THE ROYAL SOCIETY biology letters

Nuclear ribosomal DNA monophyly versus mitochondrial DNA polyphyly in two closely related mite species: the influence of life history and molecular drive

M. Navajas[1*] and P. Boursot[2]

[1]*Institut National de la Recherche Agronomique, Centre de Biologie et de Gestion des Populations, Campus International de Baillarguet, CS 30 016, 34988 Montferrier sur Lez, France*
[2]*Laboratoire Génome Populations Interactions Adaptation (UM2–IFREMER–CNRS UMR 5000), Université Montpellier II, 34095 Montpellier, France*
[*]*Author for correspondence (navajas@ensam.inra.fr).*

Recd 25.02.03; *Accptd* 14.04.03; *Online* 18.06.2003

In two very closely related but reproductively isolated mite species, *Tetranychus urticae* and *T. turkestani*, we found nucleotide diversity to be extensive for mitochondrial DNA (mtDNA) cytochrome oxidase 1 (COI) (3–4%) but extremely reduced for nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS2) (less than 0.5%). By contrast, ITS2 was shown to evolve much faster than COI between species of this genus. Furthermore, we found that these two species are polyphyletic for mtDNA but monophyletic for rDNA. Thus it appears that despite its biparental transmission and multiplicity of copies in the genome, nuclear rDNA has a smaller effective population size than mtDNA in these species. The conjunction of efficient concerted evolution and/or gene conversion in the rDNA cluster, the haplodiploidy of these species and their female-biased sex ratio could account for this apparent contradiction.

Keywords: ribosomal DNA; internal transcribed spacer; mitochondrial DNA; cytochrome oxidase 1; incongruence

1. INTRODUCTION

Inferring population or species history from gene genealogies is now common practice. It is also recognized that superimposing population history with that of a single gene can be misleading (Brower *et al.* 1996; Ross *et al.* 1999) because of the intrinsic stochasticity of the coalescence process, but also because different genes can have contrasting effective population sizes (because of differences in their mode of transmission or different selection pressures). Two of the most commonly used markers in molecular systematics are mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA), which differ dramatically in their genomic organization and mode of transmission. It is thus important to assess how these contrasting properties affect their respective abilities to reflect population history. We illustrate this aspect here by comparing intraspecific polymorphism and interspecific divergence for these two markers in two very closely related species of phytophagous spider mites, *Tetranychus urticae* and *T. turkestani*. We find polyphyly and high polymorphism for mtDNA, and the reverse for rDNA, which is in apparent contradiction with both the characteristics of the two markers and the previous demonstration of a higher mutation rate of rDNA internal transcribed spacer (ITS) than mtDNA in these species (Navajas *et al.* 1998). We discuss the possible influences of haplodiploidy and concerted evolution of rDNA to account for this observation.

2. MATERIAL AND METHODS

Table 1 summarizes the origin of the samples of *T. urticae* and *T. turkestani* used in this study and the host plants where mites were collected. The two species cannot be discriminated on the basis of external morphology, but microscopic examination of the shape of the aedeagus (male genitalia) allows unambiguous identification.

DNA extraction protocols from either fresh, frozen or ethanol-preserved females are described in Navajas *et al.* (1998). Two target fragments, the mitochondrial cytochrome oxidase 1 (COI) and the second nuclear ribosomal internal transcribed spacer (ITS2), were PCR-amplified and sequenced directly following protocols described in detail in Navajas *et al.* (1998). PCR primers amplified a 860 bp fragment of the COI gene: 5′-TACAGCTCCTATAGATAAAAC-3′ and 5′-GGAGGATTTGGAAATTGATTAGTTCC-3′. The ITS2 primers matched conserved flanking 5.8S and 28S rDNA regions (see Navajas *et al.* 1997).

The amplified ITS2 product was in some instances cloned in plasmid vector PGEMT (Promega, USA) according to the procedures recommended by the manufacturer, and the clones were sequenced in both directions as previously described (Navajas *et al.* 1999).

Phylogenetic analyses were performed using the PHYLIP v. 3.57c package (Felsenstein 1993).

