

of the otherwise extreme economy of these genomes. This phenomenon raises at least four functional and evolutionary questions (Kumazawa et al. 1996, 1998; Tang et al. 2000; Umeda et al. 2001). Why do some mt genomes, but not others, have duplicate CRs? How did mt genomes with duplicate CRs evolve? How could the nt sequences of duplicate CRs remain identical or highly similar over evolutionary time? Are duplicate CRs phylogenetic markers? Here, we present analyses of the entire and/or partial mtDNA sequences of 14 species of *Ixodes* ticks. We show that the Australasian *Ixodes* have duplicate CRs that evolved in concert in each species. Further, we address the four questions above with accumulated mtDNA sequences of metazoa with duplicate CRs and two recently published experiments on the replication of mt genomes in human cell lines with duplicate CRs.

Materials and Methods

Sequencing of the mt Genomes of *Ixodes* Ticks

Our methods of DNA extraction, PCR amplification, sequencing, sequence analysis, and annotation of mt genomes are described in Shao et al. (2001, 2004). The primers used in the present study are (1) nad1F (5'-TTTTAT-TGGCCCTTTTCGAA-3'), (2) rmlR1 (5'-CTGCT-CAATGATTTTTTAAATGCTGTGG-3'), (3) rmlR2 (5'-WWGWTTGCGACCTCGATGTTG-3'), (4) rmlR3 (5'-AAGTTACCTTAGGGATAACAGCGT-3'); (5) rmlSF1 (5'-GGCGATATGTGCATATCTAGAGC-3'), (6) rmlSF2 (5'-GTATRACCGCGRWTGCTGGCAC-3'), (7) rmlSF3 (5'-AATAATAGGGTATCTAATCC-3'), (8) rmlSF4 (5'-TACTATGTTACGACTTAT-3'), (9) tmMR1 (5'-TGGGGTATGAACCCAGTAGC-3'), (10) tmMR2 (5'-TTTGGGGTATGAACCCACTAGC-3'), (11) tmL2F1 (5'-TTCTCGCATTATATCTGCCACTT-3'), (12) tmL2F2 (5'-TCATAAAGGAAGCTTAAATTC-3'), and (13) tmQR (5'-GCACGATAAATTTTGATTTTA-3'). The combinations of primers used for each species are shown in table 1.

Phylogenetic Analyses of the nt Sequences of CRs

The sequences of the CR of *Ixodes* ticks determined in this study and the sequences of the CR of other ticks in GenBank were aligned with ClustalX (Thompson et al. 1997). For both pairwise and multiple alignments, the gap opening penalty was 15.00 and the gap extension penalty was 6.66. For multiple alignment, the delay divergent sequence was 30% and the DNA transition weight was 0.50. Neighbor-Joining (NJ), maximum-likelihood (ML), and maximum-parsimony (MP) trees were constructed with PAUP* version 4.0 (Swofford 2000). The general time reversible model and gamma distributed rates (Lanave et al. 1984) were used in ML tree construction; the instantaneous rate matrix, base frequencies, and the shape of gamma distribution were estimated by PAUP*. Bootstrap tests (1,000 replicates) were run on the NJ and the consensus MP trees. The horseshoe crab, *Limulus polyphemus* (Lavrov, Boore, and Brown 2000), was used for outgroup reference.

Results

Mitochondrial Genomes of *Ixodes* Ticks

Duplicate CRs were originally noticed in partial sequences of mt genomes of *I. holocyclus* and *I. uriae* in a phylogenetic study. To check that these CRs were not artifacts or pseudo mt genes from nuclear genomes (Lopez et al. 1994), the entire mt genomes of *I. persulcatus*, *I. holocyclus*, and *I. uriae* were amplified by PCR and sequenced. These three genomes are circular and 15,007 bp, 15,053 bp, and 14,539 bp long, respectively; all three genomes have the 37 genes and other features typical of the mt genomes of metazoa. The arrangement of 37 mt genes in these three *Ixodes* species is the same as that of the hypothetical ancestor of the arthropods (Lavrov, Boore, and Brown 2000 [fig. 1]). The mt genome of *I. persulcatus* has one CR, between the small rRNA gene (*rrnS*) and the tRNA-Ile gene (*trnI*); this position is the ancestral position of the CR for arthropods. However, *I. holocyclus* and *I. uriae* have two CRs of similar size and highly similar nt sequence: CR#1 is in the ancestral position for arthropods, whereas CR#2 is between tRNA-Leu gene (*trnL*; anticodon tag) and large rRNA gene (*rrnL*), which is novel to arthropods (fig. 1).

The genus *Ixodes* has 249 species and is the largest genus of the family Ixodidae (hard ticks, 713 species) and the suborder Ixodida (ticks, 899 species [Barker and Murrell 2004]). To examine the evolution pattern and the phylogenetic utility of the duplicate CRs found in *I. holocyclus* and *I. uriae*, the entire CR and part of the flanking genes of another 11 species of *Ixodes* were sequenced. The presence/absence of duplicate CRs in another four *Ixodes* species was tested by PCR. Sequencing and PCR test revealed that duplicate CRs were also present in another six species of *Ixodes*: *I. antechini*, *I. cordifer*, *I. cornuatus*, *I. hirsti*, *I. myrmecobii*, and *I. trichosuri* but were not present in *I. acutitarsus*, *I. asanumai*, *I. loricatus*, *I. ovatus*, *I. pilosus*, *I. ricinus*, *I. scapularis*, *I. simplex*, and *I. turdus* (table 1). Further, CR#1 of *I. myrmecobii* has two tandem repeats, CR#1a and CR#1b. All eight species of *Ixodes* ticks that have two CRs are from the Australasian lineage of *Ixodes* (*sensu* Barker and Murrell [2004]). These ticks live only in Australasia; that is, Australia, New Guinea, New Zealand, and some islands of the Pacific and Indian oceans, except for *I. uriae*, which infests sea birds that live both within and without Australasia. These ticks are referred to as the Australasian *Ixodes* hereafter. The nt sequences of the *Ixodes* ticks determined in this study were deposited in GenBank under the accession numbers shown in table 1.

