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Mitochondrial cytochrome oxidase I in tetranychid mites: a comparison between molecular phylogeny and changes of morphological and life history traits

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Abstract

Spider mites, Tetranychidae, represent one of the most cosmopolitan and economically important groups of terrestrial arthropods; however, many aspects of their evolutionary relationships remain uncertain. We sequenced part of the mitochondrial cytochrome oxidase subunit I (COI) gene in 20 species of phytophagous mites belonging to nine genera and two families (Tetranychidae and Tenuipalpidae), including several agricultural pests. As reported in insects, the sequences were extremely rich in A+T (75% on average), especially in the third codon position (95%). However, one of the genera we studied had a significantly lower A+T content (69% on average, 78% in the third codon position), showing that base composition can change substantially over short periods of time. Most interspecific differences were transversions and their number increased steadily with the number of non-synonymous differences, while the number of transitions remained constant. The phylogeny based on COI sequences was inferred using the maximum likelihood method. The results are compatible as a whole with the traditional classification based on morphological characters, but call for some minor taxonomic revisions. Some morphological characters and life history traits (mode of reproduction, adaptation to the host plant) were also analysed within this phylogenetic framework. At the family level, one can see a trend towards thelytoky becoming rarer compared to the general mode of reproduction of the group, arrhenotoky. There is also an evolutionary tendency towards a more complex mode of life, with the production of silk webs and correlated changes of the locomotion apparatus. However, in the Tetranychidae there seems to have been convergent evolution of these morphological characters together with independent development of a common adaptation to this mode of life in different genera.

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Introduction

The family Tetranychidae (Acari) consists of about 1200 described species and includes some of the world's major agricultural pests. These mites still constitute a problematic group for systematists and considerable experience is required to identify them because, due to their small size, potential diagnostic phenotypic characters are relatively few and differences often inconspicuous. Using mainly morphological criteria, several authors have attempted to study the evolution of phytophagous mites and have inferred putative relationships among some Tetranychoidea families: Tetranychidae, Tenuipalpidae and Tuckerellidae (Wainstein, 1963; Krantz & Lindquist, 1979; Mitrofanov, 1983; Lindquist, 1985). Other work combining various biological traits and morphological characters has aimed at identifying patterns of evolution within Tetranychidae (Helle *et al.*, 1970; Gutierrez *et al.*, 1979; Gutierrez & Helle, 1985, 1988).

In addition to the morphological approach, molecular data has long been recognized as an attractive marker for phylogenetic analysis. By comparing the variation patterns of DNA sequences, information about species relatedness can be obtained. Although Acari constitute an important subclass including many species of agronomical or medical importance, few genes have been sequenced in species of this group and used in phylogenetic surveys. The use of molecular information, such as ribosomal DNA sequences (Navajas *et al.*, 1992, 1994), has been attempted in mites. Mitochondrial DNA (mtDNA) sequences information is now used extensively in phylogenetic studies. The phylogenetic usefulness of several mitochondrial genes has been reviewed by Simon *et al.* (1994). In a previous study, we cloned and sequenced part of the mitochondrial cytochrome oxidase subunit I (COI) gene in the spider mite *Tetranychus urticae* Koch (Fournier *et al.*, 1994). In the present paper, mitochondrial COI sequences of 20 species of phytophagous mites belonging to the Tetranychidae and Tenuipalpidae were used to infer a molecular phylogeny as well as to examine the patterns of evolution of mtDNA sequence and of a number of morphological characters. We also analysed the evolution of several biological features that are related to adaptation of these mites to the host plant, types of defence against predators (habitat and silk production), and type of reproduction, in order to understand evolutionary trends in these traits.

Materials and methods

Biological material

The species analysed and their origin are shown in table 1. The 20 species of Tetranychoidea belong to two families. The family Tenuipalpidae is represented by a single species, *Cenopalpus pulcher* Canestrini & Fanzago and the other 19 taxa are Tetranychidae. The latter family contains eight genera belonging to the two tetranychid sub-families Bryobiinae, (genera *Petrobia* and *Bryobia*) and Tetranychinae (genera *Tetranychus, Oligonychus, Eotetranychus, Panonychus, Mononychellus* and *Eurytetranychus*). *Tetranychus urticae, T. mcdanieli* McGregor and *T. pacificus* McGregor were obtained from long-established stocks reared in the laboratory. All other species were collected in the field. In some cases, mites from the natural environment were used directly but in others they were reared for several generations in the laboratory on their natural host plant in order to increase the number of individuals.

DNA extraction, PCR amplification and sequencing

DNA was isolated either from single individuals or from pooled individuals (10–30, *Oligonychus gossypii* (Zacher), *C. pulcher* and *Eotetranychus* spp.). The material was either fresh or frozen at −20°C. To extract genomic DNA from single individuals, we used a quick method based on the chelating resin Chelex 100 (Biorad). Single individuals were coarsely crushed in a microtube and treated with 40 µl of 5% (w/v) Chelex 100. The tube was incubated at 56°C for 30 min, vortexed at high speed for 15 sec and incubated at 95°C for 15 min. The tube was then centrifuged to pellet the resin and 2 µl of resin-free extract were used as template in PCR reactions. Samples consisting of several individuals were ground with a plastic pestle in an microfuge tube with 500 µl extraction buffer (10 mM NaCl, 10 mM Tris HCl pH 8.0, 1 mM EDTA and 2% SDS) and then digested with proteinase K (50 µg/ml, 37°C for a minimum of 2 h). The samples were subsequently phenol/chloroform extracted, ethanol precipitated and the DNA resuspended in 20 µl ddH_2O and 2 µl was used as template. *In vitro* amplification was performed in 50 µl volume reactions containing 2.5 units of *Taq* polymerase (Promega), 1× *Taq* polymerase buffer (Promega), 0.2 mM of dNTP and 1.4 µM of each of the two oligonucleotide primers. Amplifications were 35 cycles of the following conditions: 92°C for 1 min, 50°C for 1 min and 72°C for 1 min. Double stranded amplification products were purified with Geneclean (Bio 101) according to the manufacturer's instructions. One third of the eluted volume was used for sequencing by the dideoxy chain-termination method (Sanger *et al.*, 1977), using the Sequenase 2.0 kit (US Biochemical Corp.) and labelling with ^{35}SdATP (Amersham). Amplification primers were designed previously from the sequence of a cloned fragment of *T. urticae* mtDNA COI (Fournier *et al.*, 1994). The primers were: 5'TGATTTTTTGGTCACCCAGAAG3' and 5'TACAG-CTCCTATAGATAAAAC3'. The two amplification primers were also used as sequencing primers.

