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UNUSUALLY LONG *HYPTIOTES* (ARANEAE, ULOBORIDAE) SEQUENCE FOR SMALL SUBUNIT (18S) RIBOSOMAL RNA SUPPORTS SECONDARY STRUCTURE MODEL UTILITY IN SPIDERS

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ABSTRACT. We report on the structure of the small-subunit ribosomal RNA (18S rRNA) sequence from *Hyptiotes gertschi* (Araneae, Uloboridae), which is the largest 18S gene sequenced in any arachnid to date. We compare this remarkable sequence to those from a range of other spiders and arachnids, and develop base-pairing models of its insert regions to determine its overall secondary structure. The *H. gertschi* sequence of 1902 bases is 86 nucleotides longer than any comparable spider sequence and contains 5 inserts between 5 and 28 bases in length, all at regions characterized as among the most variable in eukaryotic 18S genes. Inserts were also found in one of these variable regions in published sequences of 3 species of hard ticks (Acari, Ixodidae). Other arachnid taxa were remarkably uniform in 18S primary sequence length, ranging from 1802 to 1816 nucleotides. Thermodynamic modeling of the *H. gertschi* inserts suggests they are largely self-complementary, extending the stem portions of the variable regions.

Keywords: Phylogenetics, arachnids, Acari, gene inserts

The small subunit ribosomal RNA, or 18S rRNA, is one of a small set of commonly used sequences for molecular phylogenetic reconstruction of arthropod relationships (reviewed in Caterino et al. 2000). The gene coding for the 18S rRNA contains sections that are highly conserved, and these provide informative characters for the assessment of relationships between distantly related taxa, such as metazoan relationships (Giribet & Wheeler 2001; Mallatt et al. 2004). The 18S rRNA has also been used in studies of divergence between arachnid orders, as well as studies of divergence between spider genera and species (Wheeler & Hayashi 1998; Arnedo et al. 2004). In the context of arachnid molecular phylogenetics, to understand both the potential utility and drawbacks in the use of any genetic marker, it is important to have knowledge of the amount of variation in both the primary sequence and secondary structures across taxa at different levels. This is because the secondary structure can influence the rate at which different parts of the primary sequence vary.

The genes coding for ribosomal RNAs contain regions that can accumulate and lose bases through insertion and deletion events (indels) more easily than protein-coding genes, which are constrained by the requirement that they maintain an open reading frame for proper translation into a functional primary amino acid sequence. Indels can change the length of the gene and make homology assessments of individual base-pairs, and often long stretches of base-pairs, difficult. The proper methodological approach to this sequence-alignment problem is a source of controversy in phylogenetics, and opinions range from using static alignments, with regions that are difficult to align being either included or discarded (e.g., Nardi et al. 2003); to using direct optimization (Wheeler 1996), which avoids the arbitrary removal of data and possible evolutionary signal and avoids the problem of multiple-sequence alignment altogether. An additional feature of direct optimization, as implemented in the program POY (Wheeler 1999) is the ability to treat inserts as multistate characters with as many states as there are unique insert

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sequences, with a matrix of character-state transformation costs. See Giribet & Wheeler (2001) for an example of implementation of the latter method.

A very different approach, and one that has been promoted by many authors, is the use of secondary structure models to aid in homology assessment and relative weighting (Dixon & Hillis 1993; Kjer 2004). Recently, it has been proposed that elements of rRNA secondary structure can provide a basis for choice between multiple models to be used in a partitioned Bayesian analysis (Telford et al. 2005). For this technique, bases from a variety of taxa would be compared to a secondary structure model for the gene for assignment to ''stem'' and ''loop'' (and possibly transitional or ambiguous) partitions. These partitions would be individually tested for the most appropriate likelihood models, then subsequently analyzed using the partitioned, multiple-model approach.

The RNAs produced by the 18S genes form secondary structure by base-pairing with their own complementary stretches of sequence, forming helical structures commonly referred to as stems, while the non-pairing regions form loop structures that connect multiple stems, or form the terminal turn in the self-complementary stems. Because the overall secondary structure is functionally important for the ribosome, and because two complementary changes in primary sequence are required to maintain a stem structure, it has been proposed that the stems should be treated differently in phylogenetic analysis than the less-constrained loops (Dixon & Hillis 1993). To do so requires the ability to tell whether nucleotides are in a loop or a stem structure, which can be modeled using computer algorithms that develop secondary structures based on comparative or thermodynamic information (reviewed in Gardner & Giegerich 2004).

