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A fragment of the mitochondrial 16S rRNA gene as a molecular marker in phylogenetic studies on feather mites

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ABSTRACT. The use of a 200 bp fragment from the 3 '-end of the mitochondrial 1 6S rRNA gene (rDNA) as a marker in phylogenetic studies on feather mites was investigated. DNA extraction and amplification technique from ethanol preserved mites have been developed and described. Nucleotide sequences of the fragment 16S rDNA from three species of feather mites *(Pomeranzevia ninnii, Scutulanyssus obscurus, Freyana anatina)* and two ticks *(Argas reflexus, Ixodes hexagonus)* were presented and aligned; a scorpion, *Vejovis carolinianus,* was used as an outgroup. Phylogeny derived using maximum parsimony method was congruent with known phylogenetic relationships of investigated taxa.

Key words: Acarology, mites, PCR, 16S rDNA, DNA sequencing, phylogeny.

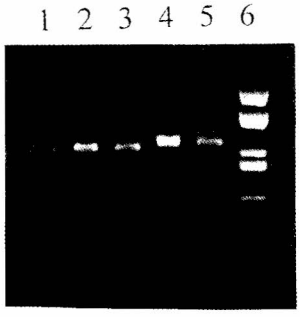
INTRODUCTION

Molecular techniques such as izoenzyme electrophoresis, immunological techniques, molecular cytogenetics, DNA-DNA hybridization, restriction site analysis of mitochondrial DNA (RFLP) and DNA sequencing have made great contributions to systematics, population biology and evolutionary studies. Techniques that use DNA sequences (DNA hybridization, RFLP analysis, and sequencing) are inherently more accurate than protein-based methods (allozyme electrophoresis and immunological techniques). Similarly, among the DNA methods, direct comparison of DNA sequence information is supposed to be the most informative. The sequences assayed have come from the nucleus and the mitochondrium (AVISE 1994, HILLIS and MORITZ 1990). Although this method provides useful phylogenetic information, it sometimes suffers in comparison to morphological studies in coverage of taxa. This is because

of high-quality tissue material needed for such studies is not readily available for some animals (e.g. small arthropods). It is especially complicated in investigations on small, not numerous individuals collected in the field and preserved for a long time in ethanol. Recent advances in polymerase chain reaction (PCR, SAIKI et al. 1988) technology have alleviated this problem. PCR amplification can be used for selection, isolation and sequencing homologous fragments DNA from different taxa.

The application of PCR technology for investigation of mite phylogeny is still a new approach. Some investigations are done in spider mites *(Tetranychidae)*. NAVAJAS et al. (1994) sequenced the ITS2 rDNA spacer and a fragment of the cytochrome oxidase I gene to asses the level of variability in intraspecific diversity in tetranychid species. FOURNIER et al. (1994) had constructed a restriction map of the mitochondrial DNA of the *Tetranychus urticae*. The most complex studies are carried out on ticks (*Ixodida*). WESSON et al. (1993) investigated the validity of species status of *Ixodes dammini* using sequence variation in the two internal transcribed spacers (ITS 1 and ITS2) of rDNA. These sequences are highly variable and can be used to assess species relationships. For resolving intermediate taxonomic levels, i.e. from species to order, sequencing of mitochondrial rRNA genes is highly informative. To test the existing phylogeny of the Ixodida, BLACK and PIESMAN (1994) sequenced the 3'-end of the mitochondrial 16S rRNA gene (rDNA) in 36 hard- and soft-tick species. In general their results indicated that examination of the 16S rDNA was useful in establishing the phylogenetic reconstruction at or below the family level. Till now there are no sequence data, published in Gene Bank that can be used for phylogenetic studies from other groups of mites, i.e. as extensive group of mites as *Astigmata*.

In this report a protocol for extracting DNA from ethanol preserved feather mites and ticks for PCR-based techniques is presented[1]. Usefulness of partial 16S rDNA nucleotide sequence as a molecular marker in feather mites was tested by investigation of the taxa with previously known phylogenetic relationships[2].



1. PCR products of the fragment 1 6S rDNA from *F. anatina* (1), *P. ninnii* (2), *S. obscurus* (3), *A. reflexus* (4) and *I. hexagonus* (5); lane 6 - pUC19 HaeIII digest

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[2]The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF005071-AF005073).

