGAG	*cytb*	this study; *Ornithodoros marinkellei*
OrmarCOX1F	CACACTCAGGAATATCCGTAGATTTAGC	*cox1*	this study; *Ornithodoros marinkellei*
OrmarCOX1R	CTTTGGACGTATGTTGAGGATGGTTGTG	*cox1*	this study; *Ornithodoros marinkellei*
OrsavCOBF	AAACCATTACCCAATGAATTTGAGGAGG	*cytb*	this study; *Ornithodoros savignyi*
OrsavCOBR	GTCGATGTTGTTGGAGATTCCTAGTG	*cytb*	this study; *Ornithodoros savignyi*
OrsavCOX1F	ATCTCCCATTCAGGCATATCTGTCG	*cox1*	this study; *Ornithodoros savignyi*
OrsavCOX1R	AATAAGGGAATTCGTTCTAGGGTTATGC	*cox1*	this study; *Ornithodoros savignyi*
OtmegCOBF	ATTCTCAGTTGATAATCCAACTCTAACTCG	*cytb*	this study; *Otobius megnini*
OtmegCOBR	GATATGGATAAATAAAGATGACTGGAAGG	*cytb*	this study; *Otobius megnini*
OtmegCOX1F	TTCCAACATTTCCCATTCTGGAATATCTG	*cox1*	this study; *Otobius megnini*
OtmegCOX1R	TAATTGCTCCAGCTAATACTGGCAGAG	*cox1*	this study; *Otobius megnini*
PamarCOBF	AACCTGCTTTCAGCAATTCCTTACATTGG	*cytb*	this study; *Antricola marginatus*
PamarCOBR	GGGAGTAATTAATGGATTGGCTATTAGGAAG	*cytb*	this study; *Antricola marginatus*
PamarCOX1F	GACTTCTACCTCCATCACTTCTACTTCTC	*cox1*	this study; *Antricola marginatus*
PamarCOX1R	CCTGGAAGAATCAGAATGTATACTTCTGG	*cox1*	this study; *Antricola marginatus*
Arlagatp6F	CAATTTTCGATCCAGCAACCTCTGCAAAT	*atp6*	this study; *Argas lagenoplastis*
Arlagatp6F2	CCCAATAATCCTTCCTGTCCTAATTACTCT	*atp6*	this study; *Argas lagenoplastis*
ArlagCO1F	CTCCCATTCAGGGATATCAGTTGACTTAGC	*cox1*	this study; *Argas lagenoplastis*
ArlagCO1R	GAAAAATGATGTATTGAAGTTTCGGTCTGT	*cox1*	this study; *Argas lagenoplastis*
ArlagCobR	TGTGGCTCCTCAAAATGATATTTGACCTC	*cytb*	this study; *Argas lagenoplastis*
ArlagcobR2	CGAAATATTAGAGGGAGCTGGAAGGTTGAT	*cytb*	this study; *Argas lagenoplastis*
ArlagCox1R	GGACATCCCGATTATTATTGATCATGCCCC	*cox1*	this study; *Argas lagenoplastis*
ArlagCox2F	TGTTGAATTTGACTCCTTTATGCTCCCCCT	*cox2*	this study; *Argas lagenoplastis*
Arlagcox3F	GCATGAGAATACTTCCAAGCCTCATTTTCC	*cox3*	this study; *Argas lagenoplastis*
Arlagnad2F	AAAAATTCTAGCCTTTTCTTCCATTACTC	*nad2*	this study; *Argas lagenoplastis*
Arlagnad2R	TGAGATTTTTATCAAAGGTTCATTTAAAA	*nad2*	this study; *Argas lagenoplastis*
Arlagnad4F	CCCCCTAAAATTGAGATTCTCAGCATAACT	*nad4*	this study; *Argas lagenoplastis*
ArlagNad4R	TAGGATGAGATGGATTAGGCTTAACCTCT	*nad4*	this study; *Argas lagenoplastis*
Arlagnad5F	TATTTTAACATCATAATTTAATAAAACAA	*nad5*	this study; *Argas lagenoplastis*