Duplicate CRs of Australasian *Ixodes* Ticks

The two CRs of the seven Australasian *Ixodes* species sequenced in this study were 349 to 476 bp long (table 2). The nt sequences of CR#1 and CR#2 of a species were 87% to 95% similar. The nt sequences of CR#1 and CR#2 of a species were more similar to each other than were the CR#1 sequences or the CR#2 sequences of different species. Consider *I. cornuatus* and *I. myrmecobii*, which are apparently sister species (fig. 2). CR#1 and CR#2 of

Table 1
Species of Ticks Studied

Species	Location of Collection	Collector or Reference	Number of CRs	Primers for CR#1 ^a	Primers for CR#2 ^b	Accession Numbers
Australasian <i>Ixodes</i> ticks						
<i>I. antechini</i> (P)	Queensland, Australia	Matthew Shaw (B1569)	2	rrnSF2 and tmMR1	nad1F and rmlR3	PCR test
<i>I. cordifer</i> (P)	Queensland, Australia	David Spratt (B2046)	2	rrnSF3 and tmMR1	tmL2F2 and rmlR3	AB161424-5*
<i>I. cornuatus</i> (P)	Tasmania, Australia	Peter McKean (B906)	2	rrnSF3 and tmMR1	tmL2F2 and rmlR3	AB161426-7*
<i>I. hirsti</i> (P)	Victoria, Australia	David Kemp (B1579)	2	rrnSF3 and tmMR1	tmL2F2 and rmlR3	AB161428-9*
<i>I. holocyclus</i> (C)	Sydney, Australia	Minoru Nakao and Masahito Fukumaga	2	NA	NA	AB075955*
<i>I. myrmecobii</i> (P)	Western Australia	Matthew Shaw (B1546)	2	rrnSF4 and tmMR1	nad1F and rmlR2	AB161432-3*
<i>I. trichosuri</i> (P)	ACT, Australia	David Spratt (B2033)	2	rrnSF3 and tmMR1	tmL2F2 and rmlR2	AB161442-3*
<i>I. uriae</i> (C)	Bonden, Sweden	Asa Gylfe and Sven Bergstrom	2	NA	NA	AB087746*
Other <i>Ixodes</i> ticks						
<i>I. acutitarsus</i> (P)	Aomori, Japan	Hiromi Fujita	1	rrnSF1 and tmQR	tmL2F1 and rmlR1	AB161176*
<i>I. asanumai</i> (P)	Lab strain, Tokyo, Japan	Fumio Hayashi	1	rrnSF1 and tmQR	tmL2F1 and rmlR1	PCR test
<i>I. hexagonus</i> (C)	NA	Black and Roehrdanz 1998	1	NA	NA	AF081828
<i>I. loricatus</i> (P)	Sao Paulo, Brazil	Marcelo Labruna (B2079)	1	rrnSF3 and tmMR2	tmL2F2 and rmlR3	AB161430-1*
<i>I. ovatus</i> (P)	Hokkaido, Japan	Minoru Nakao	1	rrnSF1 and tmQR	tmL2F1 and rmlR1	PCR test
<i>I. persulcatus</i> (C)	Hokkaido, Japan	Minoru Nakao	1	NA	NA	AB073725*
<i>I. pilosus</i> (P)	South Africa	I. Mckay (B359)	1	rrnSF2 and tmMR2	nad1F and rmlR3	AB161434-5*
<i>I. ricinus</i> (P)	Slovak Republic	J. Rehacek (B774)	1	rrnSF2 and tmMR2	nad1F and rmlR3	AB161436-7*
<i>I. scapularis</i> (P)	Lab strain, CDC, USA	Joseph Piesman (B2078)	1	rrnSF3 and tmMR2	tmL2F2 and rmlR3	AB161438-9*
<i>I. simplex</i> (P)	Queensland, Australia	Martin Schulz (B538)	1	rrnSF2 and tmMR2	tmL2F2 and rmlR3	AB161440-1*
<i>I. turdus</i> (P)	Saitama, Japan	Kazuyoshi Fujimoto	1	rrnSF1 and tmQR	tmL2F1 and rmlR1	PCR test
Metastriate ticks						
<i>Amblyomma triguttatum</i> (C)	Queensland, Australia	Shao et al., unpublished (B1545)	2	NA	NA	AB113317
<i>A. vikirri</i> (P)	NA	Murrell, Campbell, and Barker 2003	2	NA	NA	AY059179
<i>A. varanensis</i> (P)	NA	Black and Roehrdanz 1998	2	NA	NA	NA
<i>Haemaphysalis flava</i> (C)	NA	Shao et al. 2004	2	NA	NA	AB075954
<i>Hyalomma truncatum</i> (P)	NA	Murrell, Campbell, and Barker 2003	2	NA	NA	AY059271-2
<i>Rhipicephalus appendiculatus</i> (P)	NA	Murrell, Campbell, and Barker 2003	2	NA	NA	AY059216-7
<i>R. (Boophilus) microplus</i> (P) ^c	NA	Campbell and Barker 1999	2	NA	NA	AH007623
<i>R. sanguineus</i> (C)	NA	Black and Roehrdanz 1998	2	NA	NA	AF081829
Soft ticks						
<i>Carios capensis</i> (C)	NA	Shao et al. 2004	1	NA	NA	AB075953
<i>Ornithodoros moubata</i> (C)	NA	Shao et al. 2004	1	NA	NA	AB073679
<i>O. porcinus</i> (C)	Dodoma, Tanzania	Fukunaga, unpublished.	1	NA	NA	AB105451
Horseshoe crab						
<i>Limulus polyphemus</i> (C)	NA	Lavrov, Boore, and Brown 2000	1	NA	NA	NC_003057

NOTE.—CR indicates control region. C indicates complete mtDNA. P indicates partial mtDNA. Asterisks indicate sequences generated in this study.