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                    1-10       11-20      21-30      31-40
S. obscurus         AAATCATTAG TTATTTAATT GGTTACTTGT ATGAATGGGG
P. ninnii           GAATCATTAG TTATTTAATT GGTTACTTGT ATGAATGAAT
F. anatina          AGGTCATTAG CTATTTAATT GGTAGCTTGT ATGAATGAAT
I. hexagonus        TTGAAATAAG GC-TTTAAAT GGGTGC-TAA GAGAATGGTT
A. reflexus         TTGAAATAAG AT-CTTAAAT GGGTGC-TAA GAGAATGGCT
V. carolinianus     TAATCATTTG TCTTTTAATT GAG-AC-TA- --GAATGAAA

                    41-50      51-60      61-70      71-80
S. obscurus         GTACTGGCTT AATA-TTATT C----TTTTT TCTT-AATTT
P. ninnii           TTACTGAGGT AAT--TTTTT TA--GTTTTT TCTT-AATTT
F. anatina          TAACTAAGAT TTTA-TTAAT T---TTTATT TG---AATTT
I. hexagonus        TAACGATTAA TAAC-TTTCT TTAATTAATA AATT--GAAT
A. reflexus         GTACAAAGAT TTTC-TTTCT TGGATTAATT AATT-AGAAG
V. carolinianus     GGTTAGACTA TGTAATTTGG TCTTATAATA ATATTTGGAA

                    81-90      91-100     101-110    111-120
S. obscurus         ATAA--TTTT TGTTGAAAAT AACAATTGAT TCTTT-AAGA
P. ninnii           ATGA--TTTT TGTTGAAAAT AACAAAAATT TTTCT-AAGA
F. anatina          ATAG--TTTT C-TTGAAAAT ACCAAAATTT TCTTT-AAGA
I. hexagonus        TTAA-TTTTT TTGTGAAGAA GAA-AAATTT AAATTAGGGA
A. reflexus         TTGG-TTTTT AAGTGAAAAA ACTTAAATTT AATTTTGGGA
V. carolinianus     ATTTATATTT TAGTAAAAAG -CTAAAATAA G-TTTAAAGA

                    121-130    131-140    141-150    151-160
S. obscurus         C-GAAAAGAC CCTA-GAATT TTTACTAAT- ----------
P. ninnii           C-TAAAAGAC CCTA-AAATT TTTA-TAA-- ----------
F. anatina          C-TAAAAGAC CCTA-AAATT TTTA-TAATT ----------
I. hexagonus        C-AAGAAGAC CCTATGAATT TTTATTAGAT TGTGATGAGA
A. reflexus         C-AAGAAGAC CCTATGAATT TTTACTAAAC AGATGCAAGA
V. carolinianus     CGAAGAAGAC CCTATCAAGT TTTACAATAA TTATCTTATT

                    161-170    171-180    181-190    191-200
S. obscurus         ---------- CACAAGATTA GGTTGGTTGG GGAAACAAAT
P. ninnii           ---------- --CGGTTGTT ATTTGATTGG GGAAATAAAA
F. anatina          ---------- AAAAATATT- ATTTGGTTGG GGCAATAATT
I. hexagonus        ATTTAATT-- --GAAATCTA ATTTGGTTGG GGTGATTAAA
A. reflexus         A--------- -AGGTGTTTA GTTTGGTTGG GGCGATTTTT
V. carolinianus     TTCAAATTAA GAAGTTTATT GTTTGGCTGG GGCAGCAAAT
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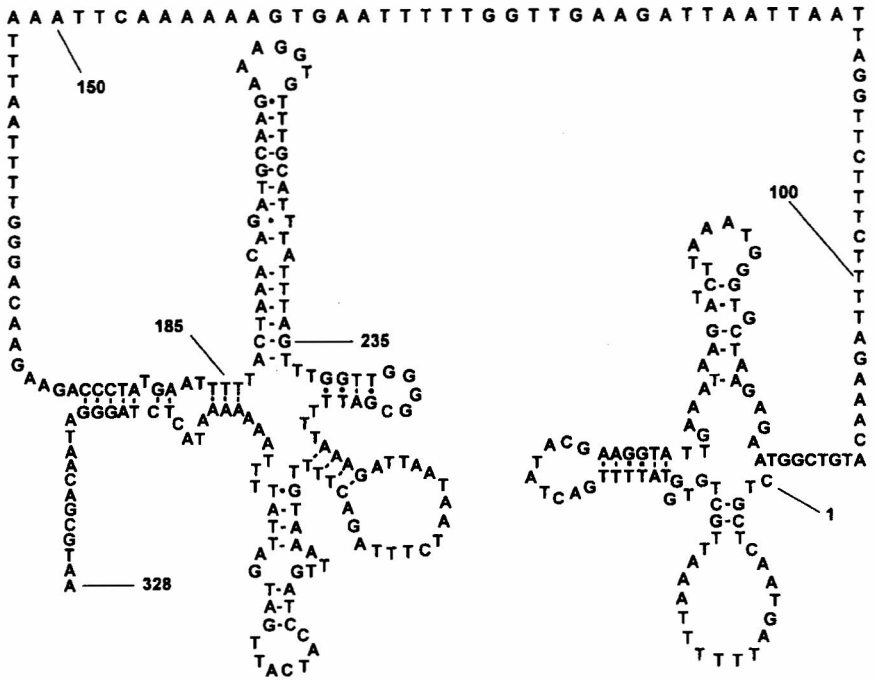
2. Alignment of the 16S rDNA fragment sequences from *Scutunalyssus obscurus*, *Pomeranzevia ninnii*, *Freyana anatina*, *Ixodes hexagonus*, *Argas reflexus* and *Vejovis carolinianus*

