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Molecular approach in spider mites (Acari: Tetranychidae): preliminary data on ribosomal DNA sequences

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ABSTRACT

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DNA sequence data were used to examine phylogenetic relationships between six species of economically important Tetranychidae mites: *Eotetranychus carpini* (Oudemans), *E. pruni* (Reck), *Tetranychus pacificus* McGregor, *T. mcdanieli* McGregor, *T. turkestani* Ugarov & Nikolski and *T. urticae* Koch. With primers directed toward conserved elements flanking the target region, the Polymerase Chain Reaction was used to amplify the ITS2 spacer of the ribosomal DNA molecule. The nucleotide sequence of a 300-bp fragment of the ITS2 was determined by direct sequencing and nucleotide divergence used for intra-generic comparison in mites. The resulting phylogenetic tree expressing interspecific relationships in genus *Tetranychus* agrees with morphological data. The study demonstrates the usefulness of the approach in the assessment of the systematics and evolution of the group.

INTRODUCTION

The family Tetranychidae (66 genera and over 1000 species) includes many agricultural pests. The systematics of the group was previously based on morphological characters which are not always easy to observe and which may be variable. Biological and cytogenetic work has led to phylogenetic hypotheses (Gutierrez et al., 1970; Gutierrez and Helle, 1985), but the scope seemed limited. Other techniques were used to improve species discrimination. A few mite species have been submitted to electrophoretic protein analysis. These studies were performed in particular on three of the agronomically most important mite species (*Tetranychus mcdanieli* McGregor, *T. pacificus* McGregor and *T. urticae* Koch). Enzymatic polymorphism was useful in the de-

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tection of inter- and intraspecific differences (Ward et al., 1982; Sula and Weyda, 1983; Grafton-Cardwell et al., 1988; Kim and Lee, 1990).

Molecular analysis in taxonomic classification provides a solid basis for phylogenetic hypotheses. Advantages of both morphological and molecular approaches of systematics were discussed by Hillis (1987). Geneticists and taxonomists have used molecular techniques over the past decade to examine intra- and interspecific variation in DNA segments. Work on restriction endonucleases was followed by sequencing leading to phylogenetic hypotheses (Miyamoto and Cracraft, 1992). The Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987; Saiki et al., 1988), amplifying specific regions in the genome using minute quantities of DNA, enables use of the DNA approach on organisms as small as mites.

Sequence comparisons of small subunits of ribosomal RNAs or their genes can be applied to compare closely and distantly related taxa (Sogin and Gunderson, 1987). Several rDNA regions have been sequenced in a broad range of eukaryotes (Hillis and Dixon, 1991). Some regions of the ribosomal genes are well conserved (coding regions) while others undergo more rapid evolution (noncoding regions referred to as "spacers"). A variable rDNA region – spacer ITS2 – was amplified using primers defined in the highly conserved flanking regions 5.8S and 28S rDNA (Fig. 1). Sequences of this 28S region are known in several eukaryotes (Qu et al., 1988).

This is a completely new approach in mites and we possess no sequence data, with the exception of a few molecular analyses of a mite neurotoxin gene in Pyemotidae (Tomaiski and Miller, 1991) and mite allergens such as Ixodidae and Pyroglyphidae (Rand et al., 1989; Tovey et al., 1989; Dilworth et al., 1991; Yuuki et al., 1991). We used PCR and chain termination sequencing of the ITS2 rDNA spacer as a first step in the molecular study of mite phylogenetic relationships to assess the level of variability in six Tetranychidae species.

MATERIALS AND METHODS

Sample preparation and DNA isolation

Mite sources are shown in Table 1. They were either collected in the field (*Eotetranychus carpini* (Oudemans), *E. pruni* (Reck)) or reared in the laboratory (*T. pacificus*, *T. mcdanieli*, *T. urticae*, *T. turkestani* Ugarov & Nikolski). Whole genomic DNA was extracted from fresh adults (10 to 30 individuals). Tissues were digested in 500 μl of extraction buffer (10 m*M* NaCl, 1 m*M* EDTA, 10 m*M* Tris, 1% SDS) and 100 μg/ml of proteinase K at 37°C for 2 h. The resulting solution was extracted twice with phenol and once with phenol/chloroform. DNA samples were concentrated and desalted on a Centricon 100 microconcentrator (Amicon).