^a Between *rrnS* and *rrnL*.

^b Between *tmL₁* and *rrnL*.

^c *Rhipicephalus (Boophilus) microplus* was formerly *Boophilus microplus* (Barker and Murrell 2004).

I. cornuatus were 95% similar, and CR#1 and CR#2 of *I. myrmecobii* were 90% similar. However, CR#1 of *I. cornuatus* and CR#1 of *I. myrmecobii* were only 81% similar, and CR#2 of *I. cornuatus* and CR#2 of *I. myrmecobii* were only 78% similar. Seven motifs, 5 to 18 nts long, respectively, were conserved among CR#1 and CR#2 of the seven Australasian *Ixodes* species we sequenced (fig. 3); these motifs were also partially conserved in the other *Ixodes* species, which have one CR (data not shown). The conservation of these motifs

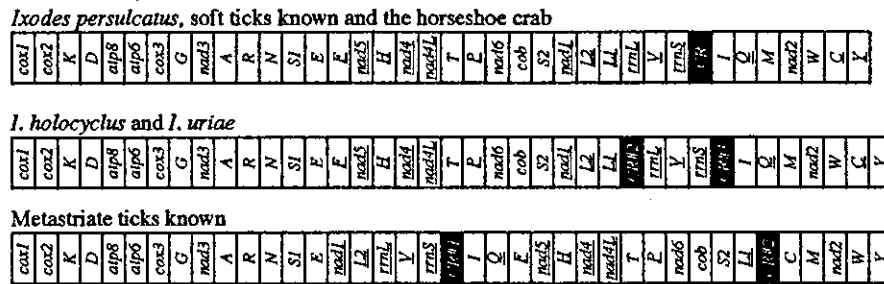


FIG. 1.—The mitochondrial genomes of *Ixodes persulcatus*, *I. holocyclus*, *I. uriae*, and metastriate ticks known. For the purpose of presentation, the circular mt genomes are linearized at the boundary between *cox1* and Y. Genes and control regions are shown as boxes. Control regions are abbreviated as CR and are highlighted in black. The names of genes are in italics: *atp6* and *atp8* for ATP synthase subunits 6 and 8, *cox1-3* for cytochrome oxidase subunits 1 to 3, *cob* for cytochrome *b*, *nad1-6* and *nad4L* for NADH dehydrogenase subunits 1 to 6 and 4L, and *rnlL* and *rnlS* for large and small rRNA subunits. tRNA genes are named with their single-letter amino acid abbreviations. The two genes for tRNA leucine and tRNA serine are differentiated by *L*₁ (anticodon tag), *L*₂ (taa), *S*₁ (tct), and *S*₂ (tga). Genes are transcribed from left to right, except those genes whose names are underlined; underline genes are transcribed from right to left.

indicates that they may have roles in the control of replication and/or transcription of the mt genome of these ticks.

Phylogenetic Trees of CR Sequences of Ticks

Our phylogenetic analyses of the CR sequences of ticks produced an NJ tree, a ML tree, 47 equally parsimonious MP trees, and a consensus 50% majority-rule MP tree. These trees were largely congruent with each other; therefore, only the consensus MP tree was shown (fig. 2). The NJ, ML, and MP trees were consistent in two groupings. First, the duplicate CRs of a species of Australasian *Ixodes* tick or metastriate tick were always grouped together. Second, the species of ticks were always grouped into four major lineages: the Australasian *Ixodes* ticks, the other *Ixodes* ticks, the soft ticks, and the metastriate ticks. These groupings concur with the current working hypothesis of the phylogeny of the subfamilies and families of ticks proposed from analyses of phenotypes, three nuclear genes (SSU rRNA, LSU rRNA, and ITS2 rRNA), and three mt genes (*rnlS*, *rnlL*, and *cox1* [Barker and Murrell 2004]). The differences among the NJ, ML, and MP trees were in the grouping of the species within the lineages of Australasian *Ixodes* ticks, other *Ixodes* ticks, and metastriate ticks, which were shown as polytomies in the consensus MP tree (fig. 2).

Species of the four major lineages of ticks in our CR-sequence tree differ in the copy number of CR and the arrangement of genes in mt genomes (figs. 1 and 2). The most parsimonious explanation for the evolution of the copy number of CR and the arrangement of mt genes in ticks is that (1) the most recent common ancestor of ticks had one CR and the arrangement of mt genes of the hypothetical ancestor of the arthropods, which has persisted in the horseshoe crab, *Limulus polyphemus* (Lavrov, Boore, and Brown 2000 [fig. 2]); (2) the CR duplicated in the lineage of the Australasian *Ixodes* ticks but no genes rearranged; (3) the CR duplicated and several genes rearranged in the lineage of the metastriate ticks; and (4) a single CR and the ancestral arrangement of genes of ticks persisted unchanged in the other *Ixodes* species and the soft ticks.

Discussion

Concerted Evolution of Duplicate CRs of Australasian *Ixodes* Ticks

Three lines of evidence indicate that the two CRs have evolved in concert in each species of the Australasian *Ixodes*: (1) the nt sequences of CR#1 and CR#2 of a species are highly similar; (2) the nt sequences of CR#1 and CR#2 of the same species are more similar to one another than either is to its namesake in other species; for example, CR#1 and CR#2 of *I. cordifer* are more similar to one another than are CR#1 of *I. cordifer* and CR#1 of any other species of tick (data not shown but see figure 3); and (3) CR#1 and CR#2 of a species were always clustered together in either the NJ, ML, or MP trees.