Here we present a comparison of 18S structures sampled from arachnid orders and spider lineages to assess the utility of secondary structure information currently available for making homology judgments across arachnid taxa, and for determining data partitions used in model-based analyses. Included are new data from the species *Hyptiotes gertschi* Chamberlin & Ivie 1935 (Araneae, Uloboridae), and remarkably similar sequences from hard ticks (Acari, Ixodidae) that demonstrate the evolutionary conservation of the overall structure of the arachnid 18s gene.

METHODS

Taxon sampling.—We sampled exemplar sequences from all arachnid orders and major spider lineages for which at least one full sequence of the entire 18S gene was available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). To sample unusually long arachnid 18S sequences thoroughly, we extended our search to include all ''partial sequences'' that came within 90 bases (~5% of the gene length) of the primer regions, since this region is highly conserved across arthropods and the missing length could be estimated despite lack of primary sequence. In taxa where 18S gene length was uniform and in the ~1800 base pair (= bp) length typical of arachnids, one individual was sampled, while all complete or nearly-complete sequences with large (> 3 bp) inserts relative to the *Aphonopelma* sp. sequence were included in the sample, since a complete secondary structure model is available for this taxon (Hendriks et al. 1988).

Among spiders, lineages included representatives from Mygalomorphae (*Aphonopelma sp.*, Theraphosidae), Mesothelae (*Liphistius bicoloripes*, Liphistiidae), Orbicularia (*H. gertschi*, Uloboridae), ''derived orb-weavers'' (*sensu* Coddington 1990), (*Nesticus cellulanus*, Nesticidae) and the RTA (retrolateral tibial apophysis) clade (*sensu* Coddington et al. 2004) (*Coelotes terrestris*, Amaurobiidae) (see Table 1). All spider sequences were from Genbank except for the *H. gertschi* data, for which new sequence data were generated in the current study. Specimens of *H. gertschi* were collected by S. Lew at the Angelo Reserve, Mendocino County (39°43′N, 123°39′W), and from Del Norte County near the Oregon border (41°59′N, 123°43′W), California, USA, in May 2003. Voucher specimens are stored in the collection of the Essig Museum of Entomology, UC Berkeley, under the voucher codes EMEC50654 and EMEC50993, respectively.

Molecular methods.—DNA was extracted from two legs from each of the *H. gertschi* specimens using a Qiagen DNeasy Tissue Kit's standard protocol. The 18S gene was directly PCR-amplified in three parts using primer pairs 1F-5R, 5F-9R, and 3F-7R (after

Table 1.—Taxa examined, with total gene length and p-distance between primary sequence of individuals and *Aphonopelma* sp. reference sequence. Length includes terminal primer regions (approximately 50 base pairs (= bp); 23 per primer + 4 bp downstream in the 3′ direction from primer 9R). * indicates partial sequences (see text), with lengths estimated by assuming uniform sequence length relative to reference sequence at missing terminal regions.

Order (lineage represented)	Taxon sampled	Genbank Accession Number	Total length (bp)	Uncorrected p-distance to reference
Araneae (Orbicularia)	*Hyptiotes gertschi* Chamberlin & Ivie 1935	DQ015708	1902	10.8%
Araneae (Mygalomorphae)	*Aphonopelma* sp.	X13457	1814	—
Araneae (Mesothelae)	*Liphistius bicoloripes* Ono 1988	AF007104	1808	2.4%
Araneae (derived orb-weavers)	*Nesticus cellulanus* (Clerck 1757)	AF005447	1816	7.8%
Araneae (RTA clade)	*Coelotes terrestris* (Wider 1834)	AJ007986	1814	5.7%
Opiliones	*Odiellus troguloides* (Lucas 1847)	X81441	1810	6.2%
Scorpiones	*Androctonus australis* (Linnaeus 1758)	X77908	1812	6.7%
Pseudoscorpiones	*Roncus* cf. *pugnax* (Navás 1918)	AF05443	1808	11.0%
Acari (Ixodidae)	*Amblyomma glauerti* Keirans, King & Sharrad, 1994	AF115372	1802	10.3%
Solifugae	*Eusimonia wunderlichi* Pieper 1977	U29492	1802	7.7%
Amblypygi	*Paraphrynus* sp.	AF005445	1810	4.8%
Uropygi	*Mastigoproctus giganteus* (Lucas 1835)	AF005446	1810	4.9%
Schizomida	*Stenochrus portoricensis* Chamberlin 1922	AF005444	1809	5.5%
Ricinulei	*Pseudocellus pearsei* (Chamberlin & Ivie 1938)	U91489	1813	5.1%
Palpigradi	*Eukoenenia* sp.	AF207648	1810	7.4%
Acari (Ixodidae)	*Sejus* sp.	AF287237	1880*	21.1%
Acari (Ixodidae)	*Lohmannia* sp.	AF287234	1887*	12.3%
Acari (Ixodidae)	*Alicorhagidia* sp.	AF022024	1872*	13.8%