Sequence availability: sequences have been deposited in the EMBL data library under accession numbers: X79901 and X80855-73.

Sequence analyses

Alignment of sequences was done by eye. Specific computer programs were written by Jacques Lagnel to perform calculations of transitions and transversions as well as synonymous and non-synonymous differences between pairs of sequences.

Phylogenetic analyses

The phylogenies were inferred and tested with several programs included in J. Felsenstein's PHYLIP 3.5c package (Felsenstein, 1993). The program DNAML was used, and we optimized the different parameters of the maximum likelihood (ML) model used to infer the phylogeny. The different parameters are: (i) base composition; (ii) the transition/transversion ratio (T); (iii) the number of categories of sites (C), each with a different relative rate of substitution and (iv) the average length of stretches of nucleotides of the same category (i.e. a measure of non-independence of adjacent

Table 1. Origin of the mite species analysed.

Family	Subfamily	Species	Host plant	Location
Tetranychidae	Tetranychinae	*Tetranychus urticae* Koch	*Phaseolus vulgaris*	Montpellier (F)
		Tetranychus kanzawai Kishida	*Manihot esculenta*	Brazzaville (Congo)
		Tetranychus mcdanieli McGregor	*Vitis vinifera*	Verzeney (F)
		Tetranychus pacificus McGregor	*Vitis vinifera*	Davis (USA)
		Tetranychus gloveri Banks	*Colocasia sp.*	Papara (Tahiti, F)
		Tetranychus neocaledonicus André	*Vigna unguiculata*	Cotonou (Benin)
		Tetranychus viennensis Zacher	*Malus domestica*	Montpellier (F)
		Oligonychus gossypii (Zacher)	*Manihot esculenta*	Brazzaville (Congo)
		Oligonychus platani (McGregor)	*Platanus acerifolia*	Montpellier (F)
		Oligonychus ununguis (Jacobi)	*Cupressus sempervirens*	Montpellier (F)
		Eotetranychus carpini (Oudemans)	*Vitis vinifera*	Montpellier (F)
		Eotetranychus coryli (Rekk)	*Corylus avellana*	Montpellier (F)
		Eotetranychus tiliarium (Hermann)	*Tilia ×europaea*	Montpellier (F)
		Panonychus ulmi (Koch)	*Malus domestica*	Montpellier (F)
		Panonychus citri (McGregor)	*Prunus laurocerasus*	Montpellier (F)
		Mononychellus progresivus Doreste	*Manihot esculenta*	Cotonou (Benin)
		Eurytetranychus buxi (Garman)	*Buxus sempervirens*	Montpellier (F)
	Bryobiinae	*Petrobia (Tetranychina) harti* (Ewing)	*Oxalis* sp.	Montpellier (F)
		Bryobia kissophila van Eyndhoven	*Hedera helix*	Montpellier (F)
Tenuipalpidae		*Cenopalpus pulcher* (Canestrini & Fanzago)	*Malus domestica*	Montpellier (F)

by the program DNAML. First, sixteen different values of the transition/transversion parameter T, ranging from 0.4 to 15, were tried, both with only the species of the genus *Tetranychus*, and with one species of each genus. In both cases T=0.5 gave the best likelihood, and this value of T was used in all further analyses. We then tried to optimize the number of categories of sites and their relative rates. As a guideline to choose initial values, we conducted a parsimony analysis using DNAPARS with all species and inspected the distribution of the number of substitutions per site. We tried five different combinations with three equifrequent categories and ended with the best combination being three categories with relative substitution rates of 0.0, 1.0 and 5.0. We then tried 16 different combinations of the frequencies of these different categories and the best combination was frequencies of 0.55, 0.3 and 0.15, respectively for the three categories defined above. We did not try to introduce correlations between adjacent sites, because it seemed inappropriate for this coding sequence. Trees were drawn using the programs RETREE and DRAWGRAM included in PHYLIP 3.5c.

Bootstrap analyses were also conducted. Due to calculation time limitations, it was impossible to analyse the bootstrapped data sets using DNAML. Instead we calculated distances between taxa in the bootstrapped datasets using the program DNADIST and the maximum likelihood option, with the optimized parameters described above. We then used the neighbour joining method (program NEIGHBOR in PHYLIP) to construct the trees and program CONSENSE to produce a majority rule consensus tree.

The program DNAINVAR was also used to test a certain number of phylogenetic results obtained by the ML method. As implemented in PHYLIP 3.5, the test allows the comparison of the three possible unrooted trees relating four taxa, based on patterns of transversion differences between taxa (Lake, 1987). The test is used as follows. Say that the hypothesis to test is that taxa A and B are sister groups when compared to taxon C. Then the test is performed running DNAINVAR with taxa A, B and C, plus a fourth taxon, D, that is known to be an outgroup. If tree ((A,B),(C,D)) has a significantly nonzero invariant but the two other trees have not, the monophyly of A and B is statistically supported.

We also used the package MEGA 1.0 (Kumar *et al.*, 1993) to calculate distances on the basis of non-synonymous substitutions and infer a phylogeny by neighbour-joining.

Morphological and life history data

The following taxonomically informative morphological characters were examined: empodium I (distal part of the leg I) of the female, tip of the peritreme (part of the respiratory system) and profile of the aedeagus (part of male genitalia). Several biological features (mode of reproduction, feeding specificity, habitat and silk production) of the species studied were also analysed.