In addition to the Australasian *Ixodes* ticks and the metastriate ticks, duplicate CRs have also been found in the mt genomes of some snakes (Kumazawa et al. 1996), sea cucumbers (Arndt and Smith 1998), *Amazona* parrots (Eberhard, Wright, and Bermingham 2001), a fish (Lee et al. 2001), a plague thrips (Shao and Barker 2003), and a sea firefly (Ogoh and Ohmiya 2004 [table 2]). Once duplicate CRs occur in an mt genome, they may evolve either in concert or independently. Independent evolution leads to the divergence of the nt sequences of the two CRs and, eventually, degeneration or deletion of one of the CRs (Bensch and Harlid 2000). Concerted evolution, however, keeps the nt sequences of the two CRs highly similar. Kumazawa et al. (1996) and Arndt and Smith (1998) proposed concerted evolution as an explanation for the high similarity of the nt sequences of the two CRs in the snakes and the sea cucumbers they studied. Black and Roehrdanz (1998) and Eberhard, Wright, and Bermingham (2001) showed that the two CRs evolved in concert in each species of the metastriate ticks and the *Amazona* parrots. Black and Roehrdanz (1998) and Eberhard, Wright, and Bermingham (2001) studied two and four species, respectively. In the present study, we studied 26 species of ticks: 15 had duplicate CRs, and 11 had one CR. Our study, together with previous studies, shows conclusively that duplicate CRs in the mt genomes of metazoa tend to evolve in concert in each species rather than independently.

Table 2
Species of Metazoa with Duplicate Control Regions in Their Mitochondrial Genomes

Species	CR#1 ^a (bp)	CR#2 (bp)	Similarity Between CR#1 and CR#2 ^b
Australasian <i>Ixodes</i> ticks			
<i>Ixodes cordifer</i>	364	390	89%
<i>I. cornuatus</i>	357	403	95%
<i>I. hirsti</i>	361	436	93%
<i>I. holocyclus</i>	352	450	87%
<i>I. myrmecobii</i>	353/352 ^c	398	90%
<i>I. trichosuri</i>	349	425	94%
<i>I. uriae</i>	388	476	91%
Metastriate ticks			
<i>Amblyomma triguttatum</i>	307	307	93%
<i>A. vikirri</i>	306	312	92%
<i>A. varanensis</i>	309	271	98%
<i>Haemaphysalis flava</i>	310	310	96%
<i>Hyalomma truncatum</i>	312	308	93%
<i>Rhipicephalus appendiculatus</i>	306	303	96%
<i>R. (Boophilus) microplus</i>	302	299	98%
<i>R. sanguineus</i>	305	303	93%
Snakes			
<i>Dinodon semicarinatus</i>	1018	1018	100%
<i>Crotalus viridis</i>	1020	1025	100%
<i>Ovophis okinavensis</i>	1033	1033	100%
Sea cucumbers			
<i>Cucumaria miniata</i>	459	410	97%
Parrots ^d			
<i>Amazona farinose</i>	1553	1457	94%
<i>A. ochrocephala oratrix</i>	1551	1838	92%
<i>A. o. auropalliata</i>	1705	1867	87%
Fish			
<i>Rivulus marmoratus</i>	887	795	95%
Plague thrips			
<i>Thrips imaginis</i> ^e	440	460	99%
Sea firefly			
<i>Vargula hilgendorfu</i> ^f	771	771	99%

^a CR#1 is at the ancestral position; CR#2 is at a novel position.

^b Similarity between CR#1 and CR#2 = (number of shared nt/length of alignable nt sequence) × 100%.

^c Length of CR#1a and CR#1b, respectively.

^d Both CRs may be in the ancestral positions for birds (see Discussion).

^e Both CRs are in novel positions for arthropods.

The presence of duplicate CRs in the mt genome of metazoa is an intriguing mutational phenomenon in light of the otherwise extreme economy of these genomes. This phenomenon raises several functional and evolutionary questions.

Why Do Some mt Genomes, but Not Others, Have Duplicate CRs?

Replication of the mt genomes of the mammals and fruitflies studied initiates at the CR (Goddard and Wolstenholme 1980; Clayton 1982; Holt, Lorimer, and Jacobs 2000). So, it is probably reasonable to speculate that mt genomes with duplicate CRs may have a selective advantage over mt genomes with one CR. For example, an mt genome with two CRs may replicate more efficiently than an mt genome with one CR (Kumazawa et al. 1996;

Arndt and Smith 1998; Umeda et al. 2001). Initiation of replication is apparently a rate-limiting step in the replication of the mt genome of *D. melanogaster* (Rubenstein, Brutlag, and Clayton 1977). If replication can initiate at both CRs, then mt genomes with two CRs could start more replication per unit time than could mt genomes with one CR. Thus, mt genomes with two CRs may "out compete" mt genomes with one CR.

Our knowledge of the replication of metazoan mt genomes is almost entirely from studies of mammals and fruitflies that have one CR. It is not clear yet how mt genomes with two CRs replicate. Nevertheless, two recent experimental studies indicate that an mt genome with two CRs may replicate more efficiently than an mt genome with one CR. Tang et al. (2000) found that the population of mt genomes in human cell lines, which was originally a mixture of genomes with one CR and genomes with two CRs, shifted over time, towards mt genomes with two CRs. This finding suggests that cells may favor mt genomes with two CRs. Tang et al. (2000) proposed that mt genomes with duplicate CRs would have a selective advantage over those with one CR if the two types of genomes were competing for a finite amount of replication factor(s). Further, Umeda et al. (2001) showed that in human cell lines that had partially duplicated mt genomes, the two CRs were equally efficient at starting replication. It is not known, however, whether the two CRs in an mt genome can be active simultaneously, and if so, whether the two CRs start replication simultaneously or sequentially.

