

DNA markers in the phylogenetics of the Acari

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Abstract: The advent of DNA amplification techniques and methods of automated DNA sequencing has greatly influenced the availability of applying nucleotide sequences in phylogenetic studies. This review presents DNA markers that have been used with success in mites and ticks to resolve questions at various taxonomic levels. Recent data suggest that well-supported phylogenies usually need genes with overlapping levels of resolution. Sequences from both nuclear and mitochondrial genomes are used for inferring phylogenetic history at various taxonomic levels. The 18S rDNA is a standard marker, especially for deep divergences (above the interfamily level). Mitochondrial loci with success recover phylogenies at intergeneric level, but also detect intraspecific relationships. Species identification is possible by using mtDNA, but the fastest-evolving rDNA spacers, ITS1 and/or ITS2, are the most frequently used for validation of a species status.

Key words: molecular markers, phylogeny, mites and ticks, rDNA, mtDNA, single-copy nuclear genes

INTRODUCTION

Molecular markers, developed over the years to detect variation between taxa, have enormously affected studies in many biological questions, ranging from population genetics to phylogenetic reconstruction and taxonomy. The advent of molecular biology techniques, which took place at the end of the 20th century – especially such innovations as DNA amplification using polymerase chain reaction (MULLIS 1986) and methods of automated DNA sequencing exerted a great influence on the availability and reasonable costs of applying molecular markers based on nucleotide sequences. Molecular markers have proven their utility in systematic and evolutionary acarology. DNA sequences have been used with success to study the phylogeny of ixodid ticks (BLACK & PIESMAN 1994, CRAMPTON et al. 1996, BLACK et al. 1997, DOBSON & BARKER 1999, MURRELL et al. 1999, 2000, 2001, 2005) as well as mesostigmatid (CRUICKSHANK & THOMAS 1999, DE ROJAS et al. 2001, 2002), sarcoptiform (RAMEY et al. 2000, DABERT et al. 2001, OTTO & WILSON 2001, SALOMONE

et al. 2002, MARAUN et al. 2004), and trombidiform mites (NAVAJAS et al. 1996, FENTON 2000, SOLLER et al. 2001). The use of molecular data in taxonomy and population genetics starts to become a standard. This enormous progress has taken less than 15 years: in 1993 only 49 DNA sequences originating from the Acari were reported in the GenBank database, whereas at the beginning of 2006 there were almost 93 000 sequences. Most of these data are expressed sequence tags (ESTs) and represent genes being expressed in mites (mainly *Dermatophagoides farinae* and *Blomia tropicalis*) and ticks (mainly representing ixodid taxa). These numbers should significantly rise when more results from the *Ixodes scapularis* Genome Project (IGP) are deposited in public databases (http://www.tigr.org/msc/i_scapularis/i_scapularis.shtml).

The development of molecular markers and an exhaustive presentation of biochemical and molecular techniques used in systematic acarology have been reviewed elsewhere (NAVAJAS & FENTON 2000, CRUICKSHANK 2002). The purpose of this review is to present molecular markers based on DNA sequences that have been used with success on mites and ticks to resolve questions at various taxonomic levels. The usefulness of these markers has been proved in many studies concerning Acari as well as other arthropods. However, it should be stressed that there are many advantages in using the same set of markers. The most important is an opportunity to combine sequences reported in databases, which have been collected for other studies, with one's own data. Data combining enables researchers to dense taxonomic sampling in their studies and to reduce the costs of sequencing.

DNA-SEQUENCES IN PHYLOGENETICS

Molecular phylogenetics is based on the assumption that the difference in nucleotide sequence between a pair of genomes should indicate how long those two genomes share a common ancestor. At present there is no doubt that the sequences of DNA molecules provide the most detailed data for phylogenetic studies. However, a problem has arisen: which fragment of which genome should be compared? Animal mitochondrial genes evolve 10 to 20 times faster than coding sequences from nuclear DNA (LI 1997). Moreover, genomic sequences have various functions, and this causes differences in rates and patterns of nucleotide substitutions. The choice of a molecular marker in a particular analysis is the most important issue, because a sequence fragment whose rate of substitution is inappropriate for the level of divergence under study can be a source of misleading data.