Giribet et al. 1999), to amplify fragments of length ~950 bp, ~850 bp, and ~1000 bp, covering the first half, second half, and an overlapping central portion of the gene respectively. The PCR protocol (modified from Hedin & Maddison 2001) used 35 cycles of 30 s at 95° C melting temperature, followed by 30 s at an annealing temperature of 52° C, followed by an extension step of 45 s at 72° C, with 3 s added to this extension for every cycle after the first. In addition, these cycles were preceded by an initial melting at 95° C for 2 min, with a 7 min final extension at 72° C. A MasterAmp PCR Optimization Kit (Epicentre Technologies) was used to choose appropriate buffers to stabilize the PCR reaction, which improved yield and quality of PCR products. Molecular extraction vouchers were stored at −80° C.

Gene size, purity and concentration were assessed by running out a portion of the PCR product on a 1.5% TBE/agarose gel. PCR products were cleaned using Qiagen QiaQuick PCR Purification Kit, and cycle sequenced in both directions using dye terminators (after Sanger et. al 1977). Cycle sequencing products were analyzed using an ABI 3730 capillary autosequencing machine. Individual sequences checked against their complementary sequences, using Sequencher 3.1.1 (Gene

Codes Corporation). This program was also used to assemble contigs of all three overlapping primer regions for each *H. gertschi* specimen, to create a single sequence for each. Additional sequences (see Table 1 for accession numbers) were acquired from GenBank for comparison.

Alignment and Comparisons.—Using Clustal X version 1.83 (Chenna et al. 2003), a static alignment of multiple sequences was created using the default settings (gap insertion cost 15, gap extension cost 6.66, transition cost 0.5 times transversion cost). Treating the *Aphonopelma* sp. sequence and model as references, gaps in the aligned sequences were compared to the secondary structure model to detect regions where insertions and deletions have occurred.

Modeling of insert regions in *H. gertschi*.—In areas with insertions > 3 bp relative to the *Aphonopelma* sp. reference sequence, secondary structures were modeled using the web-interface version of the RNAfold program in the Vienna Package (http://www.tbi.univie.ac.at/~ivo/RNA/; Hofacker 2003). This program uses a dynamic-programming algorithm with a variety of parameters to estimate the secondary structure based on minimizing the free energy of the possible stem-loop structures from the primary sequence. The parameters used include RNA base-pairing energies plus a variety of experimentally determined adjustments to this cost, including energy estimates for loops of various sizes and locations, and for single- and double-stranded multi-base motifs known to affect local stability (Mathews et al. 1999).

The expanded regions of the *H. gertschi* 18S gene were modeled using the primary sequence of the insert region plus the four complementary bases at each insertion site that could be homologized with the reference sequence and structure from *Aphonopelma* sp. Because the RNAfold program had difficulty aligning the complementary 4-base-pair ends properly in some cases, we added a complementary 5-nucleotide extension at each terminus to anchor the ends of the sequence and stand in for the rest of the structure, so that each primary sequence input took the form: 5′-GGGGG- primary sequence -CCCCC-3′. We also tested the RNAfold program's ability to predict the structures in the variable arms of the Hendriks et al. (1988) *Aphonopelma* sp. model using the same method, then calculated the percentage of base-pairs and loop members in the algorithm output that correctly matched the model.