How Did mt Genomes with Duplicate CRs Evolve?

Three mechanisms may account for mt genomes with duplicate CRs: tandem duplication, dimerization, and illegitimate recombination (reviewed in Boore [2000]). The tandem duplication mechanism starts with replication errors, such as imprecise termination and/or slipped-strand mispairing. If these errors occur in the section that has the CR, then the replication will generate an mt genome with two tandem-repeated sections, and each section contains a CR. Dimerization occurs when two linearized monomeric mt genomes join "head-to-tail" to form a large circular mt genome. A dimeric mt genome would have two CRs and two copies of each gene. Illegitimate recombination occurs when a section of one mt genome is cleaved out and then introduced into another mt genome. If this section contains the CR, then the mt genome that receives the cleaved section of genome will have two CRs. Obviously, illegitimate recombination may also cause tandem duplications if the introduced section is next to its counterpart in the receipt mt genome.

A single event of tandem duplication followed by deletions of redundant copies of genes can account for the duplicate CRs and/or gene rearrangements in the mt genomes of all of the metazoa known that have duplicate CRs, except the plague thrips and the sea firefly (see Supplementary Material online). However, different genes would have been duplicated and deleted in each of these metazoan lineages. Moreover, the two CRs are in different positions in each lineage. The mt genomes of the plague thrips and the sea firefly are highly rearranged, and,

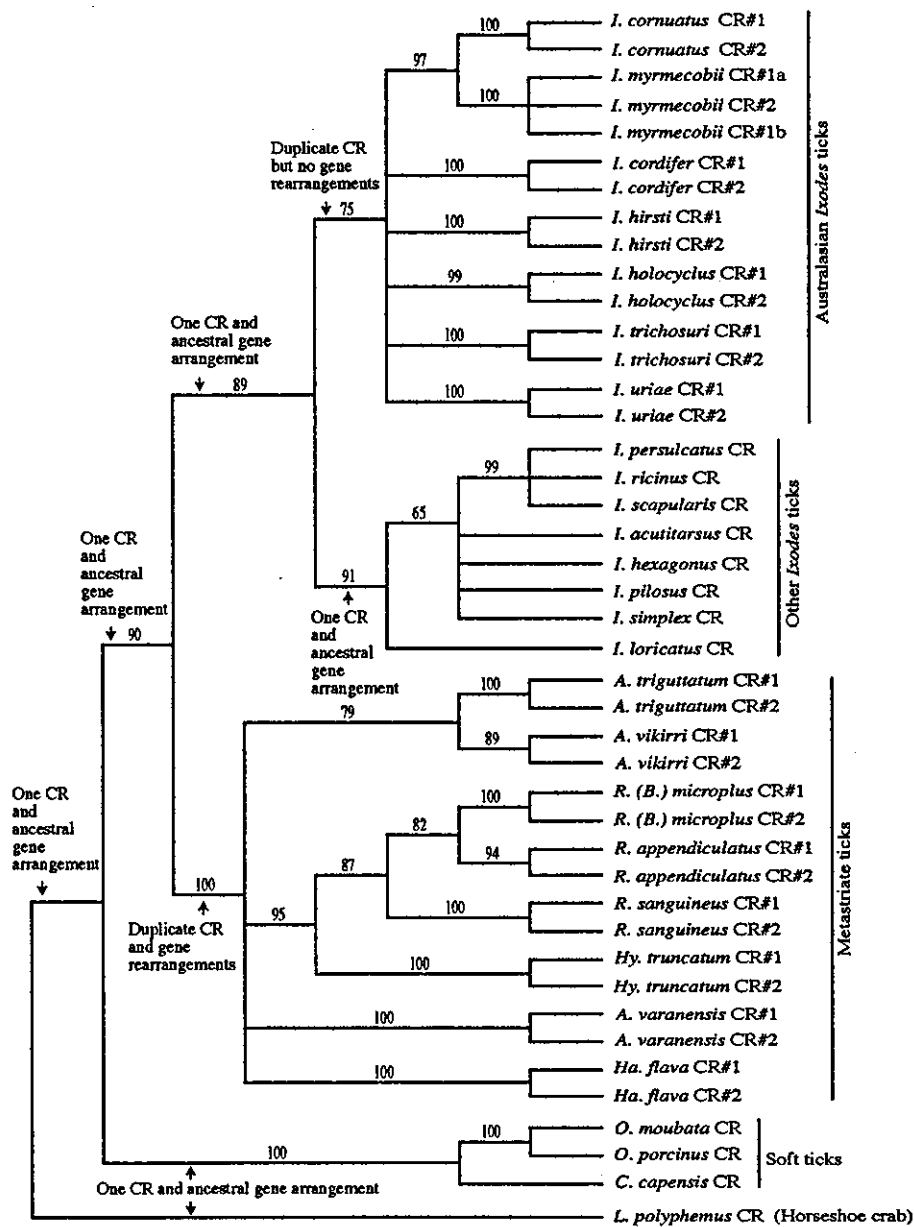


FIG. 2.—The consensus 50% majority-rule maximum-parsimony tree from the nucleotide sequences of the control regions of ticks. Bootstrap support (%; 1,000 replicates) is shown above branches. The horseshoe crab, *Limulus polyphemus*, was the outgroup. See table 1 for the full name of each species.

therefore, multiple events of tandem duplication and deletions, and/or illegitimate recombination, may have occurred in the evolution of these genomes (Shao and Barker 2003; Ogoh and Ohmiya 2004).

How Do Duplicate CRs Evolve in Concert?