3. RESULTS

(a) *Variation of COI sequences*

A mitochondrial COI fragment (849 bp) was sequenced from *T. urticae* and *T. turkestani* mites from various origins (table 1; EMBL accession numbers: submitted, available after acceptance). Comparisons of sequences revealed 97 variable positions, all involving only point mutations (EMBL accession number for sequences alignment: submitted). Intraspecific divergences ranged from 0.9% to 5.3% in *T. turkestani*, and from 2.1% to 6.2% in *T. urticae*. Interspecific divergences were of the same order, from 2.4% to 6.1%.

The maximum-likelihood phylogenetic tree obtained on the basis of the COI sequences is presented in figure 1. Whatever the position of the root, which is unknown, it appears that the two species are not monophyletic for mtDNA: the *T. turkestani* samples are split into two groups, one containing the Polish and French samples (TtP, TtFMe, TtFM and TtFMc), and the other containing the Dutch and American samples (TtNL and TtUSA). The latter *T. turkestani* samples lie on the same branch as *T. urticae* samples from Greece (TuGR) and Spain (TuS), and this grouping is very well supported by the bootstrap analysis (99%). This result was very robust to the mutation model chosen and to various phylogeny inference methods, including parsimony and various distance methods (not shown). Furthermore, likelihood ratio tests (not presented here) rejected all other possible trees among the most parsimonious that force the species to be monophyletic for mtDNA.

DOI 10.1098/rsbl.2003.0034

Table 1. Collection sites of mites.
(The asterisk indicates unknown location and *n* indicates how many individuals have been analysed for each molecule.)

species	sample abbreviation	location	country	host plant	*n* COI	*n* ITS2
Tetranychus urticae	TuET	Cairo	Egypt	*Convolvulus arvensis*	1	1
	TuTN	Sousse	Tunisia	*Malva* sp.	1	1
	TuGR	Egion	Greece	*Citrus limon*	1	1
	TuI	Palermo	Italy	*Citrus limon*	1	1
	TuS	Valencia	Spain	*Citrus aurantium*	1	1
	TuNL	Amsterdam	The Netherlands	*Sambucus* sp.	1	3
	TuF	Montpellier	France	*Sambucus* sp.	—	3
T. turkestani	TtFMe	Montpellier	France	*Euphorbia* sp.	1	2
	TtFMc	Montpellier	France	*Convolvulus arvensis*	1	1
	TtFM	Maugio	France	*Fragaria* sp.	1	3
	TtNL	Enkhuizen	The Netherlands	*Convolvulus arvensis*	1	3
	TtP	*	Poland	*Rosa* sp.	1	1
	TtUSA	Watsonville, CA	USA	*Fragaria* sp.	1	3

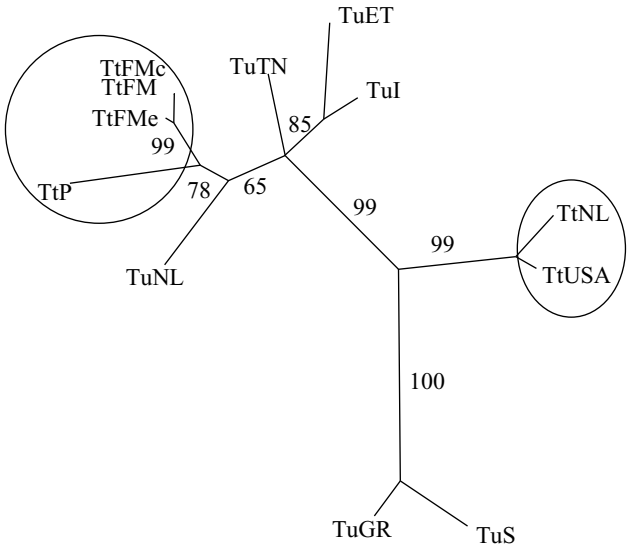

Figure 1. Phylogenetic tree inferred by the maximum-likelihood method based on mitochondrial COI nucleotide sequences of various samples of *Tetranychus urticae* and *T. turkestani* (circled). Sample abbreviations refer to those of table 1. Percentage bootstrap scores (500 replicates) are indicated along the branches.

