Genetic Variability in *Tetranychus urticae* (Acari: Tetranychidae) from Greece: Insecticide Resistance and Isozymes

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ABSTRACT Resistance to methyl-parathion, methidathion, and methomyl was studied by bioassays in *Tetranychus urticae* Koch from Greece. At LC_{50}, the resistance ratio was highly variable with methidathion (5- to 63-fold) and with methomyl (6- to 34-fold). The mortality curves with methyl-parathion showed a clear plateau at ≈20% mortality, and the resistance ratio was ≈50 fold at LC_{50} in all samples. By using isoelectric focusing on cellulose acetate membranes, electrophoretic studies of 5 loci encoding esterases (*Est-1* and *Est-2*), glucose-phosphate isomerase (*Gpi*), malic enzyme (*Me*), and phosphoglucomutase (*Pgm*) disclosed large genetic differences among the samples.

KEY WORDS *Tetranychus urticae*, insecticide resistance, genetic differentiation, isoelectric focusing.

THE TWOSPOTTED SPIDER mite, *Tetranychus urticae* Koch, a world-wide pest of many plant species, has been heavily exposed to insecticides and acaricides and has developed resistance to a large variety of compounds (Georghiou and Lagunes-Tejeda 1991). In confined environments (e.g., greenhouses), these resistances may develop extremely quickly because of the numerous generations that occur every year and the high frequency of spray applications (Cranham and Helle 1985). The evolution of resistance, in part, depends on gene flow among populations that colonize different habitats in a given area. However, no attempt has been made to study genetic exchanges between *T. urticae* populations because of the difficulty involved in investigating the genetic structure of populations of such a minute species with current electrophoretic or other techniques that use single individuals (Ward et al. 1982, Osakabe 1991, Gotoh and Takayama 1992, Osakabe and Sakagami 1993).

Our study had the following 2 objectives: (1) to determine how resistance to a few commonly used pesticides varies among populations of *T. urticae* that colonize different habitats, and (2) to investigate gene flow in this species from studies of the electrophoretic polymorphism by using the method of Kazmer (1991). This method consists of isoelectrofocusing with several stacked cellulose acetate membranes and allows investigation of up to 4 or 5 enzyme systems for each mite.

Materials and Methods

Mites. Five strains of *T. urticae* (LABOAT, ROSAT, ROSEG, AGR, and SAMB) were used. LABOAT was a mixed laboratory strain established with many collections from different regions of Greece, and was maintained on bean plants in a glasshouse at the Benaki Phytopathological Institute in Athens. Since it was established, this strain has never been exposed to pesticides. ROSAT was derived from mites collected near Athens in December 1993 on rose plants from 5 greenhouses implanted in an area of market-garden cultures; before collection, these populations were exposed for ≈10 yr to a large number of insecticides (e.g., monocrotophos, methomyl, methamidophos, heptenophos, parathion) and acaricides (e.g., fenbutatin oxide, dicofol, tetradifon, clofentezin, bifenthrine, dienochlore, abamectin). ROSEG and AGR were derived from mites collected near Egion (170 km west of Athens) in December 1993 in a rose house and on 5 trees of a nearby citrus grove, respectively. Citrus groves constitute the main crop of this region, where they are constantly treated with a mixture of dicofol and tetradifon for mites, and with methidathion for insects (e.g., white flies, aphids, scale insects). Treatments in the rose house included insecticides (monocrotophos, methidophos, methomyl, dichlorvos) and acaricides (propargite, omethoate, clofentezine, hexythiazox, dicofol, and tetradifon). SAMB is a susceptible reference strain, initially collected on *Sambucus*

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nigra (L.) and obtained in 1992 from the Laboratory of Applied Entomology at Amsterdam.

All field mites were identified by examining the male aedeagus. The strains were initiated with ≥30 individuals of all stages. Mites were maintained on detached bean leaves that were deposited on a piece of moist cotton placed on trays filled with water to prevent contamination. Mite rearing was at ≈25°C, 70 ± 5% RH, and a photoperiod of 16: 8 (L:D) h. Under these conditions, a new generation was obtained every 2 wk, and mites were transferred to new bean leaves every week. Before insecticide bioassays and electrophoreses, mites had been maintained in the laboratory for 6 and 12 generations, respectively.