Kumazawa et al. (1998) proposed two mechanisms for the concerted evolution of duplicate CRs: tandem duplication and gene conversion. The tandem duplication

FIG. 3.—Alignment of the nucleotide sequences of the duplicate control regions of seven species of Australasian *Ixodes* ticks. CR#1 is the control region between *rrnS* and *trnI*, and CR#2 is the control region between *trnL1* and *rrnL* (see figure 1). There are two copies of CR#1, in tandem, in *I. myrmecobii*: CR#1a is next to *rrnS*, and CR#1b is next to *trnI*. Dashes indicate alignment gaps. Dots indicate nucleotides that are the same as that of CR#1 of *I. cordifer*. Asterisks indicate nucleotides that are conserved among the Australasian *Ixodes* species. Gray-shaded blocks indicate conserved motifs of 5 or more nucleotides.

I. cordifer CR#1TTTTCCTTTATTTCTTTATTTTTCACC
I. cordifer CR#2TCTTACCTTC.A.A.....C
I. holocyclus CR#1TAA.A.A.....C
I. holocyclus CR#2TAACTATTACTGTATAGATTTCCTAA.....C
I. hirsti CR#1TACCTA.A.....C
I. hirsti CR#2 TGAGTAATTAATATATAATACTACTTACTTACTTACCTA.A.....A
I. erichosuri CR#1CTA.C.A.....TT
I. erichosuri CR#2TATTAAAAAACATAAAAGAGTAGTCA.C.A.....TT
I. cornutus CR#1TGA
I. cornutus CR#2TTCTTTTAA.....TGA
I. myrmecobii CR#1aTTTCATAT.A.....TAA
I. myrmecobii CR#2TTTATAAAACTTACTTAC.....TAA
I. myrmecobii CR#1bTAA.A.A.....TAA
I. uriae CR#1GTTTAAATA.....TAA
I. uriae CR#2TAACTATAGTACTA.A.....TAA
I. cordifer CR#1 CCCCATAAAGAAATAAAGGATTCAAATTTAATTCATGCTAAGGCTCATCTAATAATGAAATTTAAATA
I. cordifer CR#2T
I. holocyclus CR#1 A.TT.....A.GT.....T.....T.....T.....T.....G
I. holocyclus CR#2 A.TT.....A.GT.....T.....T.....T.....T.....G
I. hirsti CR#1AG.T.A.....T.....T.....T.....A.T
I. hirsti CR#2AGTT.A.....T.....T.....T.....A.T
I. erichosuri CR#1A.G.....ACCG.....AC.....T.....T.....T
I. erichosuri CR#2A.G.....ACCG.....AC.....T.....T.....T
I. cornutus CR#1AG.A.....A.....T.....T.....CTT.....A.C
I. cornutus CR#2AG.A.....A.....T.....T.....CTT.....A.C
I. myrmecobii CR#1aG.AT.....T.....T.....CTT.....G.AA.C
I. myrmecobii CR#2G.AT.....T.....T.....CTT.....G.AA.C
I. myrmecobii CR#1bG.AT.....T.....T.....CTT.....G.AA.C
I. uriae CR#1A.....T.A.C.....C.....T.....AT.GTA.....AACT
I. uriae CR#2A.....T.A.C.....C.....T.....AT.GTA.....AACT
I. cordifer CR#1 TGTAAAGCTTATATTTCGGCTGGGGGAAATTTTCATATGTTTGTAAATTAATGTGAAGCATAACTTCT
I. cordifer CR#2CA.....G.....T
I. holocyclus CR#1GTT.....AG.A.....CT.C.AAA.G.T.T.....AT.....GG.....AT
I. holocyclus CR#2GTT.....AG.A.....CT.C.AAA.G.T.T.....AT.....AA.....AT
I. hirsti CR#1GTT.....T.....C.....A.....Y.....CAGGT.TA.....A.....C.C.....T
I. hirsti CR#2GTT.....T.....C.....A.....Y.....AGGT.TA.....A.....C.C.....T
I. erichosuri CR#1GTT.....T.....A.....T.....AC.....G.T.A.TAA.....GA.C.F.....T
I. erichosuri CR#2GTT.....T.....A.....T.....AC.....G.T.A.TAA.....GA.C.F.....T
I. cornutus CR#1GTT.....T.....CA.....A.....CT.....A.CCAGA.T.E.....C.C.....T
I. cornutus CR#2GTT.....T.....CA.....A.....CT.....A.CCAGA.T.E.....C.C.....T
I. myrmecobii CR#1aGTT.....T.....CA.....A.....CT.....A.CCAGA.T.E.....A.C.C.....T
I. myrmecobii CR#2GTT.....T.....CA.....A.....CT.....A.CCAGA.T.E.....A.C.C.....T
I. myrmecobii CR#1bGTT.....T.....CA.....A.....CT.....A.CCAGA.T.E.....A.C.C.....T
I. myrmecobii CR#2bGTT.....T.....CA.....A.....CT.....A.CCAGA.T.E.....A.C.C.....T
I. uriae CR#1 ATC.....T.....T.A.AA.A.....CT.....C.A.AG.C.T.T.....T.....C.....GAT
I. uriae CR#2 ATC.....T.....T.A.AA.A.....CT.....C.A.AG.C.T.T.....T.....C.....GAT
I. cordifer CR#1 TCTTAAAA-TTCCTTTAAGCTATACAT-TAAATGTAATCTGTCTTAAATAGACTTCTTGGA-AGATATA
I. cordifer CR#2T.....C.....A.....T.C.....
I. holocyclus CR#1C.TTC.TGG.G.T.TA.A.C.....A.....CT.....A.A.....CT.TC.....