(b) *Variation of ITS2 sequences*

The complete ITS2 sequences (483 bp) of *T. urticae* and *T. turkestani* from various origins (table 1) were determined (EMBL accession numbers: X99881 for *T. urticae* and AJ295611 for *T. turkestani*, TtFMc). All *T. urticae* displayed the same sequence. Similar results have already been reported on this species (Navajas *et al.* 1998) on a larger sample of geographical origins distributed worldwide. In *T. turkestani*, four different sequences were found, depending on their state at three variable nucleotide positions (figure 2). No variation was found in any of the three populations from which three individuals were sequenced (TtFM, TtNL and TtUSA). The unique *T. urticae* sequence displayed from four to five differences distributed at six positions with either of the *T. turkestani* sequences. Three of these sites represent diagnostic differences between the two species (sites 339, 411 and 454; figure 2).

It thus appears that, despite their polyphyly for mtDNA, these two species have fixed divergent nuclear rDNA ITS2 sequences that can be differentiated at three diagnostic sites. However, because we sequenced the PCR products directly, some undetected variation may exist between the different copies. To check this, we cloned the PCR products and sequenced five clones from each of two *T. urticae* (TuF and TuGR) and two *T. turkestani* (TtFM and TtFMe). The five clones from each individual produced from two to five different sequences, depending on the individual. These sequences differed at a maximum of two positions from that of the PCR product which, as expected, was in all cases identical to the consensus of the different clones from the same individual. None of the clones, except for one from TtFMe, showed any variation at the sites that were found to be diagnostic between the two species. We thus conclude that intraspecific variation for ITS2 is limited compared with that of mtDNA, and that the two species show fixed differences for ITS.

Incidentally, these results allowed us to design a molecular diagnostic between these two species, based on the existence of two cleavage sites for restriction enzyme *RsaI* in *T. urticae* (positions 177 and 340), but only one in *T. turkestani* (position 177). Discrimination of the two species is possible after migration of the digested PCR products on agarose gels, which can be useful because the two species can be found on the same host plant species and are indistinguishable on the basis of external morphology.

4. DISCUSSION

It appears from our data that the speciation of *T. urticae* and *T. turkestani* is recent enough that the segregation of mtDNA lineages has not occurred, so that these sister species have not yet reached monophyly for mtDNA (figure 1). By contrast, these species have fixed alternative nuclear rDNA variants which, despite the intraspecific polymorphism detected, are monophyletic in each species, with three diagnostic nucleotide substitutions separating the two species-specific lineages (figure 2).

species	sample	sequence type	variable sites																						
			119	122	146	162	170	179	183	188	199	238	264	275	288	339	358	361	369	401	411	440	454	462
T. urticae	**all**	consensus	**T**	**T**	**A**	**C**	**A**	**A**	**A**	**T**	**G**	**T**	**T**	**T**	**G**	**G**	**C**	**A**	**A**	**T**	**T**	**A**	**T**	**T**
		clone 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
		clone 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
T. urticae	TuF	clone 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		clone 4	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
		clone 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		clone 1	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		clone 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
T. urticae	TuGR	clone 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		clone 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		clone 5	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
T. turkestani	**TtP**	consensus	.	.	.	.	.	.	.	.	.	.	.	.	**T**	**A**	**T**	.	.	.	**A**	.	**A**	.
T. turkestani	**TtNL**	consensus	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	**T**	.	.	.	**A**	.	**A**	.
T. turkestani	**TtUSA**	consensus	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	**T**	.	.	.	**A**	.	**A**	.
T. turkestani	**TtFM**	consensus	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	**T**	.	.	.	**A**	.	**A**	.
T. turkestani	**TtFMe**	consensus	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	**G**	.	.	.	**A**	.	**A**	.
T. turkestani	**TtFMc**	consensus	.	.	.	**A**	.	.	.	.	.	.	.	.	.	**A**	**T**	.	.	.	**A**	.	**A**	.
		clone 1	.	.	.	.	T	.	.	.	.	.	C	.	.	A	T	.	.	.	A	.	A	.
		clone 2	.	.	.	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	A	.	A	.
T. turkestani	TtFM	clone 3	.	.	.	.	.	.	.	C	.	C	.	.	.	A	T	.	.	.	A	.	A	.
		clone 4	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	.	.	.	A	.	A	.
		clone 5	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	.	.	.	A	G	A	.
		clone 1	.	.	.	.	.	.	.	.	A	.	.	.	.	A	G	.	.	.	A	.	A	C
		clone 2	.	.	G	.	.	.	.	.	.	.	.	C	.	A	G	.	.	.	A	.	A	.
T. turkestani	TtFMe	clone 3	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	G	.	A	.	A	.
		clone 4	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	A	.	A	.
		clone 5	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	A	.	A	.