TABLE 1

List of species examined and origin of the material

Species	Host plant	Locality
Eotetranychus carpini (Oudemans)	*Vitis vinifera* L.	Montpellier (F)
Eotetranychus pruni (Oudemans)	*Acer platanoides* L.	Montpellier (F)
Tetranychus pacificus McGregor	*Vitis vinifera* L.	Davis (Ca, USA)
Tetranychus mcdanieli McGregor	*Vitis vinifera* L.	Verzenay (F)
Tetranychus turkestani Ugarov & Nikolski	*Phaseolus vulgaris* L.	Montpellier (F)
Tetranychus urticae Koch	*Sambucus nigra* L.	Amsterdam (NL)

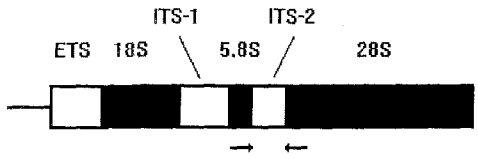

Fig. 1. Organisation of an animal rDNA replication unit (after Hillis and Dixon, 1991). Spacer ITS2 was amplified in mites by PCR. Arrows indicate the position of PCR primers. These primers are the HC2 (in the 28S region) and the LC1 (in the 5.8S region) in Depres et al. (1992).

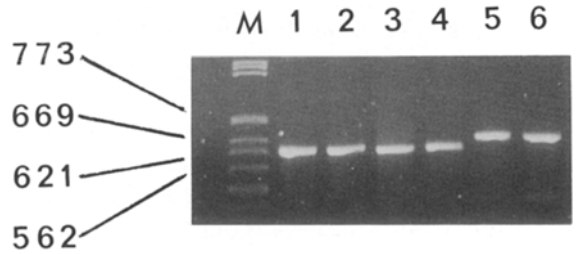

Fig. 2. Agarose gel electrophoresis of amplified ITS2 ribosomal DNA region in several species of Tetranychidae mites. Size differences were observed between the amplification products of the studied genera. Lanes 1 to 4: *Tetranychus* spp.; lanes 5 and 6: *Eotetranychus* spp. Size marker (M) noted in base pairs.

Enzymatic amplification and sequencing

The polymerase chain reaction was performed in 50-μl reactions. Amplification was carried out by denaturation at 92°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min. The cycle was repeated 30 times. Amplification products were resolved by electrophoresis in agarose gel and stained with ethidium bromide to visualize the DNA. Sequences and precise position of PCR primers are described in Despres et al. (1992) (Fig. 1).

The amplified products were directly sequenced after centrifugal dialysis

on Centricon 100 microconcentrators; 7 μl of the retentate was used for sequencing by the dideoxynucleotide chain termination method and Sequenase enzyme (United States Biochemical Co.). The products of the sequencing reactions were resolved in 6% polyacrylamide/7-*M* urea gels. Electrophoresis was carried out for 2.5 h at 40 mA. Gels were fixed in 10% glacial acetic acid/10% ethanol, dried and autoradiographed.

Sequence analysis

Sequences were aligned with the aid of the MULTALIN program (Corpet, 1988). The percent nucleotide divergences were calculated by the method of Jukes and Cantor (1969) using the program DNADIST of J. Felsenstein's package PHYLIP 3.4. A tree was constructed using the program KITSCH in PHYLIP 3.4. This program builds the tree that minimises the sum of squares of the differences between the patristic and the measured distances. This method furthermore assures a molecular clock, i.e. assumes that the rate of evolution is the same in all branches of the tree.