RESULTS

Comparisons of 18S sequence length and primary sequence divergence can be seen in Table 1. Most arachnid 18S sequences are in the range of 1800–1810 base pairs (including primer regions), with the *H. gertschi* and tick sequences being the exceptions (greater by 85 and 56–64 bases, respectively). Figure 1 shows a schematic of the Hendriks et. al (1988) model of spider 18S, with regions with *H. gertschi* and tick inserts marked with black arrows and a hatched bar, respectively.

***Hyptiotes gertschi* and tick insertions.**—The two *H. gertschi* sequences are identical, as are sequences from an additional set of amplifications of the Del Norte County specimen. However, in contrast to the marked similarity in length and structure across the arachnid exemplars from GenBank, the *H. gertschi* sequences are 86 nucleotides longer than the next-longest spider 18S (*Nesticus cellulanus*), and we estimate it to be 21 nucleotides longer than the next-longest arachnid 18S (*Sejus* sp., Acari, Ixodidae). This additional sequence is found in two large inserts—a 28-base extension of the helical arm E10-1 (Figure 2a), and 27-base extension of E10-2 (Fig. 2b)—and the rest is found as smaller inserts of 5, 6, and 13 nucleotides in helices 6, 41, and 47 respectively (Figs. 3a, b, and c). Three near-complete tick sequences were also found to have large inserts in the terminal arms of branch 10 (see Table 1).

Models of the *H. gertschi* inserts based on free-energy minimization show that the inserted sequences extend the helices in all of the cases except structure 6, where the helical stem is shortened and the terminal loop expanded. The RNAfold program also correctly replicated 90% (40 of 48) of the stem base-pairs in the Hendriks et al. (1988) model in the arms in which the inserts were found, which is comparable with the 73% base-pairing accuracy for this parameter set calculated by Mathews et al. (1999) using a variety of other well-characterized genes.

DISCUSSION

The most remarkable results from the current study were the number of sizable inser-