Insecticide Bioassays. Bioassays were done by using the slide-dip method described by Busvine (1980). We applied aqueous suspensions of commercial formulations of methyl-parathion (Methyl Bladan 40 EC [emulsifiable concentrate], 400 g/liter; Bayer, Puteaux, France), methidathion (Ultracide 20 EC, 193 g/liter; Ciba, Rueil-Malmaison, France) and methomyl (Lannate 20 SL [soluble concentrate] 200 g/liter; DuPont, Paris, France). Five replicates with at least 5 concentrations (20 mites per concentration) that gave between 0 and 100% mortality after 24 h were tested with each insecticide. With each strain a control was conducted with water. Mortality data were analyzed by probit regression (Raymond et al. [1993], based on Finney [1971]).

Electrophoreses. Genetic analysis of mites was done by isoelectric focusing (IEF) on cellulose acetate membranes as described by Kazmer (1991). Four membranes (Separax-EF; Fuji Photo Film, Tokyo, Japan) were impregnated with 4–5.6 pH gradient carrier ampholytes (Pharmacia LKB; Uppsala, Sweden), and stacked together. Sixty single adult females were squashed individually on the top membrane on a line at about 2 cm from the anode. The series of constant voltages were 500 V for 15 min, 1,000 V for 15 min, and 1,500 V for 60 min. After migration, each membrane was used to reveal a different enzyme system based on the protocols of Pasteur et al. (1988). Thirty-five enzyme systems were tested on a few mites, but activity was observed only for carbonate dehydratase, creatine kinase, esterases, fructokinase, glycerophosphate dehydrogenase, glucosephosphate isomerase, hexokinase, isocitrate dehydrogenase, malic enzyme, malate dehydrogenase, phosphoglucomutase, phosphogluconate dehydrogenase, and pyruvate kinase. Among these enzymes, 4 showed a strong activity and variations in electrophoretic mobilities. The best results were obtained using 4 stacked membranes stained, from the top to bottom, for malic enzyme (ME), phosphoglucomutase (PGM), esterases (EST), and glucosephosphate isomerase (GPI). The AGR strain was chosen as reference for naming alleles. The most common allele in this strain was arbitrarily named "100," and other alleles were named in relation to their isoelectric point (*pI*), relative to the "100" allele (the lowest numbers representing proteins with the lowest *pI*).

Deviations from the Hardy–Weinberg equilibrium were determined with the Fisher exact test as described by Louis and Dempster (1987) when ≤4 alleles were present at a locus. For ≥5 alleles, an unbiased estimation of the exact probability described by Raymond and Rousset (1995a) was computed using the Markov chain method described by Guo and Thompson (1992). Genotypic linkage disequilibrium for all pairs of loci was tested in each strain as described by Raymond and Rousset (1995b). Genetic differentiation between strains was tested statistically by comparing the allelic composition of all pairs of samples for all loci by a Fisher exact test (Raymond and Rousset 1995a). The GENEPOP software version 1.2 (Raymond and Rousset 1995b) was used for all tests. The genetic divergence between samples was estimated by the Nei (1978) unbiased genetic distance, which was used in a cluster analysis using the unweighted pair-group method with arithmetic mean algorithm. These analyses were computed with BIOSYS version 1.7 (Swofford and Selander 1981).

Results

Insecticide Resistance. Linearity of concentration-mortality curves (Table 1) was accepted ($P > 0.05$) only for the susceptible reference strain SAMB tested with methyl-parathion (slope = 2.5 ± 0.3) and for the AGR strain tested with methomyl (slope = 3.5 ± 0.3). LC_{50}s were estimated visually for strains LABOAT, AGR, and ROSAT tested with methyl-parathion, for strain AGR tested with methidathion, and for strain ROSAT tested with methomyl.