Results

Sequence variation

The sequences of the central part of the mitochondrial COI gene (a total of 340 to 390 bp) in 20 mite species are shown in fig. 1. The 340 nucleotide region sequenced in all species (nucleotides 34 to 375, fig. 1) was used in the analyses described below. The alignment was straightfor-

	10	20	30	40	50	60	70	80	90	100	110	120
Tetranychus urticae	ATCTTAATCC	TACCAGGTTT	TGGAATAATT	TCACATGTTA	TTAGTTATAA	TTTAGGTAAA	AAAGAAGTTT	TTGGTAAAAT	TGGTATAATG	TTTGCTATAA	TATCAATTGG	TTTATTAGGA
Tetranychus kanzawai	T	....	....	......A...	....	......A...	....	....	...A.....A	....	....	....
Tetranychus mcdanieli	.....TT	....	...T.....C	..G...A...	....A.....	....	........A.	....A.....	.........A	....	....	AC.T......
Tetranychus pacificus	.....TT	.......G..	...T.....	......A...	....A.....	....	........A.	....A.....	.........A	....	....	.C.T......
Tetranychus gloveri	.....T.	.T..T..A..	....	......A...	....	......G...	....	....A.....	...A.....A	....	....T.....	....C.T..T
Tetranychus neocaledonicus	.....TT	....	....	......A...	....A.....	......A...	........G.	.C..A...G.	...G.....A	....	....T.....	A.........
Tetranychus viennensis	.....TT	.......A..	...T...G.A	..T...A...	....	......A...	....	....	...A.....A	....	....	A.........
Oligonychus gossypii		.....A..	....	..T.....AT	.A..A.T...	....	........A.	....A.....	...A.....A	..C..A....	.T........	A.........
Oligonychus platani			....	..T.....	.C..C.....	....	....	....	A..A.....T	.....A....	....T.....	.........T
Oligonychus ununguis	..T.....TA	.T.....A..	....	..T.....	....A.....	......A...	....	....	A..G.....T	....	....T.....	AC.T.....T
Eotetranychus carpini	...C.T..TT	....T.....	....	..T...A...	....A.....	....	....	.C..A.....	...A.....A	....	....T.....	A...C.T..T
Eotetranychus coryli			.....	..T...A...	....A.....	......A...	....	....A.....	...A.....A	....	....T.....	....C.T..T
Eotetranychus tiliarium	...C.T..TT	....T..A..	....	..T...A...	....	.A....A...	....	....A.....	...A.....A	....	....T.....	A.....G...
Panonychus ulmi				..T...N...	....A.T...	.G........	....	....G.....	A..A.....A	....	....T.....	C...C.....
Panonychus citri		..	.......G..	..T.....	....A.....	......A...	........G.	.......G..	A..A.....A	..C.......	....	.C.T......
Mononychellus progresivus	.....T.	.......A..	C..T.....	..T...A...	....	....	........A.	........T.	A..A.....A	........G.	....	....
Eurytetranychus buxi	..T.....TT	.G..T.....	...T..T...	....	.C....TC..	......G...	........C.	....	....	..C.......	.G..T.....	...G.....T
Petrobia harti				....	....A.T.TT	.AC...A...	........A.	....A.....	...A...T.A	....	....T.....	AA.T......
Bryobia kissophila	..T.....TT	.......A..	...T..G...	........AT	.A....T.T.	..CT......	....	....A...G.	.........T	....	GC..T.....	AG.T.....G
Cenopalpus pulcher	.....TA	.T..T..A..	......T...	..T.NATC.T	.A..A.T.TT	..GT......	....	.C..A....C	...A.....T	........T.	.T..T.....	AG....G..T

	130	140	150	160	170	180	190	200	210	220	230	240
Tetranychus urticae	TTTATTGTTT	GAGCACATCA	TATATTTACA	GTAGGTATAG	ATGTTGATAC	TCGAGCTTAT	TTTACAGCTG	CCACTATAAT	TATTGCTATC	CCTACTGGAA	TTAAAATTTT	TAGTTGATTT
Tetranychus kanzawai	....	....T..C..	....	....	....	....	....	.T..A.....	.........T	....	....	....
Tetranychus mcdanieli	....	....T.....	....	..T.....	....	A.....A...	....	.T..A.....	......A..T	....	....	...A......
Tetranychus pacificus	....	....C..C..	....	..T..A....	....	......A...	....	.T..A.....	......A..T	....	....	....
Tetranychus gloveri	....	....	....	..T.....	....A.....	....	....	.A........	......G..T	....	....	...A......
Tetranychus neocaledonicus	........A.	....T.....	...G.....	.....A....	....A.....	....	....	.T........	......A..T	....	....	......G...
Tetranychus viennensis	...G.A....	....T.....	.........T	....	....A.....	......A...	.....T....	.T........	.........T	..A..A....	....	...A......
Oligonychus gossypii	........A.	.G..T..C..	....	.....A....	....A.....	A.........C	........A.	.T........	.........T	..A.......	....G.....	....
Oligonychus platani	..CG....A.	....T.....	....	....	..A.......	A.........	....	.T........	....	....	....	...A..G..C
Oligonychus ununguis	...G.....	....T.....	.........T	.....A....	..A.......	...G......	....	.T........	....	....	....G.....	...A......
Eotetranychus carpini	....	.G........	.........T	.....A....	....A.....	....	........G.	.T........	.........T	..AN......	....	C.........
Eotetranychus coryli	....	....T.....	....	..T.....	....	......A...	........A.	.T........	....	..A.......	....	....
Eotetranychus tiliarium	....	....T.....	.........T	....	....A.....	....	..C.....A.	.T..A.....	....	..A..A..T.	....	....
Panonychus ulmi	...G.A....	....T.....	....	.....G....	....A.....	....	.....T....	.T........	....	..A.......	....	...A......
Panonychus citri	...G.A..G.	....T.....	....	.....A....	....A.....	....	........A.	.T........	....	..A.....T.	....	....
Mononychellus progresivus	...G....A.	....T.....	.........T	A.T.......	....	....	........A.	.T..A.....	....	.....A..T.	....	...A......
Eurytetranychus buxi	...G.A....	.G.....C..	.........T	....	....	....	........G.	.T..A.....	.........T	........T.	....	......G..C
Petrobia harti	........A.	....T.....	....	....	....A.....	....	.....T..A.	.A........	......AG.T	..A..A....	....	....
Bryobia kissophila	..CG....A.	....T..C..	....	.....A..G.	....	A..G......	.....G..A.	.T..A.....	......AG.T	........T.	....	C.....G..C
Cenopalpus pulcher	....	....T.....	.........T	.....AT...	..A.......	...TT.....	.....T....	.T........	.......G.T	....	....	....