Arlagnad5R	ATAATATTAGTTCTAGCTATTGGGCTACCT	*nad5*	this study; *Argas lagenoplastis*
Arlagnad5R2	TATTAATTTTAGTGTTTTCTATATGAATT	*nad5*	this study; *Argas lagenoplastis*
ArlagND1F	TAAATTCAAACAAAAAACTCCCAAACCACC	*cox1*	this study; *Argas lagenoplastis*
ArlagRRNLR	GGATTAAGATGCTTTTATGATGAAGAGGTC	*nad1*	this study; *Argas lagenoplastis*
ArlagrThrR	AGAGAGTCCCAAAGATGGTTTACAAAACCA	tRNA-Thr	this study; *Argas lagenoplastis*
Arlagseq12SF	TTATAATAATAGGGTATCTAATCCTAGTCT	12S rRNA	this study; *Argas lagenoplastis*
ArlagseqCOBR	AATGGTGGTTCGACTGGGCATGAGCCAATG	*cytb*	this study; *Argas lagenoplastis*
ArlagseqCOX1F	ATTTCCATTACGTACTATCAATAGGTGCAG	*cox1*	this study; *Argas lagenoplastis*
ArlagseqCOX1R	GGGGCATGATCAATAATAATCGGGATGTCC	*cox1*	this study; *Argas lagenoplastis*
ArlagtGlnF	CAAAATTTAACGTGCCATTAACACCAAAG	tRNA-Gln	this study; *Argas lagenoplastis*
ArlagtGlnR	CTTTGGTGTTAATGGCACGTTAAATTTTG	tRNA-Gln	this study; *Argas lagenoplastis*
ArlagtHisF	TCTCATCTTTGAACCCACAATTCAACATT	tRNA-His	this study; *Argas lagenoplastis*
ArlagtTrpR	ATCTGGTTAGATATATTATGCTTTGAAGGC	tRNA-Trp	this study; *Argas lagenoplastis*

Table S2. 18S rRNA and 28S rRNA sequences retrieved from GenBank.

Higher Taxon	Species	18S	28S
Metastriata	*Amblyomma americanum*	AF291874	AF291874
	Amblyomma auricularium	FJ464426	
	Amblyomma boeroi	FJ464420	
	Amblyomma dubitatum	FJ464425	
	Amblyomma cajennense	JX573119*	JX573127*
	Amblyomma elaphense	JN863721	JN863722
	Amblyomma glauerti	AF115372	AF120305
	Amblyomma maculatum	L76344	AF120306
	Amblyomma neumanni	FJ464424	
	Amblyomma parvitarsum	FJ464423	
	Amblyomma parvum	FJ464422	
	Amblyomma pseudoparvum	FJ464421	
	Amblyomma sphenodonti	DQ507238	JN863726
	Amblyomma triguttatum	AF018641	
	Amblyomma tuberculatum	L76345	AF120307
	Amblyomma variegatum	L76346	AF120308
	Amblyomma vikirri	AF018642	
	Amblyomma fimbriatum	AF018644	JN863725
	Amblyomma latum	L76347	AF120304
	Bothriocroton concolor	AF018643	JN863723
	Bothriocroton glebopalma	AF115370	AF120303
	Bothriocroton hydrosauri	AF115371	
	Bothriocroton oudemansi	DQ668033	
	Bothriocroton undatum	AF018645	JN863724
	Dermacentor andersoni	L76340	AF120311
	Dermacentor marginatus	Z74480	
	Dermacentor nitens	KC769621	KC769642
	Haemaphysalis doenitzi	JQ346682	
	Haemaphysalis flava	JX573120*	JX573128*
	Haemaphysalis formosensis	JX573121*	JX573129*
	Haemaphysalis hystricis	JX573122*	JX573130*
	Haemaphysalis humerosa	JX573123*	JX573131*
	Haemaphysalis inermis	L76338	AF120309
	Haemaphysalis leachi	AF018647	
	Haemaphysalis leporispalustris	JX573124*	JX573132*
	Haemaphysalis longicornis	JQ346680	
	Haemaphysalis parva	JX573125*	JX573133*
	Haemaphysalis petrogalis	AF018648	
	Haemaphysalis punctata	Z74478	
	Haemaphysalis sulcata	JX573126*	JX573134*
	Hyalomma dromedarii	L76348	AF120313
	Hyalomma lusitanicum	Z74482	
	Hyalomma rufipes	L76349	AF120314
	Hyalomma truncatum	DQ813264	
	Rhipicephalus (Boophilus) annulatus	Z74481	
	*Rhipicephalus (Boophilus) australis**	KC769614	KC769635
	Rhipicephalus (Boophilus) microplus	AF018656	AF200189
	*Rhipicephalus (Boophilus) microplus Cambodia**	KC769615	KC769636
	*Rhipicephalus (Boophilus) microplus China**	KC769616	KC769639
	*Rhipicephalus (Boophilus) microplus Brazil**	KC769619	KC769640