Some features of an ideal sequence marker for phylogeny reconstruction have been taken into consideration by CRUICKSHANK (2002). The sequence for comparisons should be an easy to align single-copy gene, its substitution rate should be high enough to provide a sufficient number of informative characters, as well as it should be low enough to avoid reversion homoplasy derived from saturation of mutations at variable sites. Unfortunately, the ideal fragment of the sequence that guarantees proper reconstruction of phylogenetic relationship at every taxonomic level does not exist. The high rate of base substitution in the mitochondrial genome (compared to nuclear DNA) limits mtDNA markers to low and medium taxonomic levels (CUROLE & KOCHER 1999). The mitochondrial genome is effectively a single copy, which could exclude the possibility of comparisons of paralogous copies of genes. However, nuclear mtDNA (*numt*), which includes copies of mitochondrial genes transposed into

the nuclear genome, is known from many eukaryotic genomes, including arthropods (RICHLY & LEISTER 2004). They exhibit various degrees of identity with their mitochondrial copies, and usually can be identified on the basis of mutations leading to the loss of function of a gene product (PONS & VOGLER 2005). On the other hand, nuclear genes considered as single-copy ones might have an unrecognized paralogous copy or functional constraints of their products may reflect in the sequence conservation (GAUCHER et al. 2001). As a result, different nuclear genes may yield different phylogenies. Therefore, sequences with overlapping levels of resolution should be used for inferring phylogenetic history at various taxonomic levels.

NUCLEAR RIBOSOMAL GENES

The utility of genes coding for ribosomal RNA (rDNAs) is found in the ubiquitous presence and relative conservation of many regions of their nucleotide sequences. In eucaryotes, nuclear rRNA genes form multigene families, in which all members have identical or nearly identical sequences which makes them easy to amplify and sequencing. A cluster of rDNA consists of the gene coding for 18S rRNA (small subunit ribosomal RNA, SSU rRNA), 2 internal transcribed spacers (ITS1 and ITS2) separated by the 5.8S rRNA gene, and the gene coding for the 28S rRNA (large subunit ribosomal RNA, LSU rRNA). Additional 2 external transcribed spacers (ETS) are located upstream of the 18S rDNA and downstream of the 28S rDNA. A non-transcribed spacer (NTS) separates adjacent copies of the rDNA repeat unit. Both spacers (ETS and NTS) are also called intergenic spacers (IGSs). Despite the numerous copies of nuclear genes coding for rRNA, they tend to have the same sequence, however the IGS regions accumulate more substitutions and show heterogeneity (HILLIS & DIXON 1991). Each member of a rDNA cluster has been tested for phylogenetic utility. Due to their high variability and the large-sized IGS regions are used less frequently as phylogenetic markers. On the other hand, the short and conserved 5.8S rRNA gene has shown relatively few informative characters and also was abandoned (HILLIS & DIXON 1991, LI 1997, ROKAS et al. 2002). The gene coding for 18S rRNA and the internal transcribed spacers (ITS1 and ITS2) have proved to provide the most useful systematic characters (DOOLITTLE 1999).

Small subunit ribosomal RNA gene (18S rDNA)

Due to its ubiquity, size, and generally slow rate of evolution, the 18S rRNA gene is the most frequently sequenced of all molecular markers in the field of molecular phylogeny of all taxa (OLSEN & WOESE 1993, VAN DE PEER et al. 2000). The gene coding for 18S rRNA can provide informative characters for assembling relationships of evolutionary distant taxa (> 100 Mya) (OUVRARD et al. 2002). For the sake of multiple copies and relatively short length (ca. 1.8 kb), the whole sequence of 18S rDNA is easy to amplify by PCR using universal primers designed on the previously known sequences. Some regions in 18S rDNA are conserved, which enables to align them easily. On the other hand, other sequences having high substitution rates are variable and make the alignment ambiguous. The secondary structure data of 18S rRNA available at the Comparative RNA website (www.rna.icmb.utexas.edu) and programs for nucleic acid folding and hybridization prediction (*mfold*, <http://www.bioinfo.rpi.edu/applications/mfold/old/rna/>) are used to

estimate the secondary structure of regions of 18S rRNA and the structural context of problems in the alignment.

