MITOCHONDRIAL DNA SEQUENCES CODING FOR A PORTION OF THE RNA OF THE SMALL RIBOSOMAL SUBUNITS OF *TETRAGNATHA MANDIBULATA* AND *TETRAGNATHA HAWAIENSIS* (ARANEAE, TETRAGNATHIDAE)

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ABSTRACT. A region of mitochondrial DNA coding for most of the third domain of the 12S rRNA of the ribosomal small subunit has been sequenced from two spiders in the genus *Tetragnatha* (Araneae, Tetragnathidae): a circumtropical species *T. mandibulata* and an endemic Hawaiian species *T. hawaiensis*. The secondary structure of the spider ribosomal RNA shows strong similarity to that of insects. Across this region, the two *Tetragnatha* sequences are 22% different. The *T. mandibulata* sequence is 36% different from the homologous segment in *Drosophila yakuba* and 51% different from the same segment in *Homo sapiens*. The spider sequences are sufficiently variable to be useful in studying genetic relationships among at least some of the species in this genus.

A powerful approach to studying genetic relatedness of species involves DNA sequence comparisons which can be used to estimate branching order of phylogenetic trees as well as evolutionary distance between extant taxa (Felsenstein 1988). Recent application of the polymerase chain reaction and direct sequencing has accelerated efforts to examine a wide range of taxa with DNA comparisons (Kocher et al. 1989; Martin et al. 1990). To date the use of this method in studying spiders has not been reported. Here we describe a procedure we have used to amplify and to determine the sequence of nucleotides of a 279-base-pair region of mitochondrial DNA (mtDNA) from two species of the genus *Tetragnatha* (Araneae, Tetragnathidae).

Sequencing DNA has the following advantages over other techniques of genetic comparison: (1) it has greater resolving power over a hierarchical range of intraspecific to intergeneric comparisons, (2) sequences are easily compared with known sequences from other species, and (3) functional information on products encoded by DNA allows strong inferences on the selective importance of mutations observed, allowing character weighting for sites that are not selectively neutral (Kocher et al. 1989). Mitochondrial DNA was chosen for this work because its matriarchal inheritance and lack of recombina-

tion make it often more instructive than nuclear DNA in comparing taxa (Wilson et al. 1985). This DNA evolves rapidly at the sequence level in arthropods (DeSalle and Templeton 1988; Palumbi and Benzie 1991) and has proven useful for comparing recently-evolved taxa in Hawaii (DeSalle et al. 1987).

In order to determine nucleotide sequence in a particular mtDNA segment, the segment must first be amplified either by genetic cloning or by using the polymerase chain reaction (Mullis and Faloona 1987; Saiki et al. 1988). The latter method depends upon knowledge of oligonucleotide sequences that flank the segment of interest and that serve as primers for enzymatic amplification. Kocher et al. (1989) have described universal (highly-conserved) oligonucleotide sequences flanking a 300-base portion of the third domain of the 12S ribosomal RNA gene that can be used to amplify mtDNA from animals as diverse as humans and invertebrates. The conservation of these primers makes them useful to investigators sequencing the DNA of species for which there is no previous sequence information (Simon et al. 1990). We first used insect-specific primers for the 12S rRNA region, slightly modified by C. Simon (pers. comm.) from the original primers of Kocher et al. (1989), to amplify and sequence the DNA of *Tetragnatha mandibulata*

Walckenaer. We then designed a spider-specific primer based on this sequence to amplify and sequence the same region from a Hawaiian endemic species *T.hawaiensis* Simon.

MATERIALS AND METHODS

All solutions used were either sterilized or prepared using sterile deionized, distilled water, and all glass- and plastic-ware were sterile with the exception of the Centricon tubes (see below). The chelicerae and a front leg of each spider were placed in 70% ethanol as voucher specimens. Total (genomic) DNA was prepared by homogenizing a single spider in a 1.5 mL Eppendorf tube in 200 μL of 25 mM Tris HCl (pH 7.5), 100 mM EDTA, 2% SDS, and 200 μg/mL Proteinase K, followed by incubation for 1–2 hr in a water bath at 65 °C. The homogenate was extracted first with phenol, previously-equilibrated with 1 M Tris HCl buffer (pH 7.5), then with 25:24:1 phenol/chloroform/isoamyl alcohol 1 to 4 times (until all the protein-containing white interface was removed), and finally with 24:1 chloroform/isoamyl alcohol. One-half volume of a 7.5 M solution of ammonium acetate was added to the extract to achieve a final concentration of 2.5 M and mixed well before adding 2½ volumes of 95% ethyl alcohol and mixing again. This solution was incubated at room temperature for 15 min to allow for DNA precipitation. The DNA was pelleted by centrifugation at 14,000 x g for 15 min at room temperature, washed with 500 μL of 70% ethyl alcohol, dried under a vacuum, and resuspended in 25 μL water. Five μL of this preparation was electrophoresed on a 0.8% agarose gel in Tris-borate buffer and stained with ethidium bromide as described in Sambrook et al. (1989) to ensure that high molecular-weight DNA was present. The preparation is stable indefinitely when stored at −20°C.