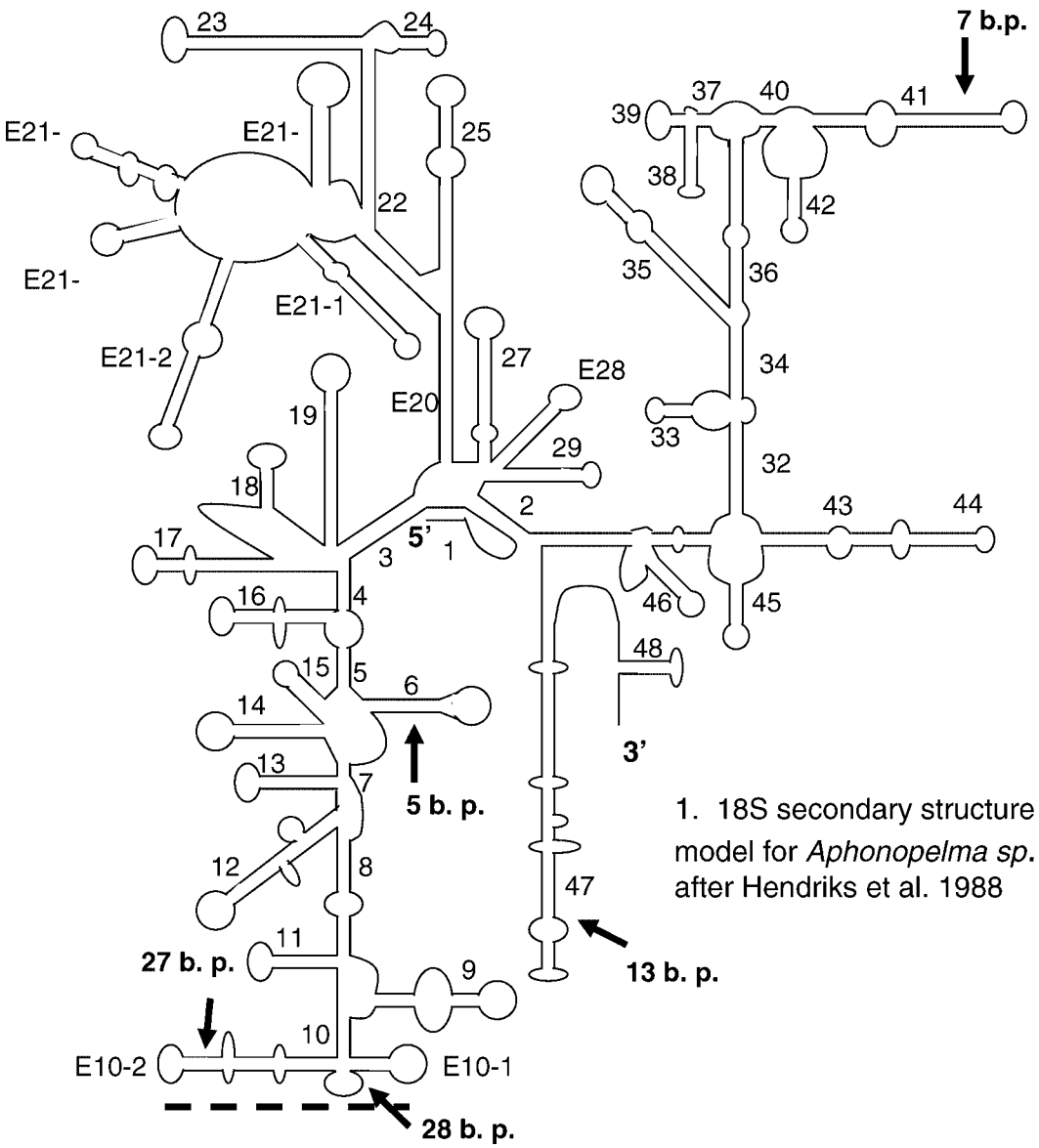
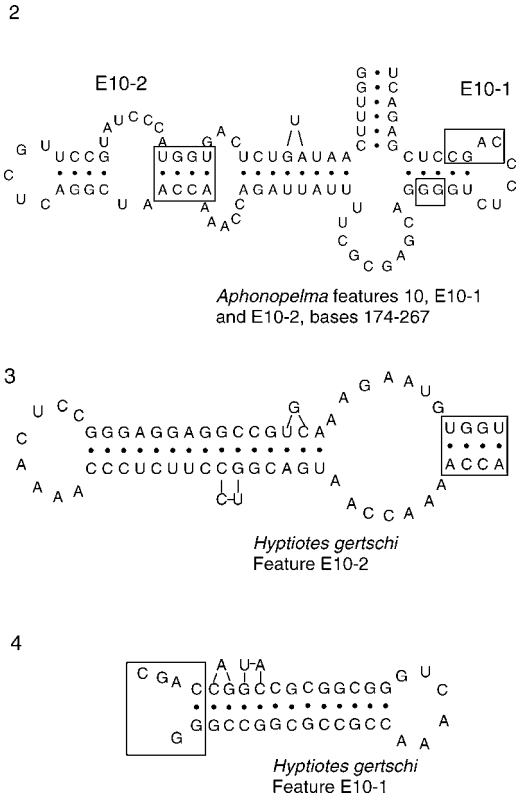
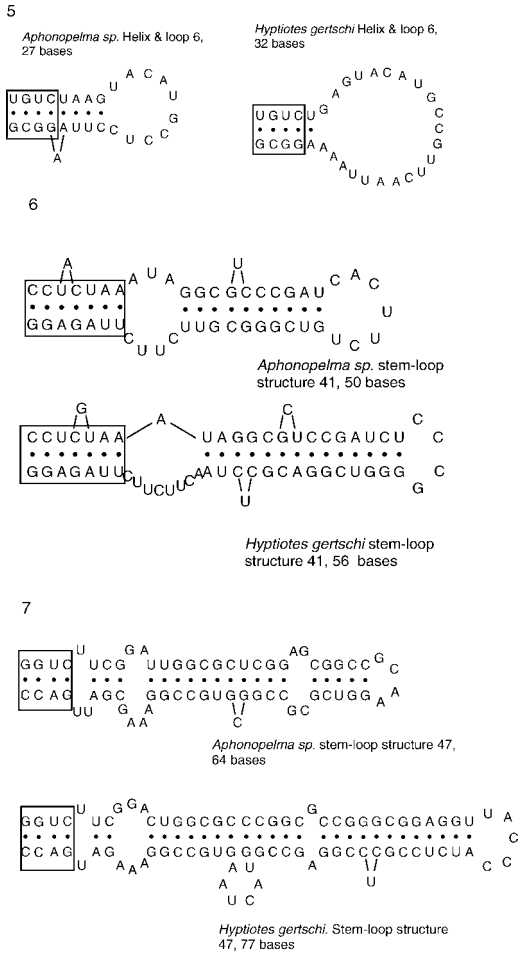