I. holocyclus CR#2C.TT.TAG.G.T.T.A.C.....A.....T.....A.A.....AA.TT.....
I. hirsti CR#1C.C.C.TTG.G.T.TG.A.C.....A.....G.....A.CTA.....A.A.....CT.TC.....
I. hirsti CR#2C.C.C.TTG.G.T.TG.A.C.....A.....G.....A.CTA.....A.A.....CT.TC.....
I. erichosuri CR#1C.A.TTT.TT.G.T.TA.....C.T.A.....A.CT.....A.A.....CT.TC.....
I. erichosuri CR#2C.A.TTT.TT.G.T.TA.....C.T.A.....A.CT.....A.A.....CT.TC.....
I. cornutus CR#1C.C.TC.....TT.CG.....AA.A.C.....A.....G.....G.....CT.....T.....GC.TCAGC.....C
I. cornutus CR#2C.C.TC.....TT.CG.....AA.A.C.....A.....G.....G.....CT.....T.....GC.TCAGC.....C
I. myrmecobii CR#1aC.C.TC.....T.CG.....G.A.C.....A.....G.....C.....C.....C.TC.T.T.....T
I. myrmecobii CR#2C.C.TC.....T.CG.....G.A.C.....A.....G.....C.....C.....C.TC.T.T.....T
I. myrmecobii CR#1bC.C.TC.....T.CG.....G.A.C.....A.....G.....C.....C.....C.TC.T.T.....T
I. myrmecobii CR#2C.C.TC.....T.CG.....G.A.C.....A.....G.....C.....C.....C.TC.T.T.....T
I. uriae CR#1C.....GTT.....T.AACA.C.....AG.G.....CTA.....A.....GCTGT.....AC.C.C.C
I. uriae CR#2C.....GTT.....T.AACA.C.....AG.G.....CTA.....A.....GCTGT.....AC.C.C.C
I. cordifer CR#1 CTTAAGCTATTAAAT-AAATGAATTCGTATACGTGCCCCCTTAT-ATAAAGAGTGAATCATATCTC
I. cordifer CR#2T.....T.....AA.....T.....T.....T.....T.....T.....T.....T
I. holocyclus CR#1TAA.....GAAC.....T.....CA.....TT.A.T.....A.....T.T.T.....TCA
I. holocyclus CR#2TAA.....GA.....T.....AA.....TT.TG.....TA.....TT.....AAC
I. hirsti CR#1TAA.....GA.....T.....CA.....CAA.TA.....T.A.....T.T.A.T.....T
I. hirsti CR#2TAA.....GA.....T.....CA.G.....CAA.TA.....T.....T.....T
I. erichosuri CR#1TAA.....GA.....T.....CA.....TA.....A.....T.T.....T.A
I. erichosuri CR#2TAA.....GA.....T.....CA.....TA.....A.....T.T.....T.A
I. cornutus CR#1C.AGC.T.GA.....T.G.....CA.....TTT.TT.....A.....T.....AGA
I. cornutus CR#2C.AGC.T.GA.....T.G.....CA.....TTT.TT.....A.....T.....AGA
I. myrmecobii CR#1aA.GTT.T.GAT.....T.....CA.....T.....T.....T.....A.....T
I. myrmecobii CR#2A.GTT.T.GAT.....T.....CA.....T.....T.....T.....A.....T
I. myrmecobii CR#1bA.GTT.T.GAT.....T.....CA.....T.....T.....T.....T.....T
I. uriae CR#1T.CG.....GA.....A.....T.....TA.A.T.....TT.TAT.....GST.....T.....T.A.T
I. uriae CR#2T.CG.....GA.....A.....T.....TA.A.T.....TT.TAT.....GST.....T.....T.A.T
I. cordifer CR#1 TTUSCAGAAAATACAATTTCRAAAAANAATTAANAANAATA
I. cordifer CR#2AA.A.G.....G.A.....ACCTTGGGGGGT.....TT.TAATTAACACAAAT
I. holocyclus CR#1 AGAA.T.....TTAC-GA.....TTG.GT.T
I. holocyclus CR#2 CG.TG.AG.GCTACT.G.....TTTTCG.....A.TTC.....TAA.TCTTGGATTTTCTTAATTAACACAAAA
I. hirsti CR#1A.....G.ATTTA.A.C.T.CYTT.APTT.....A.....
I. hirsti CR#2T.G.....TATT.CG.....TTT-T.CYTT.....TTT.....AACTCTTAAAAATTTCTAAGACAAACAA
I. erichosuri CR#1 A-TT.T.TTT.....T.A.A.T.....T.T.T
I. erichosuri CR#2 A-AA.A.....TCTT.....C.....TT.....T.TTCA.TTT.....TA.AATACTAATTTTCTAAATAAAGACAAA
I. cornutus CR#1T.A.T.T.ATTT.....AATTT.C.T.....A.....T.....Y
I. cornutus CR#2TTA.....G.....ATTTAAAA.....T.....TT.....TGG.....A.AGCCCTTTATCATCTT.TTTCTAAAAAGAC
I. myrmecobii CR#1aTTA.....T.....A.A.AGA.....T.TT.G.....
I. myrmecobii CR#2TTA.....C.C.A.A.AA.....T.T.T.CT.....TTT.....AATTTAAATAAACCAGATA
I. myrmecobii CR#1bTA.A.....TT.TA.T.....AAT.CYTTG.....TA.....T.....
I. uriae CR#1 AGTA.TT.....T.P.G.T.....G.ATGTC.TT.....TC.....TTR.AAATAAATTTTCTAGT
I. uriae CR#2 AAT.T.A.....T.T.....TAA.AA.....GST.TTCT.C.....CTTTA.ATCCCTCATGATCTGAGTTCAGACGGGCTGA
I. cordifer CR#1
I. cordifer CR#2
I. holocyclus CR#1 AAATAAATGCTTACTAAATAT
I. holocyclus CR#2
I. hirsti CR#1
I. hirsti CR#2
I. erichosuri CR#1
I. erichosuri CR#2
I. cornutus CR#1
I. cornutus CR#2
I. myrmecobii CR#1a
I. myrmecobii CR#2
I. myrmecobii CR#1b
I. uriae CR#1
I. uriae CR#2 GCGAGTGGTTCATCTTATTTCTAATTAAGACAAAGTAAATTTACTTCTTTTITTTGAT