One μL of a 1:10 dilution in water of this DNA and 2.5 units of DNA polymerase from *Themus aquaticus* (Perkin-Elmer Cetus) were incubated in 100 μL of buffer containing 67 mM Tris HCl (pH 8.8), 3 mM magnesium chloride, 16.7 mM ammonium sulfate, each of four deoxynucleoside triphosphates at 200 μM , and each of two primers at 1 μM according to the protocol of Saiki et al. (1988). Thermal profile for 45 cycles was as follows: (1) DNA melting for 1 min at 94 °C, (2) annealing for 1 min at 50 °C, and (3) polymerization for 2 min at 72 °C to amplify the double-stranded sequence. The primers and their

location in the *Drosophila yakuba* mtDNA sequence of Clary and Wolstenholme (1985), were 12St-L (14503), a *Tetragnatha*-specific primer designed by H. Croom: 5'-GGTGGCATTT-TATTTTATTAGAGG-3' and 12Sbi-H (14214), an insect-specific primer designed by C. Simon: 5'-AAGAGCGACGGGCGATGTGT-3'. One μL of the double-stranded product of the first amplification was cycled under the same conditions as above except that only one primer was added to the incubation mixture. This produced a single-strand sequencing template, which was processed in a Centricon 30 microconcentrator (Amicon) to concentrate and purify the DNA product. The template was then sequenced by the dideoxy chain termination method of Sanger et al. (1977) as described by Engelke et al. (1988), using the primer that had not been used in the second amplification. DNA from three individuals of each species was sequenced in both directions, and no intraspecific variation was observed.

Homologous sequences of DNA from different taxa were aligned to minimize deletions or additions using software written by S. R. Palumbi and C. Parrish. Pairwise percent differences were calculated by counting only sites where both species have nucleotides in our aligned sequences. One strand of spider DNA was folded to show its secondary structure using the folded sequences of insects as a guide (Clary and Wolstenholme 1987; Simon et al. 1990).

RESULTS AND DISCUSSION

The DNA sequences from the two *Tetragnatha* species were compared with the 12S rRNA genes from both *Homo sapiens* and *Drosophila yakuba* (Fig. 1). The two *Tetragnatha* sequences are 22% different from each other, 36% different from the homologous segment in *Drosophila* (Clary and Wolstenholme 1985), and 51% different from the same segment in *Homo* (Anderson et al. 1981). In comparison, the *Drosophila* and *Homo* segments differ by 45%. These values are based solely on pairwise nucleotide differences, ignoring insertions and deletions, using the alignments in Fig. 1. It is difficult to align nonconserved regions of DNA from distantly-related groups, so other alignments may yield slightly different percentages. Likewise, the differences here are uncorrected for multiple mutations at the same site, which has the effect of

Tetragnatha mandibulata   `AACATGTTTATTAATCGACATTACACGATTATTTT`
Tetragnatha hawaiensis   `..T.......A.......ATC..........C...`
Drosophila yakuba   `...C.....TG.......T.A.C......GGACC.`
Homo sapiens   `.G.C....CTG.......T.AAC.C....C.ACC.`

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TACTTTTTTATAA----ATTTTATATACCTCCGTCC--AGAATAAATTTTTAATA---TTT
C......AATA.T................G....TT...........AAAA..-.......
.....AAA.T.GTAATC.G..........GT...TATC......TT..A.A.GA.TAA.AA
C..CACC.CT.GC..TC.GCC........G..A..TTC..C.A.CCC.GA.G.AGGCTACA

TATTCAAAATAATATTATAATAATTTA-----GGTAAAGGTGTAGACTTTAAATTAGT-TT
...........AT..C..GA......................A...A..T.AG..A..A
.......T.-.T.T.A....A.....TATCA.A.C.........CT.A..TT.A....AA
A.G.A.GCGC..GTACCC.CGT.AAG.CGTTA...C.........C.CA.G.GG.G.CAAG

AAATGTGTTACATTAAAAATTATTT----AAGAATTATTTTTTATAA----CAATATGA
......A............AA..........G....AAC.AA.C.T....AGT.T..A..
T....G......A....-T...........ACGGATAAAA....G.AA..A...T.T...
.....G.C......TTCT.CCCCAGAAAACTACGA..GCCC....G.AACTT..GGGTC..