	*Rhipicephalus (Boophilus) kohlsi**	KC769618	KC769637
	*Rhipicephalus (Boophilus) geigyi**	KC769620	KC769638
	Rhipicephalus appendiculatus	AF018653	
	*Rhipicephalus appendiculatus**	KC769617	KC769641
	Rhipicephalus bursa	AJ003816	
	Rhipicephalus haemaphysaloides	DQ839552	
	Rhipicephalus pusillus	Z74483	
	Rhipicephalus sanguineus	AJ003815	AF120312
	Rhipicephalus zambeziensis	AF018654	
	Rhipicentor nuttalli	AF309949	
Prostriata	*Ixodes acutitarsus*	AF115364	
	Ixodes affinis	L76350	AF120288
	Ixodes auritulus	AF018649	AF120290
	Ixodes cookei	L76351	AF120292
	Ixodes corwini	AF115365	
	Ixodes hexagonus	JN018307	JN018404
	Ixodes holocyclus	AF018650	AF120294
	Ixodes kopsteini	L76352	AF120293
	Ixodes luciae	AF115367	
	Ixodes persulcatus	AY274888	
	Ixodes pilosus	AF018651	AF120289
	Ixodes ricinus	Z74479	
	Ixodes simplex	AF018652	
	Ixodes tasmani	AF115368	AF120295
	Ixodes uriae	AF115369	AF120296
Nuttalliellidae	*Nuttalliella namaqua*	JF751071	
Argasidae	*Antricola (Parantricola) marginatus**	KC769606	KC769622
	*Antricola (Antricola) mexicanus**	KC769603	KC769626
	Argas lahorensis	L76354	
	*Argas miniatus**	KC769610	KC769631
	Argas monachus	KC769609	
	Argas persicus	L76353	
	Argas sp. SpringbokSA- QMS95171*	KC769611	KC769632
	*Carios mimon**	KC769599	KC769627
	Carios puertoricensis	L76357	
	*Nothoaspis amazoniensis**	KC769600	KC769624
	*Ornithodoros braziliensis**	KC769604	KC769629
	Ornithodoros coriaceus	AF096274	
	Ornithodoros fonsecai	KC769608	KC769633
	*Ornithodoros marinkellei**	KC769601	KC769625
	Ornithodoros moubata	L76355	
	Ornithodoros rondoniensis	KC769602	KC769623
	*Ornithodoros rostratus**	KC769605	KC769628
	*Ornithodoros savignyi**	KC769612	
	Otobius megnini	L76356	AF120297
	*Otobius megnini**	KC769607	KC769630
Holothyrida	Allothyridae gen. sp.	AF115373	AF120299
	Allothyrus cf. *australasiae*	AY620910	AY626589
	Allothyrus cf. *constrictus*	AY620911	AY626590
	Allothyrus sp.	AF018655	
	Allothyrus sp. LamingtonNP-QMS95173*	KC769613	KC769634
	Diplothyrus lecorrei	GU392116	

	Neothyridae sp.	GU392115	
	Sternothyrus braueri	AY620912	AY626591
Opilioacarida	*Caribeacarus armasi*	GU392113	GU392120
	Opilioacarus texanus	AF115375	AF120302
	Opilioacarid SJD-2001	AF287235	
Xiphosura	*Limulus polyphemus*	L81949	AF212167
	Tachypleus tridentatus	HQ588745	JN018314

* indicates sequences submitted in this study.

Table S3. Results of the Ktreedist analysis of mitochondrial genes. K-score, scale factor and number of symmetric differences are given for the DNA (left) and amino acid (right) sequence of each mitochondrial protein-coding, compared to a concatenated alignment of all thirteen genes. The dashed line indicates the first cutoff, including only the genes with <20 symmetric distances (mtDNA3 and mtAA4), and the dotted line indicates the second cutoff, excluding genes with >28 symmetric differences (mtDNA10 and mtAA10).

Gene	K-score	Scale factor	Symmetric differences	Protein	K-score	Scale factor	Symmetric differences
cox1	2.17469	0.25986	12	*nad1*	0.5842	0.92703	10
cox3	3.28694	0.39106	16	*nad5*	0.2954	0.70512	14
nad1	3.08396	1.06837	16	*cox1*	0.53319	2.27375	16
cox2	3.08742	0.10709	20	*nad4*	0.96759	0.61930	16
cytb	4.58477	0.50250	20	*nad2*	0.64051	0.58402	22
nad5	2.65369	0.47865	20	*cox2*	0.58033	1.37973	25
nad2	3.23638	0.84211	22	*nad3*	0.7868	0.78320	25
nad4L	6.07636	0.13351	22	*cox3*	0.63179	0.98809	26
nad3	5.5068	0.69267	24	*cytb*	0.77918	1.13095	26
atp6	3.63232	1.18338	27	*nad6*	0.56926	0.57738	29
nad6	4.45055	1.99905	33	*atp6*	0.80253	0.93793	30
nad4	4.94698	0.60105	38	*nad4L*	0.77955	0.65946	32
atp8	8.75632	0.17589	63	*atp8*	1.14895	0.22158	51

Table S4. Comparison of bootstrap support between DNA analyses including (123) and excluding (12) third codons.