RESULTS

The ITS2 spacer of ribosomal DNA was amplified in the six Tetranychidae species listed in Table 1. Size differences were observed between the amplification products of the two studied genera (Fig. 2). A fragment of 200 to 300 nucleotides of the 3′ end of the ITS2 region was sequenced. Pairwise comparisons of nucleotide positions (Fig. 3) showed a low substitution rate within each genus and good alignment is thus possible. In contrast, there were considerable divergences between the two genera and sequences were aligned independently at generic level (Fig. 3A and B). Percent of nucleotide divergences between the *Tetranychus* species varied from 1.4% between *T. urticae* and *T. turkestani* to 1.7% between *T. mcdanieli* and *T. pacificus* (with 3 and 2 insertions/deletions respectively between the two pairs of species). These divergences reach 10% between *T. urticae* and *T. pacificus* (4 insertions/deletions). In the genus *Eotetranychus*, the nucleotide substitution rate between the studied species is 7.6% (8 insertions/deletions).

Sequences of the genus *Tetranychus* were processed in a phylogenetic tree to express the relationship between species (Fig. 4). Two clusters clearly appear from this analysis: *T. urticae* and *T. turkestani* on one side and *T. mcdanieli* and *T. pacificus* on the other side.

Fig. 3. Comparison of the mite sequences of the 3′ end of the ITS2 ribosomal DNA region. Sequences of the two studied genera are aligned independently: (A) *Tetranychus* spp. (B) *Eotetranychus* spp. Tetranychidae species were aligned by reference to the consensus sequence (with identities denoted by hyphens and deletions represented by dots).

A

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                1       10        20        30        40        50        60
 consensus      GATCAAAAATATTGATTAAGTTGATTAATGTATATT  TGTTGTTTGCAAGCAA GCAAC

T. urticae      ------------------------------T----TG-----------------.-----
T. turkestani   ------------------------------A----TG-----------------.-----
T. mcdanieli    ------------------------------A----GT----------------A------
T. pacificus    ------------------------------A----GT----------------A------

                      70        80        90       100       110       120
 consensus      GTAAATCTACTTTAA GTTTGCACAA TTT  C  TTGCA TAC TTCTTAGGTCTGCTT

T. urticae      ---------------T----------A---TG-CT-----T---.----------------
T. turkestani   ---------------A----------A---TG-CT-----T---.----------------
T. mcdanieli    ---------------C----------T---GC-AA-----A---.----------------
T. pacificus    ---------------C----------T---GC-AA-----A---C----------------

                     130       140       150       160       170       180
 consensus      TAACAGA ATGAAATAG TACTATTTGTATG TT  TACAAGTGCA  GAAGATTCATCA

T. urticae      -------C--G------C----------C-A--TA----------A.--A-------C-
T. turkestani   -------.--A------C----------T-A--TA----------A.--A-------C-
T. mcdanieli    -------.--G------A----------T-T--AC----------GT--A-------C-
T. pacificus    -------.--G------A----------T-T--AC----------GT--G-------A-

                     190       200       210       220       230       240
 consensus       TTA CAGGTTAG  GATCACCAT GTTATCTG AATACGACTTTAGCGTCGTCAGATAG

T. urticae      G---A--------TT---------A--------C----------T----------------
T. turkestani   G---A--------TT---------A--------C----------.----------------
T. mcdanieli    T---G--------GA---------T--------T----------T----------------
T. pacificus    T---G--------GA---------T--------T----------T----------------

                     250       260       270       280       290       300
 consensus      GCGACTAACCTTAGAATCTCATGCTAGTATCTAT CATATATACTG TTGCAGAGATGAAAT

T. urticae      ----------------T----------------TT----------C-T--------------
T. turkestani   ----------------T----------------T.----------C-T--------------
T. mcdanieli    ----------------T----------------T.----------T-T--------------
T. pacificus    ----------------C----------------AT----------T-G--------------
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B

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                1       10        20        30        40        50        60
 consensus      AGA C A    AGTAAAAAGGTT GGAAAAGT CACA    CACACGC CAATTAAG TT