MATERIALS AND METHODS

DNA was extracted from 3 species of feather mites: *Scutulanyssus obscurus*, *Pomeranzevia ninnii (Avenzoariidae)* and *Freyana anatina (Freyanidae)* and from two species of ticks: *Argas reflexus (Argasidae)* and *Ixodes hexagonus (Ixodidae)*.



3. Secondary structure of 328 nucleotides in the 3 -end ofthe mitochondrial 16S rRNAgene in *Argas reflexus* (after BLACK and PIESMAN 1994, modified). Feather mites contained about 20 nucleotides less at the stem loop structure located between positions 185 and 235

DNA was isolated from 20 alcohol preserved feather mites (body length 0.5 mm) and from one leg of the tick. Before homogenization specimens were washed with ether/ ethanol (1:1) and dried on a filter paper. Feather mites were homogenized between two pieces of mat glass. Tissue material was incubated in 50 µl of digestion buffer (10 mM TrisHCl pH 8.5, 1.5 $MgCl_2$, 50 mM KCl, 0.5% Triton X-100) containing 200 µg/ml proteinase K (Serva) at 56°C for 1.5-72 h. The time of incubation has depended on the period of the ethanol preservation specimens. Then the sample was incubated at 95°C for 10 min. to inactivate the protease.

Amplification was accomplished with two primers 16S+I (ctgctcaatgatttttttaaa ttgctgtgg and 16S-2 (ttacgctgttatccctagag) designed by BLACK and PIESMAN (1994) and yielded a product about 320 bp. PCR were done in 50 µl of reaction mixture (10 mM TrisHCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X- 100, 200 µM each dNTP and I µM primers) in 500 µl microcentrifuge tubes. The tubes were exposed at a distance of 5 cm to ultraviolet light (260 nm) for 10 min. to destroy contaminating template DNA. Then mite template (1.5 µl) was added. The tubes were placed in a MicroCycler E (Eppendorf) and heated at 95°C for 5 min. After first denaturation 2