To date, more than 200 sequences of the 18S rDNA of mites and ticks have been released in the GenBank database (<http://www.ncbi.nlm.nih.gov/>). These data were used mainly for resolving the phylogeny of tick subfamilies (CRAMPTON et al. 1996, BLACK et al. 1997, MANGOLD et al. 1998, DOBSON & BARKER 1999, KLOMPEN et al. 2000, MURELL et al. 2001) and relationships among the major lineages of the anactinothrichid mites (MURELL et al. 2005). Studies using the 18S rDNA for mite taxa are currently conducted and their results have not been published yet. Basing on published sequence data, one can only guess the subject and range of the studies: they concern such problems as the phylogeny of parasitiform mites, the relationships between allergy-causing dust mites, the phylogeny of Sarcoptiformes or the phylogeny of parasitic mites of the cohort Psoroptidia with emphasis on feather mites.

Large-subunit ribosomal RNA gene (28S rDNA)

To date there is no full-length sequence of 28S rDNA for a species of Chelicerata in the GenBank database. This is because the gene coding for the LSU RNA (28S rDNA) is the longest molecule of rRNA genes (ranging from 3000 to 5000 bp). The 28S rRNA has a mosaic structure of about a dozen conserved “core” segments and hypervariable “expansion” segments (HANCOCK & DOVER 1988). The expansion segments range in length from 10 to several hundred base pairs and evolve up to 10 times the rate of the core segments. In theory the expansion segments can be used successfully to infer phylogenetic relationships among closely related taxa, and many authors extensively sequence 1–2 segments along with other DNA markers. However, researchers differ in choosing between domains using the 28S rDNA sequence data. Thus the D1 domain (along with a fragment of 18S rDNA) has been tested for inferring phylogeny in ticks (CRAMPTON et al. 1996), whereas the D2 domain has been used for genotyping the Eriophyidae (GOOLSBY unpubl.), and the D7 region was applied to study the evolution of haplodiploidy in dermanyssine mites (CRUICKSHANK & THOMAS 1999). The D3 domain is one of the most frequently sequenced and studied fragments of the 28S rDNA in mites and ticks, and its application ranges over a wide variety of phylogenetic levels. The nucleotide sequence of D3 and its flanking regions (fragments of conserved core segments) have been applied to evaluate phylogenetic relationships among sexual and asexual oribatid mites and for inferring the phylogeny of Oribatida (MARAUN et al. 2003, 2004), as well as for establishing the systematics of the *Acarus siro* complex (WEBSTER et al. 2004) or resolving the phylogeny of the extant chelicerate orders (WHEELER et al. 1998). The evolution and variability of this relatively short sequence fragment (ca. 120–200 bp) was in detail investigated in populations of *Ixodes* ticks (MCLAIN et al. 2001). However, other authors have sequenced 2 fragments of 28S rDNA, D3-D5 and D9-D10, among other markers for inferring the phylogeny of parasitiform mites (KLOMPEN unpubl.). This lack of consistency in choosing one proper and universal molecular marker from the 28S rRNA gene may result from the weak resolution of the 28S rDNA on its own. In other studied arthropod taxa the 28S rDNA data are used occasionally, with results that are in broad agreement with those for 18S rDNA.

Internal transcribed spacers (ITS1, ITS2)

Spacer regions in the nuclear rDNA array can be used to infer phylogeny among closely related taxa that have diverged within the last 50 Mya (HILLIS & DIXON 1991). Moreover, spacers evolve much more rapidly than the rRNA genes, thus the variation in these regions can be used to identify species or strains. Conserved flanking regions of the 18S and 28S rDNAs facilitate amplifications of the ITS1 and ITS2 sequences using PCR. Unfortunately, high substitution rate of spacers have an adverse effect on their utility due to intraspecific or even intraindividual diversity of sequences (MCLAIN et al. 1995, FENTON et al. 2000, DE ROJAS et al. 2002, DABERT et al. 2005). Because of this diversity, heterogenic copies of amplified ITS fragments have to be cloned in plasmids and then individually sequenced, which makes the studies more laborious and expensive.

Despite this difficulty, ITS sequences have been used with success for resolving many phylogenetic problems in the Acari, especially for determination of species status (WESSON et al. 1993, BERRILLI et al. 2002, LOHSE et al. 2002, WEBSTER et al. 2004, DABERT et al. 2005), but also to investigate the history of a species (NAVIA et al. 2005).