E. pruni        ---T-A-....-----------C--------A----ACCA-------G--------C--
E. carpini      ---A-T-GAGT-----------T--------T----....-------A--------T--

                      70        80        90       100       110       120
 consensus      GT TGTG  TGAC  TCAAACATTTGCTTGAACGAT AAAACCATATGCACATAAAGA A

E. pruni        --G----CG----CT---------------------.---------------------A-
E. carpini      --A----..----..---------------------G---------------------T-

                     130       140       150       160       170       180
 consensus      C  ACAGA  CATGTATTGTTGATTATAT TCACCTTAAATTGCAATCGAGACCCA TAT

E. pruni        -..-----AC-------------------C-------------------------T---
E. carpini      -GC-----GA-------------------T-------------------------G---

                     190       200       210
 consensus      AG GAGA GCCTTTTCTTTG CGTCATATTA

E. pruni        --C----.----------.----------TC
E. carpini      --T----A----------A----------AT
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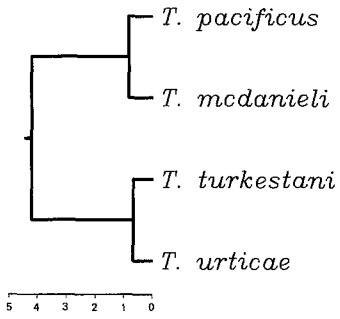

Fig. 4. Phylogenetic tree obtained from four species of *Tetranychus* mites. The sequences considered are those of Fig. 3. The scale below the tree gives the percent of nucleotide divergences.

DISCUSSION

The variability of sequences enabled good separation of the various species and appeared to be suitable for examining phylogenetic relationships within each genus. In contrast, the differences between genera were too great to enable proper alignment of sequences. This important sequence divergence between genera was accompanied by a difference in size of about 100 bp between the products of amplification (Fig. 2). Clearly, more conserved regions of the genome must be analysed to resolve the phylogenetic relationships at this higher taxonomic level.

The phylogenetic tree (Fig. 4) of the genus *Tetranychus* is in agreement with the morphology. Apart from a slight difference in the shape of the aedeagus, *T. mcdanieli* and *T. pacificus* appear to be very similar and display little sequence divergence. Although a clear distinction can be made between *T. urticae* and *T. turkestani* in the shape of the aedeagus, they are morphologically and molecularly close. This is in agreement with the geographical origin of the species studied: *T. urticae* and *T. turkestani* probably originated in Eurasia, while *T. mcdanieli* and *T. pacificus* are from North America. In contrast however, *E. pruni* and *E. carpini* display relatively substantial nucleotide divergence and are from the same area and appear to be morphologically very close, according to conventional criteria, except for a slight difference in the shape of the peritreme end. One might therefore wonder whether the systematics of all the *Eotetranychus* species close to this group should perhaps be revised.

It is interesting to note that while the studied *Tetranychus* species are polyphagous, the studied *Eotetranychus* live on one or only a few plant genera. This feature and the sequence analysis above raises the more general question of whether specialisation on a host plant can lead to speciation in phytophagous mites. Our data also raise the question of the reliability of morphological characters for the understanding of the evolution of the group.

In this study, the DNA from several individuals of each species was pooled and the resulting sequence represents the consensus sequence of the species. It will subsequently be interesting to analyse the intraspecific variability of the DNA fragment studied.

The sequences of various rDNA domains are becoming available for a wide range of organisms. However, the data presented here for several Tetranychidae species are the first ITS2 sequences studied in mites. We took advantage of the conservative fragments of the ribosomal gene to initiate the examination of DNA sequences from taxa about which we have no previous sequence information. Our approach provides new features for use in conjunction with traditional morphological criteria and with biological features for the assessment of the systematics and evolution of the group.

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REFERENCES

Corpet, F., 1988. Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res., 16: 10881–10889.

Depres, L., Imbert-Establet, D., Combes, C. and Bonhomme, F., 1992. Molecular evidence linking hominid evolution to recent radiation of Schistosomes (Platyhelminths, Trematodes). Mol. Phyl. Evol., in press.