Figure 1.—General model for secondary structure of arachnid 18S ribosomal DNA. Small numbers refer to general stem-loop numbering system after (Nelles et al. 1984). Those features preceded by an ''E'' are specific to eukaryotic 18S sequences. Arrows and bold numbers refer to locations and size (in bases relative to tarantula model) of *H. gertschi* inserts, respectively. Hatched bar represents region with large inserts found in 3 tick taxa.

tions in the *H. gertschi* sequences and, in particular, the shared expansion of stem E10 found in both *H. gertschi* and ticks. In *H. gertschi* all insertions took place in known ''variable regions'' of the eukaryote 18S structure; helices 6, E10-1 and -2, 41, and 47 belong to regions that have been characterized as variable across a range of taxa (Van de Peer 1996). The RNAfold models show that much of the inserted *H. gertschi* sequence is self-complementary and these insertions function to extend the stem regions, rather than increase the loop size, except in Helix 6. This extension and maintenance of complementary



Figures 2–4.—Region of arachnid 18S gene with unusually large inserts. 2. Basic structure for helices 10, E10-1 and E10-2 in arachnids. Primary sequence and model after Hendriks et al. (1988); 3. Expanded E10-2 stem-loop structure in *H. gertschi*; 4. Expanded E10-1 stem loop in *H. gertschi*. Boxes represent areas of homology where expanded arms attach to conserved, adjacent parts of the structure.



Figures 5–7.—Smaller inserts and secondary structure model comparisons. 5. Structure 6 with 5 base pair insertion in *H. gertschi*; 6. Structure 41 with 6 inserted bases in *H. gertschi*; 7. Structure 47 with 13 inserted bases in *H. gertschi*.

stems has remarkable parallels with the findings of Hancock & Vogler (2000), who revealed a similar pattern of complementary stem expansion in the evolution of hypervariable regions of 18S in tiger beetles.

A variety of factors suggest the *H. gertschi* sequence represents the functional 18S rRNA gene rather than a pseudogene copy or experimental artifact. The presence of insertions in areas amplified by three different primer pairs, the replication of the sequence in individuals from two populations, the maintenance (and, in some cases, increase in length) of the base-pairing in stem structures, and the non-random, self-complementary sequence found in both large and small inserts, make it likely that we have sequenced a functional gene and not a pseudogene or an experimental artifact. One would expect to see decay of pseudogene sequence relative to the selectively-constrained functional gene (Giribet & Wheeler 2001), and this decay (in the form of random mutations) should be equally distributed throughout the sequence rather than restricted to known variable regions. Though some metazoans, such as helminths, are known to carry two functional copies of the 18S gene with differing sequences (Carranza et al. 1996), we found only single bands in our agarose gel visualizations of all three PCR products, and no second allele or double-peak patterns were seen in the raw sequence data,

despite repeating the amplification and sequencing processes, suggesting the existence of a single, uniform 18s sequence in *H. gertschi*.

How exceptional is the *H. gertschi* 18S rRNA? It seems to be an anomaly for spiders, which, based on those sampled in the current study, generally vary little even between the most distantly related lineages. Along with the tick data, these anomalous sequences show that the presence of large inserts in arachnids, though rare, is consistent with a general eukaryotic 18S evolutionary tendency—overall structure is maintained, while rare inserts appear in known variable areas. It is worth noting that only the *H. gertschi* gene contains expansions of several variable regions while the tick inserts are restricted to the E10 region.

With only two populations of *H. gertschi* sampled in the current study, we cannot establish the phylogenetic distribution of the 18S rDNA extension, whether it occurs in other *Hyptiotes* species, other uloborid genera, or their sister group, the Deinopidae. Such extensions have not been described from other Orbicularia, the sister-group of the deinopoid (Uloboridae + Deinopidae) clade, but may be more widespread within the deinopoids. The large number of inserts found in *H. gertschi* in this study suggests that there might be variation in number and size of inserts found in different taxa, since it seems unlikely that all these inserts appeared simultaneously. It would be interesting to map any such variation onto the existing phylogeny of Deinopoid genera (Coddington 1986) to determine the pattern of evolution of the 18S gene in greater detail. Alternatively, if variation seems to be limited to a subset of *Hyptiotes* or a small number of uloborid genera, the inserts themselves could be evaluated as phylogenetic characters within these groups.