Fig. 1—*Continued below*

	250	260	270	280	290	300	310	320	330	340	350	360
Tetranychus urticae	ACTACAATTT	TAAATTCTCA	TATTAACTTT	AATATTTCTA	TATATTGATC	AATAGGATTT	TTAATTATAT	TTTCTATTGG	AGGATTTACA	GGAATTGTAG	CTTCAAATTC	ATGTTTAGAT
Tetranychus kanzawai	.....T...A	....C..A..	.....T...	..C.....	....C...G.	....	....	....C.....	....	........G.	.......C..	....
Tetranychus mcdanieli	.....T....	.......A..	.....T...	.....C....	........G.	T.........	....	....	...T......	....	....	....
Tetranychus pacificus	........CA	.......A..	.....T...	..C.....	....C.....	T.........	........G.	...A......	...G......	....	....	....
Tetranychus gloveri	G....T..AA	.T.....A..	.....GT..C	.GA.....A.	....	....	....	....A.....	G..T......	....	....T..C..	....
Tetranychus neocaledonicus	..A..T...A	.T.....A..	.....T...	....CA..A.	....	....	....	....A.....	....	....	.A..T..C..	T.........
Tetranychus viennensis	.....T...A	.T........	.....T...	........AT	.TAT....G.	TT........	C.........	....	...G......	....	....T.....	....
Oligonychus gossypii	.....T...A	.T.....A..	C.....T..A	........A.	.T......G.	T.........	....	.....G.A..	....	......A.T.	....T.....	T.........
Oligonychus platani	T....T...A	....	C.........	......AGA.	....C.....	...G......	....	....	....	......A.T.	....T.....	....
Oligonychus ununguis	T....T..CA	....	C.....T...	......AGA.	....	T.........	....	....	T.........	..G...A.T.	....T.....	....
Eotetranychus carpini	.....T...A	.T.....A..	.....T...	....	.T.TA.....	....	....	....	T.........	......A.T.	....	T.........
Eotetranychus coryli	.....T...A	.T.....A..	.....T...	.....CC...	.T.TA.....	T.........	....	....	T..T......	..G...A.T.	....	T..C..G...
Eotetranychus tiliarium	.....T...A	.T.....A..	.....GT...	....	.T.TA...G.	C.........	....	....	....	......A.T.	.C........	T.........
Panonychus ulmi	..A.....AA	.T.....A..	.....T...	......G.A.	.T........	....	....	....	T..C.....T	......A.T.	....T..C..	T.........
Panonychus citri	..A..T..AA	.T........	.....T...	........A.	.T..C.....	T.........	........G.	....A.....	...T.....T	......A.T.	....	T.........
Mononychellus progresivus	.....T..CA	.T........	.....T...	.........G	.C........	....	....	....	...T......	.....CA.T.	....	T.........
Eurytetranychus buxi	.....T...A	.T..C..G..	...C.TT...	TC..G...C.	...TA...G.	TT.G..G...	....	.....T.A..	G..C..C...	.....C..T.	....T..C..	......G...
Petrobia harti	G....T..AA	.T........	CT.A.TTA.A	....A.G..T	..ATA...G.	....	....	....	...T.....T	......A.T.	..G.T.....	T.........
Bryobia kissophila	T....T...A	.T.....A..	.T.A.TT...	...G.A...T	...TA...G.	T.........	....	....	...T......	..T..CA.T.	..G.T..C..	T.....G...
Cenopalpus pulcher	T..T.T...C	.T........	.T.A.TT..A	.GATC.A..T	.T.T....GT	TT....T...	..GT......	..A.......	......A...	..T...A.TC	A...T.....	T.C.......

	370	380	390
Tetranychus urticae	ATTAATTTAC	ATGATTCATA	CTATATTGTA
Tetranychus kanzawai	....	....C.....	T..C......
Tetranychus mcdanieli	....	.....A....	...C..N...
Tetranychus pacificus	......C.T.	.....A.T..	T..C......
Tetranychus gloveri	....	.....A.T..	....
Tetranychus neocaledonicus	......C.T.	.....A.T..	T.NN...NG.
Tetranychus viennensis	....	.....A....	T.....N...
Oligonychus gossypii	....	.....A.T..	T.....
Oligonychus platani	...TC.....	.....A.G..	T........T
Oligonychus ununguis	...TC.....	....CA.T..	T........T
Eotetranychus carpini	...TC.....	.....A....	T......
Eotetranychus coryli	...TC.....	.....A.T..	.....
Eotetranychus tiliarium	...TC.....	.C..CA.T..	T......
Panonychus ulmi	...TG.....	.....A.T..	
Panonychus citri	...GC.....	.....A....	T...
Mononychellus progresivus	..CTC.....	....CA	
Eurytetranychus buxi	....	.C...A.T..	...CTAG
Petrobia harti	...TC.....	.....A.G..	T........T
Bryobia kissophila	..CTC.....	.....A.T..	T.........
Cenopalpus pulcher	...TTA..T.	.....A.T..	T...G...

Fig. 1. Nucleotide sequences of the partial cytochrome oxidase I gene in 20 species of mites. Dots indicate bases identical to those of the first species.

ward and involved no insertions/deletions. The number of nucleotide differences in pairwise comparisons of species ranged from 18 (5%) to 86 (25%).