Figure 2. Sequence variation of rDNA ITS2 in *Tetranychus urticae* and *T. turkestani*. Only variable sites are shown and numbered as in EMBL submitted sequence (accession number X99881). Dots indicate identity with the first sequence. Two types of sequence are presented: those determined directly from the PCR products (in bold) and those determined after cloning these products (in regular type), in which case clones from the same PCR product are numbered. Abbreviations of collection sites are as in table 1.

The polyphyly for mtDNA could result from secondary hybridization as has been invoked in other studied cases (see, for instance, Wilson *et al.* 1985; Arnold 1992; Vogler & DeSalle 1994; Tang *et al.* 1996). However, repeated attempts to cross *T. turkestani* and *T. urticae* in our laboratory have always failed, although it could be shown that mating occurred (A. Migeon and M. Navajas, unpublished data). Thus hybridization appears an unlikely explanation for the polyphyly of these two species.

Mitochondrial DNA nucleotide diversity averages 4.3% in *T. urticae* and 3.3% in *T. turkestani*. It is only 0.2% and 0.5%, respectively, for rDNA, including the variation among clones. High mitochondrial haplotypic diversity contrasting with monomorphism of the ITS2 was also reported in, for instance, the mosquito *Anopheles funestus* (Mukabayire *et al.* 1999), but such observations could merely result from a higher mutation rate of mtDNA. However, in a previous study (Navajas *et al.* 1998), we had found a substitution rate of mtDNA that was 2.5 times lower than rDNA between species of a group of five tetranychid species closely related to those studied here. Therefore, it appears that the reduced rDNA polymorphism that we observe here witnesses a smaller effective population size as compared with mtDNA, rather than a lower mutation rate. Assuming the neutrality of mutations, theory predicts that nucleotide diversity is proportional to effective population size and mutation rate, and given the levels of diversity measured for these two markers and the estimated ratio of their mutation rates, we can estimate the ratio of effective population sizes: $N_{mit}/N_{rDNA} = (4.3/0.2) \times 2.5 \approx 54$ for *T. urticae* and $(3.3/0.5) \times 2.5 \approx 16$ for *T. turkestani*. This fits well with our observation of an apparently more rapid coalescence to species monophyly for rDNA, but it may seem surprising that a nuclear sequence has a smaller effective population size than the maternally transmitted clonal

mtDNA (and even more surprising when it is a repeated sequence).

Setting aside selection, several factors could be responsible for this difference of effective population size. One is the haplodiploidy of these arrhenotokous species: haploid males transmit only one copy of the nuclear genome. If N_m and N_f are the male and female effective population sizes (respectively, in number of individuals), then mitochondrial effective size equals N_f, while nuclear single-copy effective size equals (in number of alleles; Wright 1969):

$$N_{nuc} = \frac{9N_mN_f}{2N_m + N_f}.$$

We then get the following relationship between the mitochondrial and nuclear single-copy effective population sizes:

$$\frac{N_{mit}}{N_{nuc}} = \frac{2}{9} + \frac{1}{9}\frac{N_f}{N_m},$$

from which it appears that mitochondrial effective population size is expected to become greater than nuclear population size when N_f/N_m becomes greater than seven. Although sex ratios are essentially variable in arrhenotokous species, a general excess of females is observed in nature in these mite species. Females reared in the laboratory typically hatch one male egg for every six female eggs. As females pupate, numerous males gather around them and compete for copulation, resulting in a higher variance of male than of female reproductive success, and also contributing to the relative reduction of male effective population size. Of course, the fact that rDNA is present in multiple copies in the genome should tend to inflate its effective size as compared with a single-copy nuclear gene such as is considered in the calculations above, but it is not known to what extent this is counterbalanced by the effects of concerted evolution, or even eventually reversed by the operation of biased gene conversion. It is thus difficult at this stage to quantify the relative effects of biased sex ratio and concerted evolution in determining the relative effective population sizes of mtDNA and rDNA, but it is likely that these two aspects account for much of the apparent discrepancy between the two markers. The comparison with single-copy nuclear genes could shed further light on this question.

Acknowledgements

The authors thank F. Rousset for helpful comments on the theoretical approach and J. Lagnel for technical assistance in sequencing.

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