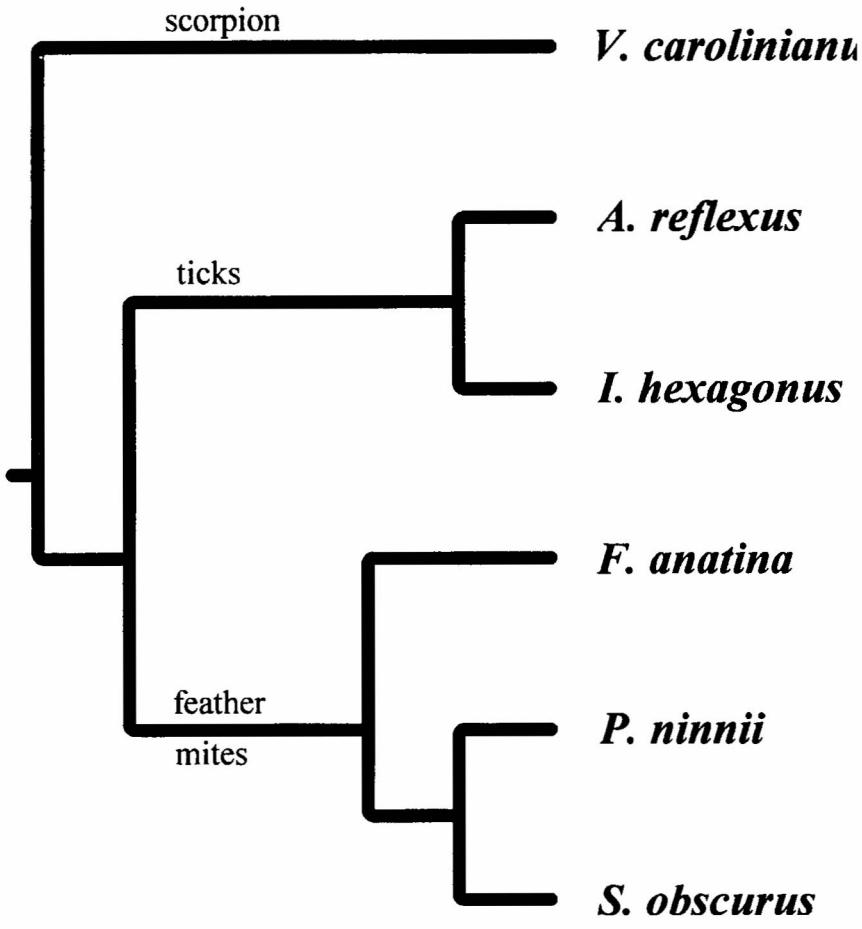
units of Taq DNA polymerase (PrimeZyme, Biometra) were added. Amplification was completed with a program consisting of 10 cycles of 30 sec at 95°C, 1 min. at 48°C, and 30 sec at 72°C. This was followed by 32 cycles of 30 sec at 95°C,30 sec at 54°C, and 30 sec at 72°C. A final extension reaction was carried out for 5 min. at 72°C and the reaction mixture was stored at 7°C. Negative controls (non template) were run simultaneously. Amplified DNA was run on a 1.5% low melting agarose (NuSieve, FMC). DNA was purified from agarose by glass wool filtration. From 1 to 8 µl of the filtrate (10 ng DNA) was used as a template for direct cycle sequencing using *fmol* Sequencing System (Promega). The original PCR primers were used for sequencing. Sequences were read manually from autoradiographs. These were initially aligned using DNASIS 2.1 (1995) and then manually shifted. Maximum-parsimony analysis was performed with PHYLIP 3.57C (1995) using DNAPARS.

RESULTS

The length of the amplified 16S gene region was from 301 bp (in *Scutulanyssus obscurus)* to 328 bp (in *Argas reflexus)*, Fig. 1. Nucleotide sequences of A. *reflexus* and *A. hexagonus* were almost congruent with the sequence published in Gene Bank; there were two substitutions, which correspond with population variability in this sequence fragment. The frequencies of adenine, cytosine, guanine, and thymine were 0.366, 0.069, 0.159 and 0.406 respectively. For comparison a fragment of 193 nucleotides was chosen. This fragment corresponds to nucleotides 51-244 of the published *A. reflexus* sequence (Acc. L34322, BLACK and PIESMAN 1994). With gaps added for alignment, 200 sites were used in the analysis, Fig. 2. The region of the sequence in which alignment was ambiguous corresponds to stem-loop structure located between nucleotides 185 and 235 in *A. reflexus*, Fig 3. Feather mites contain about 20 nucleotides less at this site than ticks. In the analysis a scorpion, *Vejovis carolinianus*, was used as an outgroup (Acc. U09398, AVISE 1994). Maximum parsimony tree derived from variation in the fragment of the 16S rDNA nucleotide sequence is shown in Fig. 4.

DISCUSSION

The method of mite DNA extraction presented here is simpler than those proposed in literature (KALISZEWSKI et al. 1992, HUBBARD et al. 1995, ZAHLER et al, 1995). In this method phenol/chloroform extraction to eliminate proteinase was omitted. The presented method permits to avoid contaminations in template DNA, in particular arising from previous amplifications. To avoid the carry-over of polymerase inhibitors in ticks, i.e. blood, DNA was prepared from the distal fragments of the tick legs. To increase the yield of the digestion of alcohol preserved mites by the proteinase K, one extraction with ether/ethanol (1: 1) was introduced. This method of DNA extraction permitted to successfully amplify fragments of DNA from mites that had been preserved in ethanol for even 20 years.



4. Maximum-parsimony tree obtained from sequence variation in the fragment of 16S rDNA. It is completely congruent with known phylogenetical relationships between test taxa

The fragment chosen for sequence comparisons was shorter than those presented in literature (BLACK and PIESMAN 1994). A truncated fragment was sufficient for resolving analyzed taxa (from subfamily to order level). Using maximum-parsimony method only one tree was obtained that agreed with the known phylogenetic relationships between studied taxa.

The method of sequence variation comparisons provides new possibilities in mite phylogenetic investigations. Particularly, it may be a powerful and versatile tool when morphological characters are limited in number or can be variously interpreted.

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