mechanism starts with a replication error. Replication of a strand that starts in one CR, say CR#1, pauses at the other CR, CR#2. Then the newly synthesized fragment is unwound from the template strand. The two ends of this fragment reanneal to CR#1, and the rest of the fragment forms a loop. Replication of this strand then restarts, and a new strand with three CRs (CR#1, CR#2, and CR#1 with CR#2 in the loop) is synthesized. If the loop is deleted, then replication of the next strand will generate an mt genome with two identical or nearly identical CRs. The gene conversion mechanism involves homologous recombination. The crossing over of nicked strands between the two CRs of an mt genome forms a Holliday structure, which leads to two heteroduplex intermediate CRs. Subsequent DNA repairs replace the nt sequence of one CR with that of the other and lead to two identical CRs in an mt genome.

The tandem duplication mechanism is a less plausible explanation, in our view, for the concerted evolution of duplicate CRs than is the gene conversion mechanism. For the tandem duplication mechanism to account for the concerted evolution of duplicate CRs in each species, the same replication errors must occur over and over again, and independently in each species. This circumstance is less likely. Further, as discussed above, each tandem duplication event could potentially lead to rearrangement of mt genes in a species. However, we did not find differences in mt gene arrangement among the seven species of the Australasian *Ixodes* ticks (two species sequenced entirely; five species sequenced partially), among the eight species of the metastriate ticks (four species sequenced entirely and others sequenced partially), or among three species of the *Amazona* parrots (all sequenced partially [table 2]). Rather, gene conversion is a more plausible mechanism for the concerted evolution of duplicate CRs. Indeed, recombination (homologous and/or nonhomologous) may be an indispensable part of the mtDNA replication and repair machinery of metazoa (Rokas, Ladoukakis, and Zouros 2003). Further, gene conversion can account for the high similarity of the nt sequences of the CRs in each species and the conservation of the gene arrangement among species of the Australasian *Ixodes* ticks, the metastriate ticks, and the *Amazona* parrots, respectively.

Are Duplicate CRs Phylogenetic Markers?

Mitochondrial genomes with duplicate CRs may be a synapomorphy for the Australasian *Ixodes* ticks (fig. 2). Further, duplicate CRs, together with gene rearrangements, is probably a synapomorphy for the metastriate ticks. Kumazawa et al. (1996) suggested that duplicate CRs might be a synapomorphy for the snakes they studied from three genera of two families. Taken together, these studies indicate that duplicate CRs are informative phylogenetic markers at low taxonomic levels such as within a genus, within a family, or among families.

Intriguingly, Mindell, Sorenson, and Dimcheff (1998) and Bensch and Harlid (2000) found that birds from four orders had a novel mt gene arrangement. This novel gene arrangement had a control region (CR) plus a degenerate

control region (NC), whereas the typical mt genome of birds has only one CR at the position of NC. These authors showed that the novel gene arrangement evolved by convergence in these lineages of birds and, therefore, was not a synapomorphy. The *Amazona* parrots also had a novel gene arrangement (Eberhard, Wright, and Bermingham 2001). However, instead of a CR and an NC, the *Amazona* parrots had two CRs, CR#1 and CR#2, in the positions of CR and NC in the mt genomes of the four orders of birds studied by Mindell, Sorenson, and Dimcheff (1998) and Bensch and Harlid (2000). The two CRs share high similarity of nt sequences and have evolved in concert (Eberhard, Wright, and Bermingham 2001).

The novel gene arrangement of birds with a CR and an NC was thought to have evolved independently at least five times in the four orders of birds by tandem duplications followed by deletions of genes (Mindell, Sorenson, and Dimcheff 1998; Bensch and Harlid 2000). As discussed above, tandem duplication is a mechanism that involves replication errors. If the novel gene arrangement in birds evolved five times independently, then the same replication errors, same tandem duplications and, subsequently, deletions of the same genes, would have to occur five times independently in the four orders of birds. This circumstance is unlikely, in our view.

Boore (2000) proposed a much more parsimonious explanation for the evolution of the novel mt gene arrangement in the four orders of birds. A gene arrangement with duplicate CRs, CR#1 and CR#2, such as that of the *Amazona* parrots, may be ancestral for all birds. The two CRs may have evolved in concert in some lineages of birds, whereas in other lineages, they may have evolved independently. In the lineages of birds that have two CRs that have evolved independently, one of the two CRs would eventually be deleted or would degenerate; this would lead to either the typical or the novel gene arrangement of birds observed by Mindell, Sorenson, and Dimcheff (1998) and Bensch and Harlid (2000). The apparent convergent evolution in birds of genomes with a CR and an NC suggests that duplicate CRs may not be a reliable phylogenetic marker at high taxonomic levels, such as among orders.

Conclusion

The Australasian *Ixodes* ticks we studied have duplicate CRs that have evolved in concert in each species. To date, mt genomes with duplicate CRs have been found in eight lineages of metazoa. Our analyses of the accumulated mtDNA sequences of metazoans with duplicate CRs and two recent experiments on replication of mt genomes in human cell lines with duplicate CRs indicate that mt genomes with duplicate CRs may have a selective advantage in replication over mt genomes with one control region. Tandem duplication followed by deletion of redundant copies of genes is the most plausible mechanism for the generation of duplicate CRs in mt genomes. Once duplicate CRs occur in an mt genome, they tend to evolve in concert rather than independently, probably by gene conversion. However, in some lineages of metazoa, it seems that gene conversion may not always occur over evolutionary time and, therefore, the two CRs

may evolve independently. Duplicate CRs may be useful phylogenetic markers at low taxonomic levels, such as within a genus, within a family, or among families, but not at high levels, such as among orders of metazoa.

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