A detailed analysis of the patterns of substitution and evolution of this gene will be presented elsewhere. A few characteristics however are worth mentioning here because they have an important bearing on the use of the molecule for phylogenetic analysis. The sequences are extremely rich in A+T (75% on average in the 20 species), and especially so at the third codon position (95%). The base composition does not vary significantly from one species to another, with the notable exception of *Eurytetranychus buxi* (Garman), which has a significantly higher G+C content at the third codon position (22.1% in *E. buxi*; 5.8% on average, statistical analysis presented elsewhere).

This biased base composition has important consequences on the substitution patterns of the mite COI sequences. Among differences between all pairs of studied species, 68% are transversions and 38% are synonymous transversions at the third codon position. In fig. 2 we have plotted the number of transition and transversion differences observed in all pairwise comparisons of species, against the number of non-synonymous differences, which is expected to be well correlated to divergence time. It can be seen that there is no correlation between the number of transition differences and divergence time. On the contrary, the number of transversions increases steadily with the number of non-synonymous differences, although a slight saturation is visible at the third codon position.

Phylogenetic inference

The phylogeny obtained using the maximum likelihood model with the parameters optimized as described in the experimental procedures paragraph is shown in fig. 3. The species *E. buxi* was not included because of its peculiar nucleotide composition and because current phylogeny inference techniques, including the ML algorithm used, do not allow for variations in base composition among branches. As expected, *C. pulcher*, which belongs to the family Tenuipalpidae, is the most distantly related to all the other species, which are part of the family Tetranychidae. Within the Tetranychidae, the two species of the subfamily Bryobiinae (*Bryobia kissophila* van Eyndhoven and *Petrobia* (*Tetranychina*) *harti* (Ewing)) are most distantly related to the Tetranychinae, but their monophyly is not clear in the ML analysis. The monophyly of the Bryobiinae was tested using Lake's invariants. This was performed by including in the analysis with DNAINVAR the two Bryobiinae species, *C. pulcher* as the outgroup and one of the Tetranychinae. Of the 16 different tests performed with the 16 Tetranychinae, five supported the monophyly of the Bryobiinae, three rejected it and the remaining eight tests did not allow a decision. Thus the monophyly of the Bryobiinae remains uncertain on the basis of these data but is not ruled out. Within the Tetranychinae, three of the genera in which more than one species was studied appear monophyletic. However this is not the case of the genus *Oligonychus*, because although *Oligonychus platani* (McGregor) and *O. ununguis* (Jacobi) are clearly sister taxa (they appear together in 98% of the 1000 bootstrap analyses), *O. gossypii* is not grouped with them. Maximum likelihood analysis groups this species with the genus *Tetranychus*, although outside of it. However, this position does not seem stable because it was found in only 10% of the 1000 bootstrap analyses. The branching order of the different genera of Tetranychinae suggested by the ML analysis also needs to be tested.

That *Eotetranychus* is external to the other Tetranychinae was tested with Lake's invariants by including *P. harti* as the outgroup, one *Eotetranychus* and two other Tetranychinae of two different genera. Fifteen of the many possible combinations of this type were tested; five of the tests favoured the external position of *Eotetranychus* and two contradicted the hypothesis. The grouping of the three genera *Panonychus*, *Oligonychus* (*O. platani* and *O. ununguis*) and *Mononychellus* was tested by comparing either pair and the outgroup and one *Tetranychus* or one *Eotetranychus* and calculating Lake's invariants. Although the pair *Panonychus–Oligonychus* is always accepted by the test, the grouping of *Mononychellus* with either *Oligonychus* or *Panonychus* is not supported (6 tests for and 11 against, over 33 tests performed). Finally, the monophyly of the genus *Tetranychus* was tested and especially the possibility that *Tetranychus viennensis* Zacher might not belong to it because it is the species most distantly related to the others within this genus. Lake's invariants tend to reject the hypothesis that *T. viennensis* is a *Tetranychus* (three tests against and one for). For reasons of comparison, the same test was carried out with *Tetranychus neocaledonicus* André instead of *T. viennensis*, and the former was never found not to belong to the genus *Tetranychus* by Lake's invariants. This reinforces our suspicion that *T. viennensis* might not belong to the genus where it is classified. All the above-mentioned hypotheses of monophyly of different groups of species or genera were tested by letting DNAML compare alternative trees and perform the maximum likelihood ratio test, but no significant results were obtained (details of the use of the test can be found in the documentation of PHYLIP). The phylogenetic analyses based on non-synonymous substitutions only (using the program MEGA) gave the same topology as the ML analysis (not shown).

Variation of morphological and life history traits

Three anatomical features are shown in fig. 4 for each of the species studied: empodium, peritreme and aedeagus. The empodium is classically used as the basis for distinguishing between genera of Tetranychidae and the two other characters are used for species determination. The shape of the empodia is mainly homogeneous in each genus. The main traits of the morphological classification based on these characters can be summarized as follows. Firstly, the empodia of Tenuipalpidae and Bryobiinae both have tenent hairs which are not present in Tetranychinae, and Bryobiinae also have a pad-like empodium with developed claws. Tetranychinae species secrete silk and some even spin webs; adaptation to locomotion on silk strands or web structures is by elongation of the empodium followed either by the division of the tip (*Mononychellus*, *Eotetranychus*) or by the addition of proximoventral hairs on a more or less pronounced empodial claw (see Gutierrez & Helle, 1985 for details on morphological characters). This claw is strong in *Panonychus* and *Oligonychus*, generally reduced to a minute spur in *Tetranychus*, but apparently absent in *T. viennensis*. Secondly, peritremes of the less derived tetranychid mites terminate in anastomosing sacs; these are also reported to occur in several tenuipalpid species. The more evolved taxa