Dilworth, R.J., Chua, K.Y. and Thomas, W.R., 1991. Sequence analysis of cDNA coding for a major house dust mite allergen Der f II. Clin. Exp. Allergy, 21: 25–32.

Grafton-Cardwell, E.E., Eash, J.A. and Granett, J., 1988. Isozyme differentiation of *Tetranychus pacificus* from *T. urticae* and *T. turkestani* (Acari: Tetranychidae) in laboratory and field populations. J. Econ. Entomol., 81: 770–775.

Gutierrez, J., Helle, W. and Bolland, H.R., 1970. Etude cytogénétique et réflexions phylogénétiques sur la famille des Tetranychidae Donnadieu. Acarologia, XII(4): 732–751.

Gutierrez, J. and Helle, W., 1985. Evolutionary changes in the Tetranychidae. In: W. Helle and M.W. Sabelis (Editors), Spider Mites. Their Biology, Natural Enemies and Control, Vol 1A. Elsevier, Amsterdam, pp. 91–107.

Hillis, D.M., 1987. Molecular versus morphological approaches to systematics. Ann. Rev. Ecol. Syst., 18: 23–42.

Hillis, D.M. and Dixon, M.T., 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q. Rev. Biol., 66: 411–453.

Jukes, Y.H. and Cantor, C.R., 1969. Evolution of protein molecules. In: H.N. Munro (Editor), Mammalian Protein Metabolism. Academic Press, New York, pp. 21–32.

Kim, S.S. and Lee, S.C., 1990. Development of acaricidal resistance esterase isozyme of *Tetranychus urticae* (Acarina: Tetranychidae). Korean J. Appl. Entomol., 29: 170–175.

Miyamoto, M.M. and Cracraft, J., 1992. Phylogenetic analysis of DNA sequences. Oxford University Press, 400 pp.

Mullis, K.B. and Faloona, F.A., 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol., 155: 335–350.

Qu, L.H., Nicoloso, M. and Bachellerie, J.P., 1988. Phylogenetic calibration of the 5′ terminal domain of large rRNA achieved by determining twenty eucaryotic sequences. J. Mol. Evol., 28: 113–124.

Rand, K.N., Moore, T., Sriskantha, A., Spring, K., Tellam, R., Willadsen, P. and Cobon, G.S., 1989. Cloning and expression of a protective antigen from the cattle tick *Boophilus microplus*. Proc. Natl. Acad. Sci. USA, 86: 9657–9661.

Saiki, R., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239: 487–491.

Sogin, M.L. and Gunderson, J.H., 1987. Structural diversity of eukaryotic small subunit ribosomal RNAs: evolutionary implications. Endocytobiology III. Ann. N. Y. Acad. Sci., 503: 125–139.

Sula, J. and Weyda, F., 1983. Esterase polymorphism in several populations of the two-spotted spider mite, *Tetranychus urticae* Koch. Experientia, 39: 78–79.

Tomaiski, M.D. and Miller, L.K., 1991. Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. Nature, 352: 82–85.

Tovey, E.R., Johnson, M.C., Roche, A.L., Cobon, G.S. and Baldo, B.A., 1989. Cloning and sequencing of a cDNA expressing a recombinant house dust mite protein that binds human IgE and corresponds to an important low molecular weight allergen. J. Exp. Med., 170: 1457–1462.

Ward, P.S., Boussy, I.A. and Swincer, D.E., 1982. Electrophoretic detection of enzyme polymorphism and differentiation in three species of spider mites (*Tetranychus*) (Acari: Tetranychidae). Ann. Entomol. Soc. Am., 75: 595–598.

Yuuki, T., Okumura, Y., Ando, T., Yamakawa, H., Suko, M., Haida, M. and Okudaira, H., 1991. Cloning and expression of cDNA coding for the major house dust mite allergen Der f II in *Escherichia coli*. Agric. Biol. Chem., 55: 1233–1238.