AAGAGGATTTATAAGCTACTTTTTAATTAAAATTTTAACTTGAATTAAAAA--TAAATGCG
..A.............-..TAA...T........A...T....................TA
.G.T.....GGT..TA.AA..--.TAA.G.T.AA...T....-..TT.GCTC....ATAT
.G.T......GC..TA.AC.A-AG.G..G.G.GC.T.G.....-C.GGGCCC.G..GCGC
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Figure 1.—Comparison of Mitochondrial 12S Ribosomal DNA from *Tetragnatha mandibulata*, *Tetragnatha hawaiensis*, *Homo sapiens*, and *Drosophila yakuba*. Dots represent positions that are identical to the top sequence, and dashes represent gaps in the sequences required to maximize alignment. The sequence from the *Drosophila* is that of Clary and Wolstenholme (1985) between their base pairs 14236 and 14502. The *Homo* DNA sequence is that of Anderson et al. (1981) between their bases 1201 and 1475.

making the more distantly-related taxa appear deceptively similar.

When using the polymerase chain reaction with genomic DNA, one must always consider the possibility that nontarget nuclear or mitochondrial DNA has been amplified. Using the method of Palumbi and Wilson (1990), we separated mtDNA from nuclear DNA of 25 specimens of *T. mandibulata* on a cesium chloride gradient before amplifying and sequencing the mtDNA fraction. The sequence obtained was identical to that in Fig. 1. In order to verify that the spider sequences code for the third domain of 12S rRNA, a single strand was folded to generate the secondary structure stabilized by hydrogen-bonding between complementary bases. In all of the taxa studied to date (Dams et al. 1988; Simon et al. 1990), the folded structure of this domain forms helical paired stems and unpaired loops. The structure obtained from *T. mandibulata* (Fig. 2) is essentially the same as those of the other known taxa. Conservation of secondary structure, despite the large overall sequence differences among taxa (Fig. 1), suggests we have sequenced a func-

tional ribosomal gene. The third domain of the small rRNA encoded by nuclear DNA is both larger than, and has a structure distinct from, that represented in Fig. 2 (Woese et al. 1983; Dams et al. 1988).

We have sequenced most of the homologous region from 19 other spiders: *Aphonopelma chalcodes* Chamberlin (Araneae, Theraphosidae), *Doryonychus raptor* Simon (Araneae, Tetragnathidae), and 17 endemic Hawaiian *Tetragnatha* taxa. In the case of the *A. chalcodes*, cesium chloride gradient purified mtDNA was used for the amplification instead of genomic DNA. We found each of these sequences to be more similar to the spider sequences in Fig. 1 than to those of any other known taxa (Croom and Palumbi, unpublished). Such similarity suggests that neither of the sequences reported in this paper is from contaminating DNA.

Interestingly, 83% of all bases in the two *Tetragnatha* sequences are either A or T. In the strand shown in Fig. 2, the frequencies for bases are: 39% A, 43% T, 8% C, 10% G. The percent AT across this region is 79% for *Drosophila* and
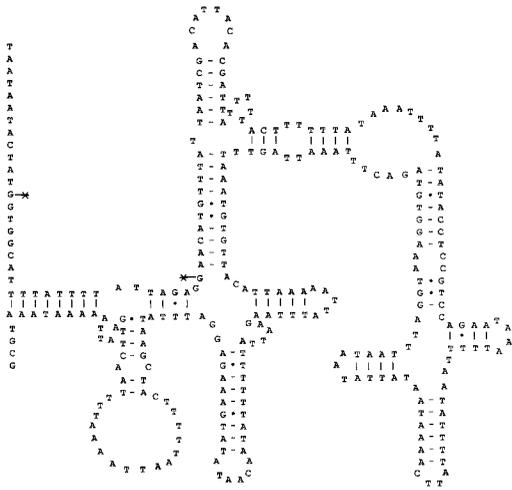
Figure 2.—Mitochondrial DNA of *Tetragnatha mandibulata* folded to show the secondary structure of the third domain of 12S ribosomal RNA for which it codes. Dashes represent hydrogen bonds between A and T or C and G, and dots represent the weaker hydrogen bonds between T and G. The portion of the sequence between the asterisks * is that of the primer 12St-L. Folding was based on the structure of Simon et al. (1990).

53% for *Homo*. This is consistent with the observation that all known arthropods have high AT content in this domain (Simon 1991).

The third domain of rRNA is highly conserved across many taxa (Kocher et al. 1989). Hence, the large difference (22%) between these two *Tetragnatha* species is surprising but not without precedent. Palumbi and Benzie (1991) have found similar percent diversity in the homologous mtDNA region among species of shrimp in the genus *Penaeus*. In addition, we have found 3–13% variation in the homologous DNA from 18 different endemic Hawaiian tetragnathids that appear (on morphological grounds) to have been derived from a single introduction to the islands (Gillespie, unpublished). Such high diversities imply that these species have either diverged for a long period or that their sequences have diverged at a rapid rate. We are currently using these sequences, as well as those coding for mitochondrial proteins, to conduct systematic analysis of this group which has undergone explosive radiation (Gillespie, in press) in the Hawaiian archipelago.

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