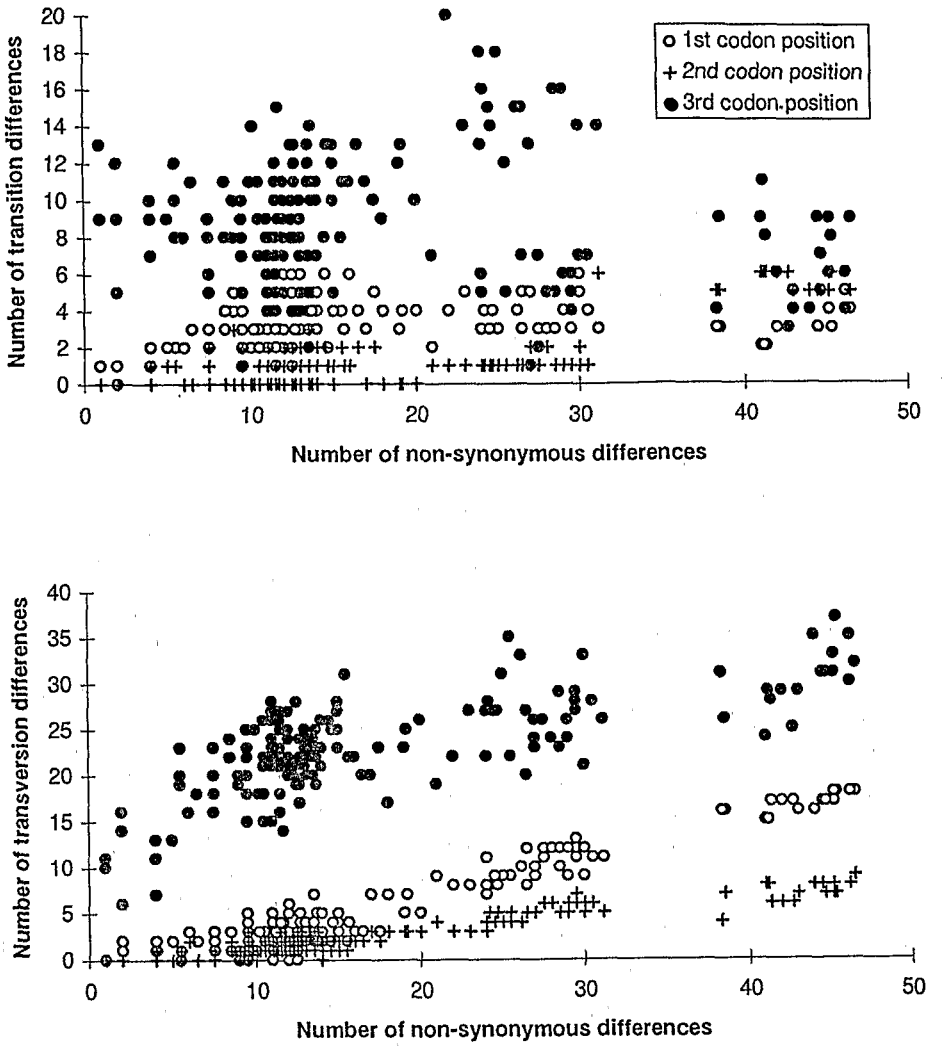

Fig. 2. Plot of the number of transition (upper graph) and transversion (lower graph) differences by codon position against the number of non-synonymous differences in pairwise comparisons of the cytochrome oxidase I sequences of the 20 mite species listed in table 1.

of the family have peritremes ending in a simple bulb or in a single hook formed by 4–6 chambers. The mtDNA phylogeny reported here is overall concordant with this evolutionary trend. *O. gossypii*, with hook-shaped peritremes, stands out from the two other *Oligonychus* studied, whose peritremes end in a bulb. In addition, *T. viennensis* has anastomosed peritremes reminiscent of those of less derived species and clearly differing from the consensus form of the genus *Tetranychus*. Thirdly, in the Bryobiinae, the aedeagus generally consists of a shaft narrowing to form a slender stylet. This aspect is replaced by a wide variety of shapes in the Tetranychinae. The shape of the aedeagus is generally homogeneous in each genus, although two exceptions are worthy of mention. In *O. ununguis* and *O. platani*, the aedeagus bends downwards whereas it bends dorsally in *O. gossypii*. Among the *Tetranychus*, *T. viennensis* also has a distinctive aedeagus with a sharp dorsal bend and small anterior angulation.

Several life history traits of the mite species studied are also shown in fig. 4: mode of reproduction, feeding specificity, habitat and silk production. Although arrhenotoky is the most usual reproductive system in Tetranychinae, it is interesting to note the closeness of the species of the genera *Panonychus*, *Mononychellus* and *Oligonychus* (*O. ununguis* and *O. platani*) which, without being thelytokous, include the only thelytokous species of the subfamily Tetranychinae (Ehara & Gotoh, 1992; Gutierrez *et al.*, 1991; Gutierrez & Helle, 1985). With regard to host plant specificity, it would appear that polyphagy is a dominant trend in the species of the genus *Tetranychus* studied here which, with the exception of *T. viennensis*, live on herbaceous plants; while the data on habitat show the intrageneric homogeneity of *Oligonychus*, *Eotetranychus* and *Tetranychus*. The data on silk production confirm the evolutionary trends mentioned above which clearly separate the Bryobiinae (without silk) from the Tetranychinae (with silk).