	Node	mtDNA3	mtDNA3 ex. 3	mtDNA10	mtDNA10 ex. 3	mtDNA5	mtDNA5 ex. 3	mtDNA13	mtDNA13 ex. 3
A	*Allothyrus* sp.	77	76	86	**91**	41	42	83	**91**
B	*Nuttalliella namaqua*	93	94	**95**	93	25	28	72	77
C	*Ixodes*	**100**	**100**	**100**	98	72	78	94	97
D	*Amblyomma* s.s. + Rhipicephalinae	50	52	**100**	99	91	**100**	97	99
E	Argasidae	**100**	**100**	**100**	**100**	**100**	99	**100**	**100**
F	Argasinae	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
G	*Ar. lagenoplastis* + *Ar. miniatus*	6	11	69	68	73	**92**	68	62
H	*Argas* sp. + *Ar. africolumbae*	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
I	Ornithodorinae	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
J	*Otobius*	89	92	**96**	86	72	71	94	84
K	*Ornithodoros* s.s.	58	75	**93**	**93**	59	80	67	66
L	*Or. rostratus* + *Or. brasiliensis*	98	78	**100**	95	87	55	68	99
M	Neotropical Ornithodorinae	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
N	*Antricola*	96	98	**100**	**100**	**100**	**100**	**100**	**100**
O	*Or. fonsecai* + *Or. capensis*	45	**60**	19	24	8	10	16	20

Table S5. Results of the Approximately Unbiased (AU) test of confidence in tree selection on the 24 clades differing among the 13 tested topologies. The dashed line separates clades which are present in the unconstrained tree (Figure 3) from alternate clades.

	Topology constraint (Clade)	**p-value [a]**	**-lnL difference [b]**
1 (D)[c]	*Amblyomma* s.s. + Rhipicephalinae (monophyly of Amblyocephalus sensu Burger et al. 2013a)	0.999	-61.6
2 (J)	*Otobius megnini* sister to *Ornithodoros* s.s. + Neotropical Ornithodorinae	0.997	-28.4
3 (B)	*Nuttalliella namaqua* sister to Ixodidae	0.915	-11.0
4	*Amblyomma* s.s. + Rhipicephalinae + *Haemaphysalis* + *Am. sphenodonti* (paraphyly of Haematobothrion sensu Burger et al. 2013a)	0.802	-10.6
5 (A)	*Allothyrus* sp. sister to Mesostigmata + Ixodida	0.761	-7.3
6	*Or. (S.) marinkellei* + *No. amazoniensis* + *Antricola*	0.890	-6.0
7	*Or. (Al.) capensis* sister to rest of Neotropical Ornithodorinae	0.574	-2.6
8	*No. amazoniensis* + *Antricola* spp.	0.431	-0.8
9 (P)	*Or. (S.) marinkellei* + *No. amazoniensis*	0.696	0.8
10	*Or. (S.) marinkellei* + *Antricola* spp.	0.513	2.6
11 (O)	*Or. (Al.) capensis* + *Or. (Al.) fonsecai* (*Alectorobius* monophyly)	0.513	2.6
12	*Or. (Al.) fonsecai* + *Or. (S.) marinkellei*	0.175	6.0
13	*Or. (Al.) fonsecai* + *Or. (S.) marinkellei* + *Antricola*	0.175	6.0
14	*Allothyrus* sp. sister to Ixodida	0.239	7.3
15	*Haemaphysalis* + *Am. sphenodonti* + *Bothriocroton* (monophyly of Haematobothrion sensu Burger et al. 2013)	0.198	10.6
16	*Nuttalliella namaqua* sister to Argasidae + Ixodidae	0.114	11.0
17	*Nuttalliella namaqua* sister to Argasidae	**0.035**	12.5
18	*Or. (Al.) capensis* + *Or. (S.) marinkellei*	0.059	15.5
19	*Or. (Al.) fonsecai* + *No. amazoniensis* + *Antricola*	0.059	15.5
20	*Otobius megnini* sister to Neotropical Ornithodorinae	**0.002**	28.4
21	Rhipicephalinae + *Haemaphysalis* + *Am. sphenodonti* + *Bothriocroton* (paraphyly of Amblyocephalus sensu Burger et al. 2013a)	**< 0.001**	61.6
22	*Or. (Al.) capensis* + *Or. (S.) marinkellei* + *No. amazoniensis*	**< 0.001**	92.5
23	*Or. (Al.) fonsecai* + *Antricola*	**< 0.001**	92.5
24	*Or. (Al.) fonsecai* + *Or. (S.) marinkellei* + *Or. (Al.) capensis*	**< 0.001**	234.3

[a] p-values indicating support in the AU test ($p > 0.95$) are underlined and p-values indicating rejection in the AU test ($p < 0.05$) are in bold type.

[b] the difference in -lnL values between the given clade and the next highest scoring alternate clade

[c] letters in brackets are the node labels in Figure 3 and Table 4