Once the taxonomic range and amount of variation of these inserts has been characterized, hypotheses about causal biological, physiological or ecological factors allowing such inserts to occur can be tested rigorously. Comparative studies could also be performed with the wide range of metazoan taxa which show similar inserts, such as myriapods, proturans, and helminth worms, to find general correlations between 18S size and phenotypes (Carranza et al. 1996; Giribet & Wheeler 2001).

While the RNA folding algorithm used may not predict intra-molecular folding dynamics of rRNA accurately, it provides a repeatable method for producing a plausible structure for sequence data which lacks obvious homologs from related taxa for comparison. The algorithm also gives a reasonable basis for judging whether an insert extends a helix, a loop, or both, particularly with short sequences such as those modeled in this study. With changes affecting sequence length concentrated in the terminal ends of known stem-loop structures, the *H. gertschi* and tick 18S genes appear to be the exceptions that prove the rule; bases come and go, albeit very rarely, but the helical backbone and location of stem and loop structures of the small subunit ribosomal RNA have remained conserved throughout arachnid evolution.

Because the expanded sequence has only been found in a single spider species, it is impossible to argue that this is evidence of any trend in 18S evolution in spiders. However, the increase in size of the 18S gene seen here differs from the documented trend (relative to other metazoans) toward reduction in size in the hypervariable region of the mitochondrial 16S gene (Smith & Bond 2003) and in the arms of some tRNA genes (Masta & Boore 2004) of spiders. It should be noted that the cited trends are in mitochondrial genes, whereas 18S is part of the nuclear genome, and the two genomes may be subject to different selective pressures or constraints.

To understand the broader pattern of 18S size change, it is useful to look at the evolution of the 18S gene in other metazoan taxa. Giribet & Wheeler (2001) showed that the general pattern of 18S evolution across a much broader sampling of taxa, including hexapods, chelicerates, myriapods, and crustaceans, is one of conserved length in the 1800 bp range, with occasional increases in size. Deletion events appear to be exceedingly rare. The data presented here are in keeping with those findings, although the total length for the *H. gertschi* 18S gene is greater than that of any of the 49 chelicerate taxa reported by Giribet & Wheeler (2001) or of any full arachnid 18S sequence currently found in the Genbank database. Complete or nearly-complete sequences are required for secondary structure modeling since there is long-range complementary base-pairing in 18S secondary

structure (Telford et al. 2005). Unfortunately for the pursuit of whole-gene comparisons and structural modeling, many arachnid studies have used only half of the 18S gene, (e.g., Wheeler & Hayashi 1998; Arnedo et al. 2004), and there is a sizable and important spider lineage, the haplogynae, for which no complete 18S sequence is available.

As for the overall utility of 18S data for arachnid phylogeny, this data set shows that the Hendriks et al. (1988) model is sufficient for locating structural changes in the 18S gene for all known arachnid genotypes, though the value of modeling based solely on primary sequence using thermodynamic predictions on a single taxon is limited. In the majority of cases, alignment is trivial with a mean of less than 1% sequence length divergence between most taxa. This similarity in sequence-length across Araneae is also corroborated by data from a family-level study of the RTA clade by the authors (unpublished data). For data partitioning, relative-weighting, and model-choice purposes, the number and locations of the stem-loop structures of the 18S gene remain largely identical to that of most sequenced eukaryotes. Determination of stem and loop regions should be achievable with some confidence for the large majority of arachnid cases, using the Hendriks et al. (1988) model as a guide.

In exceptional situations where homology assessments or stem-loop status of individual bases are difficult, such as within the tick-specific inserts seen here, removing the insert data may be defensible, for two reasons: first, the tendency of insertions to occur independently in the same areas across widely divergent taxa (such as spiders and ticks having inserts in the E10 region) could plausibly add homoplasy to a data matrix and place taxa in incorrect groupings. Second, because the insertions tend to be small relative to the more easily homologized portions of the rRNA (much smaller, for instance, than the $>$ 200 bp insertions found in many myriapods, see Giribet & Wheeler 2001), and only occur in a small number of taxa, there is likely to be little reduction in phylogenetic signal from the sequence if an insert region is excluded from analysis.

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