Discussion

Mode of evolution of mite mtDNA

The base composition of the mite COI gene is very similar to what has been described in several insects (Clary

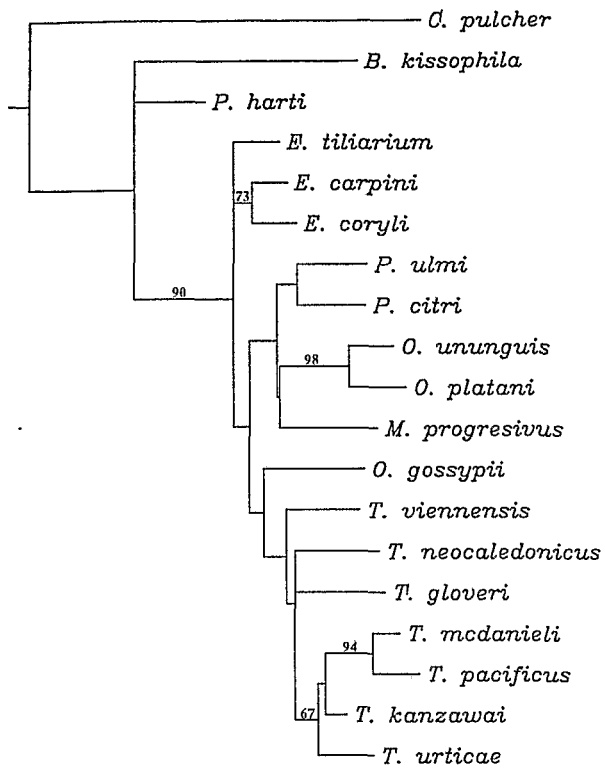

Fig. 3. Phylogenetic tree obtained from the cytochrome oxidase I sequences using a maximum likelihood model. The bootstrap values (1000 resamplings) greater than 50% are indicated.

& Wolstenholme, 1985; Crozier & Crozier, 1993; Mitchell *et al.*, 1993). The genetic code inferred on the basis of COI sequences seems to be a conservative feature of insects and mites as well. This and other features will be analysed and detailed elsewhere. Here we will focus on the characteristics of these sequences that have a bearing on our phylogenetic analysis.

First of all, significant base composition variation occurs between genera, *Eurytetranychus* showing a significantly higher G+C content than the other mite species studied here. Among insects such variations have been reported, but only between extremely divergent species (Jermiin & Crozier, 1994). However, the change of base composition that we report here in mites concerns relatively closely related taxa, that is, genera inside the same family. Thus, although there seems to be continuous pressure in insects and mites to maintain a high proportion of A+T, secondary relaxation of this constraint appears to have occurred several times, and has happened in a relatively short time in the case of mites. This feature has not allowed us to include *Eurytetranychus* in the phylogenetic analysis, as already mentioned.

A consequence of the strong compositional bias is the nature of the nucleotide differences between species. Most differences are attributable to transversions with a majority of A–T changes. This does not mean however that transition substitutions are improbable, and the rapid saturation of transition differences that can be seen on fig. 2 can be interpreted as the consequence of both a high transition substitution rate and a strong bias on base composition. In fact, comparisons of very closely related taxa, as well as intraspecific comparisons not presented here, show that transition substitutions are more frequent than transversions by a factor 2 to 4. This phenomenon is well documented in *Drosophila* (DeSalle *et al.*, 1987), and mammals (where transitions are about ten times as frequent as transversions, Irwin *et al.*, 1991). On the other hand, transversion differences accumulate at an almost steady rate as a function of the number of non-synonymous differences, although a slight saturation is visible at the third codon position (fig.2). For this reason this gene appears suitable to conduct a phylogenetic analysis at the taxonomic level considered here, but would not allow comparisons of more distantly related taxa.

Phylogenetic relationships: morphological versus molecular evolution

Base composition bias, relative frequencies of different types of substitutions and variations of substitution rates at different positions are important parameters to take into account in order to properly reconstruct the phylogeny of the sequences. However, accurate estimation of these parameters cannot be performed by a simple examination of the sequences, without knowledge of their phylogeny. We chose maximum likelihood precisely because it allows an optimization of a number of parameters to be done by taking the phylogeny into account. In attempting to optimize the parameters of the Felsenstein ML model for our dataset, we explored a large spectrum of values for these parameters, so that we feel confident that the values chosen are close enough to the optimum. One thing we did not do because of computation time limitations was to test the interactions between variations of the different parameters (we allowed only one parameter to vary at a time). However, the many calculations we performed with different parameter values showed that the method appears robust to parameter variations.

The phylogenetic relationships found using the ML approach agree well with family, subfamily and genus subdivisions previously defined by classical taxonomy. Overall, the present data provide good support for the monophyly of the subfamily Tetranychinae and for that of its genera, with the notable exception of *Oligonychus*, the monophyly of which is clearly rejected, although the exact position of *O. gossypii* could not be determined with confidence. The monophyly of the subfamily Bryobiinae also requires demonstration. Within the Tetranychinae we also failed to properly resolve the relative branching order of the different genera although the phylogeny obtained by the ML method is identical to that based on non-synonymous substitutions. Thus, although the results make sense, longer sequences are clearly needed to gain more confidence. This will require characterization of additional sequences in these organisms.

Although analysis of COI sequences is compatible with the main lines of classification established on the basis of morphological criteria alone, it also requires the revision of several points in the taxonomy of the Tetranychidae. In a general manner, empodium shape serves as the basis for distinction between genera. However, this character has its limits in certain cases and other criteria such as shape of the aedeagus and the peritreme could be conclusive. The three *Oligonychus* species studied have homogeneous empodium