NUCLEAR PROTEIN-CODING GENES

Amino acid sequences of proteins were some of the first “true” molecular markers used in phylogenetic studies. However, insufficient knowledge about homology of the sequences used for comparisons has led to incongruence among phylogenetic trees based on morphological data and those reconstructed from comparisons between sequences of paralogous genes (often members of a multigene family, i.e. globins). To avoid this problem, much effort has been made to find single-copy nuclear genes that represent unambiguous homology and have phylogenetically informative characters. A number of protein-coding genes of nDNA have been explored for use in arthropods. The gene encoding elongation factor 1 α (EF-1 α) is one of the most prevalent among them. EF-1 α is one of key elements in protein synthesis and catalyzes the binding of aminoacyl-tRNAs to the A-site of the ribosome in eucaryotes. EF-1 α belongs to the slowest-evolving proteins known (GAUCHER et al. 2001), and along with a homologous protein in bacteria (EF-Tu) it has been used to root the universal tree of life (BALDAUF et al. 1996). There are two general approaches in using EF-1 α sequence data. The first relies on using a combination of exon and intron sequences derived from the genomic DNA in order to increase its phylogenetic utility. However, synonymous saturation, coupled with amino acid sequence conservation, limits the utility of exon sequences at intermediate levels, and moves this marker towards deep-level phylogenies as well as towards species discrimination. The second approach, based on sequence data from the complete coding sequence of the gene by using RT-PCR (REGIER & SHULTZ 2001, DANFORTH et al. 2004) is unfeasible to apply to long-preserved and/or less numerous small-sized specimens. Moreover, the results received from these data are often ambiguous (KLOMPEN 2000, CRUICKSHANK et al. 2001, GIRIBET et al. 2001, REGIER et al. 2005).

Similar problems concern other protein-coding genes of nuclear DNA: the largest subunit of RNA polymerase II (POL II), the α subunit of the sodium-potassium

ATPase, or the elongation factor 2 (EF-2) genes used for resolving the phylogeny of many arthropod groups, often gave results different from those inferred from other data (GIRIBET et al. 2001, ANDERSON et al. 2004, REGIER et al. 2005). However, POL II genes have proved that their sequences contain strong phylogenetic signals in ticks at the generic and higher levels (FANG et al. 2002). All the above-mentioned genes considered as having a single copy, have in fact more copies in the genome. More detailed studies showed the existence of paralogous gene copies, some of them being expressed (HEDIN & MADDISON 2001, DANFORTH et al. 2004).

MITOCHONDRIAL DNA

Mitochondrial DNA has higher rate of base substitution than most nuclear genes, so mtDNA sequences are a useful phylogenetic marker for clades that have diverged relatively recently, i.e. no more than a few million years ago (CUROLE 1999). Nevertheless, the use of much longer sequences, even of the complete sequence of the genome, has encouraged attempts to study deep divergences. Sequencing of large fragments or even the entire mitochondrial genome is possible because animal mitochondrial genome is with some exceptions a circular and relatively small molecule (14–17 kb in mites and ticks) that can be amplified in a few long fragments. To date, 14 complete DNA sequences of mitochondrial genomes of the Acari (among 20 sequences from Chelicerata) have been released in the GenBank database. Among them, 10 sequences belong to Ixodida, 3 genomes are from Prostigmata, and 1 from Mesostigmata. The high variability and a strong bias in mites towards amino acids encoded by AT-rich codons make it more difficult to design universal primers for amplifying specific regions in mtDNA of the Acari. Nevertheless, to date more than 2100 partial sequences of the mite and tick mtDNAs have been published in the GenBank database. Basing on the 16S rDNA, BLACK and PIESMAN (1994) have resolved the phylogeny of hard- and soft-tick taxa and initiated progress in studies based on rDNA sequence data for other groups of Acari. Similarly, other studies concerning also protein-coding genes of mtDNA from mites (NAVAJAS et al. 1996, 1998) have provided acarologists with data about nucleotide sequences for the designing of primers for PCR amplification of homologous fragments of mtDNA in other taxa.