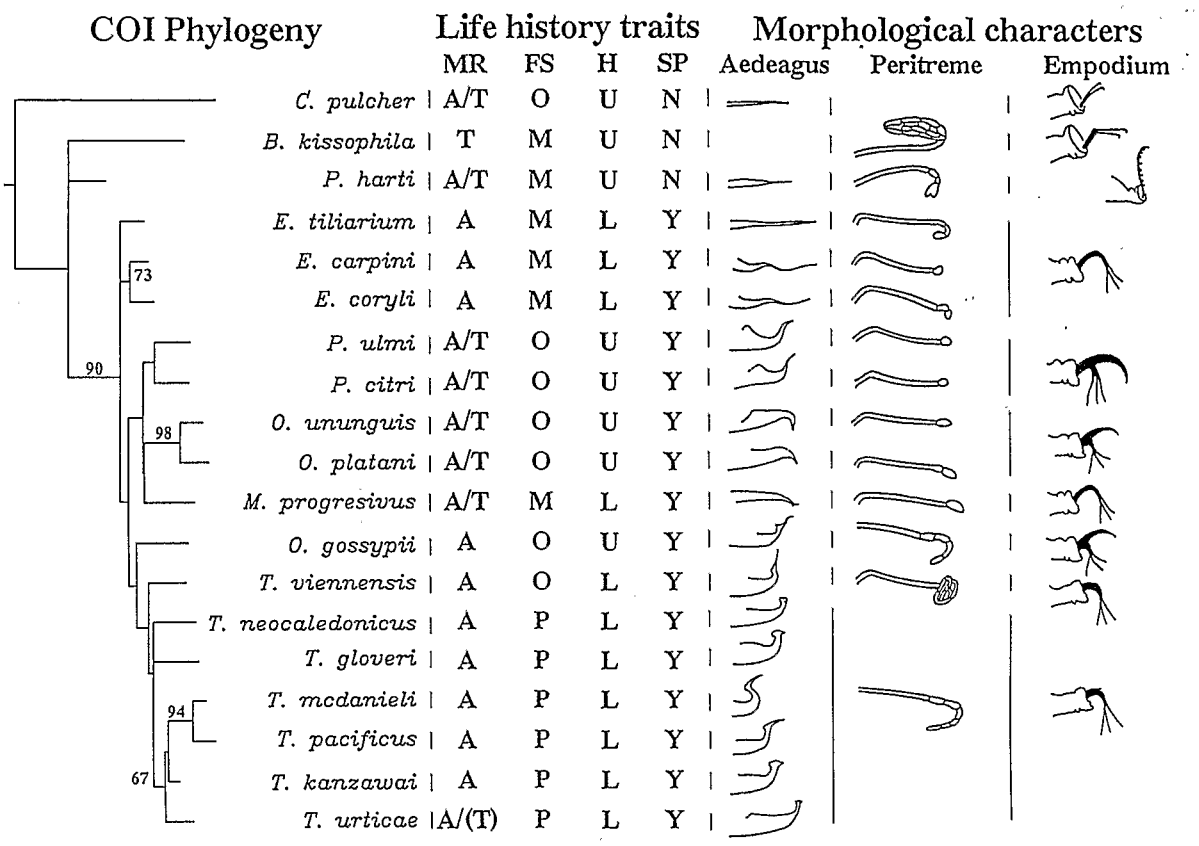

Fig. 4. Life history traits and morphological characters studied in several species of mites (homologous structures of empodia are in black). The distribution of characters is compared to the phylogenetic tree of fig. 3.
MR (mode of reproduction): A=arrhenotoky; T=thelytoky; A/T=thelytoky is reported in some species of the genus; A/(T)=thelytoky has been observed in some strains. FS (feeding specificity): O=oligophagous; M=monophagous; P=polyphagous. H (habitat): U=upper side of the leaves; L=lower side of the leaves. SP (silk production): N=no; Y=yes.

shape but the aedeagus clearly distinguishes the species *O. gossypii* from the two others (fig. 4). This species has already been placed in a different subgenus than the two other *Oligonychus* on the basis of morphological characters. This phylogenetic break in the genus is also reflected by the mtDNA divergence of both subgroups. A second case of unsuitability of morphological characters is raised by the data reported here. Phylogenetic analysis places *T. viennensis* outside the other species analysed in the genus. This led us to carefully re-examine the empodium, and an absence of a mediodorsal spur was observed on all the legs of specimens of both sexes, whereas the presence of the spur characterizes the genus *Tetranychus*. This differentiation of *T. viennensis* noticed *a posteriori* is supported by a distinct aedeagus shape in males. In addition, the species appears to have conserved an ancestral peritreme shape only found elsewhere in the genus in a near species, *Tetranychus savenkoe* Rekk. This range of characters would appear to favour the rehabilitation of the genus *Amphitetranychus*, created by Oudemans (1931) to classify the two latter species and subsequently considered as a subgenus by Wainstein (1960). In these cases of uncertainty with regard to the importance to be awarded to the morphological characters used, comparison of molecular data and morphological characters can make it possible to reinterpret the taxonomic criteria used and to identify the characters that are most pertinent to the systematics of a group.

Evolution of life history traits

Attempts made to incorporate ecological data into phylogenetic reconstruction for insect groups, as reviewed by Miller & Wenzel (1995), become available for spider mites. Of the two Tetranychidae subfamilies, the Bryobiinae have, in general, retained more plesiomorphic characters, such as life on the upper surface of the leaf and no silk production. In contrast, the derived Tetranychinae are generally oligophagous or polyphagous, some reside on the underside of the leaf and all are able to produce silk (Gutierrez & Helle, 1985, 1988). Phylogeny at the family level (fig. 4) is compatible with directional evolution of some of these traits (habitat, feeding specificity and spinning behaviour) and with the evolution of the related morphological characters. Empodium structure and shape are correlated with behavioural and locomotion characteristics: the empodium and the two claws on each side of it have evolved from a type adapted to movement on stems and the upper surface of leaves to a type adapted to movement along silk threads or webs. However, among the Tetranychidae, life on the lower leaf seems to have evolved several

times and to have led to convergent evolution of empodium shape.

Type of reproduction is an important trait in the life history of species. Distribution of the mode of reproduction characteristic of each species within the family has been evaluated (fig. 4). Comparison with phylogenetic data suggests that an evolutionary trend for thelytoky to become rarer has operated since the origin of the family. In the Bryobiinae, numerous genera contain both arrhenotokous and thelytokous species but approximately 40% of species reproduce by thelytokous parthenogenesis. In the Tetranychinae however, arrhenotokous parthenogenesis is the rule, except in some species of the genera *Panonychus*, *Oligonychus* and *Mononychellus*, and some strains of *Eurytetranychus* and *Tetranychus* which are thelytokous. Examination of higher taxa indicates that arrhenotoky is ancestral in Tetranychoidea (Norton *et al.*, 1993). The data reported here are compatible with the hypothesis of repeated appearance of thelytoky, particularly as this type of reproduction can appear at any time. Even in the genus *Tetranychus* where arrhenotoky seems fixed, there is genetic potential for evolution of thelytoky.

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