Mitochondrial gene content and gene arrangement

It was supposed that gene arrangement comparisons could be a powerful tool for phylogenetic studies, especially for those focused on ancient relationships (BOORE & BROWN 1998). Arthropods are one of the best-studied groups for mtDNA gene order. Among the few rearrangements that have been found in arthropod mtDNAs, all have been translocations of tRNA genes (BOORE 1999). However, recent reports using comparisons of the whole mtDNA sequences from ticks have shown that mitochondrial gene order is not conserved in all arthropods (BLACK & ROEHRDANZ 1998, SHAO et al. 2004), and may be highly rearranged (SHAO et al. 2005) even among closely related species (CAMPBELL & BARKER 1998). Recent results from other arthropods suggest that gene rearrangements in the mitochondrial genome are more common than was supposed (COVACIN et al. 2006).

Mitochondrial rDNAs and protein-coding genes

Like in other animals, mtDNA from ticks encodes 13 subunits of the enzymes of oxidative phosphorylation, the 2 rRNAs of mitochondrial ribosome, and 22 tRNAs. The whole mtDNA molecule is engaged in gene coding except for a long non-coding sequence, called a control region, which contains regulatory elements for replication of the mitochondrial genome. Among these 37 genes, only a few have been sequenced for mites and ticks (12S and 16S rDNAs, fragments coding for cytochrome oxidase subunits, and a fragment of the cytochrome *b* gene). However, the large subunit of ribosomal RNA (16S rDNA) (over 800 partial sequences published in the GenBank) and cytochrome oxidase I gene fragments (*cox1*, *COI*; over 500 partial sequences) are the most frequently used markers for inferring relationships at the low and intermediate level. Although most of published mtDNA sequences from mites and ticks are relatively short fragments, they have been used with success for resolving many phylogenetic and taxonomic problems. Sequences from mtDNA have proved to be one of the most useful phylogenetic tools in mites and ticks for inferring relationships between closely related species (NAVAJAS et al. 1994, NORRIS et al. 1997, DABERT et al. 1999, SALOMONE et al. 1996, SKERRATT et al. 2002) as well as at the intermediate level (BLACK & PIESMAN 1994, DABERT et al. 2001).

Sequence analysis of mtDNA markers causes similar problems as in the case of nuclear genes, especially when the comparison concerns more diverged taxa: rDNA sequences that evolve much faster than nuclear ones need to be aligned on the basis of the secondary structure information, and protein-coding genes, although they are easy to align, disturb the analysis due to saturation at synonymous sites. The choice of the gene fragment for analysis has formerly depended on accessibility of primers for PCR amplification. Since more sequence data from mtDNA of various taxa are available, the choice of the marker can be more conscious. For example, in the Acari the *COI* gene (about 1500 bp in length) to date has been sequenced in 2 non-overlapping fragments. It seems that the use of the 5'-end fragment of the gene is a better solution because this fragment of *COI* has been chosen as a DNA-barcode sequence for animal species identification (HEBERT et al. 2003, HANNER 2005). New, non-destructive methods of DNA isolation, basing on incubation of whole specimens in the lysis buffer and later using exoskeletons for preparing voucher slides, allow describing of a species along with its molecular data. The accumulated knowledge of nucleotide sequences of the *COI* genes in various taxa will enable identification of species or attribute an analyzed specimen to the proper group.

CONCLUSIONS

A review of this length cannot summarize all sequence-based markers used in phylogenetics and every case in which these data have been used to date. This paper focused on the DNA markers that have been widely tested and whose utility has been proved not only in mites and ticks, but also in other arthropods. Well-supported phylogenies usually have been resolved by using more than one locus, more often than not using both nuclear and mitochondrial sequences (i.e. KLOMPEN et al. 2000, GIRIBET et al. 2001, ROKAS et al. 2002). The 18S rDNA is the standard marker for metazoan phylogenetics, especially for deep divergences (above the interfamilial level).

On the other hand, mitochondrial loci successfully recover phylogenies at the inter- and intrageneric level as well as intraspecific relationships. Species identification is possible with the use of mtDNA (for example, *COI*), but the fastest-evolving rDNA spacers, ITS1 and/or ITS2, have been the most frequently used for validation of a species status.

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