In the presence of methyl-parathion, the Greek strains displayed a striking similarity (Fig. 1); all presented a clear plateau at ≈20% mortality, and similar mortalities at concentration equal to 10,000 mg/liter or higher ($\chi^2 < 3.0$, df = 2, $P > 0.05$). Resistance ratios were between 31 and 56 at LC_{50}, and between 34 and 54 at LC_{90}. In the presence of methidathion and methomyl, all Greek strains also were more resistant than the susceptible SAMB strain. In contrast to our observations with methyl-parathion, the strains were largely different from each other. Thus, resistance ratios (RR) at LC_{50} were higher in ROSAT and ROSEG strains collected on rose trees (RR = 63 and 38 with methidathion, and RR = 15 and 26 with methomyl, respectively) than in LABOAT (RR = 8 with methidathion and methomyl) or AGR strains (RR = 5 and 6 with methidathion and methomyl, respectively). The LC_{50}s for methidathion and methomyl were significantly correlated ($r = 0.999$, $P > 0.95$): classification of the strains (excluding ROSAT tested only on few individuals) in order of increasing LC_{50} was thus the same with both in-

Table 1. Characteristics of resistance to insecticides in *T. urticae* strains from Greece

	n°	Slope ± SE	LC_{50} (mg/liter)	LC_{50} 95% CI	χ^2	RR	RR 95% CI
Methyl-parathion							
SAMB	500	2.54 ± 0.27	180	151– 210	2.35	—	—
LABOAT	1,200	—	8,500[a]	—	—	47	—
AGR	1,100	—	9,500[a]	—	—	53	—
ROSEG	1,000	1.59 ± 0.32	5,550	3,210– 9,580	34.17	31	20–46
ROSAT	80	—	10,000[ab]	—	—	56	—
Methidathion							
SAMB	600	2.91 ± 0.71	32	10– 95	9.95	—	—
LABOAT	900	1.97 ± 0.36	243	153– 385	40.27	8	3–17
AGR	600	—	160[a]	—	—	5	—
ROSEG	700	1.94 ± 0.33	1,207	693– 2,079	10.25	38	15–91
ROSAT	140	—	2,000[ab]	—	—	63	—
Methomyl							
SAMB	700	1.61 ± 0.25	1,134	656– 1,937	14.23	—	—
LABOAT	600	2.35 ± 0.43	9,111	6,104–13,695	11.04	8	5–12
AGR	700	3.47 ± 0.34	6,895	6,268– 7,592	2,46	6	4– 9
ROSEG	700	2.4° ± 0.37	38,000	19,803 ± 43,564	15.08	26	16–41
ROSAT	140	—	17,000[ab]	—	—	15	—

n, Number of mites tested.
[a] Estimated visually.
[b] One replicate only.

secticides; i.e., SAMB, AGR, LABOAT, and ROSEG.

Genetic Differentiation. Among the 4 enzyme systems studied, electrophoregrams of each mite displayed ME multiple bands. Although at least 2 loci were clearly involved and both polymorphic, we could not interpret the electrophoretic patterns genetically; therefore, this locus was not considered further. Two loci encoding esterases were identified by the ability of the proteins to hydrolyze preferentially α- (*Est-1* locus) or β- (*Est-2* locus) naphthyl acetate. *Est-1* had 2 phenotypes: P, which corresponded with the presence of a band with the same electrophoretic mobility in all strains, and A, which corresponded with its absence. *Est-2* showed many different genotypes with 1 (homozygotes) or 2 (heterozygotes) bands, and all bands had similar staining intensities. *Pgm* electrophoregrams showed 1 band in homozygotes and 2 in heterozygotes. Homozygotes at the *Gpi* locus also had a single band, but heterozygotes displayed an elongated band (the 3 expected dimeric proteins having very similar *pI* were not clearly separated). In total, 2, 8, 2, and 4 alleles were identified at the *Est-1, Est-2, Gpi,* and *Pgm* loci, respectively (Table 2).

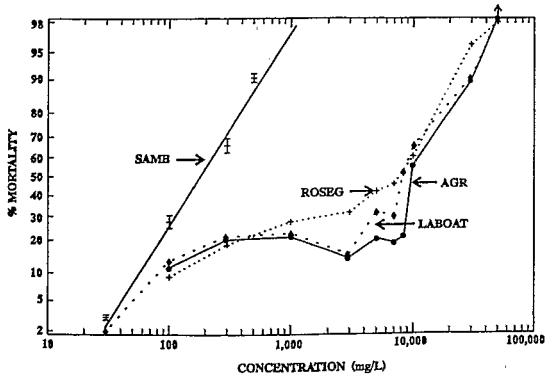

Fig. 1. Concentration–mortality curves of *T. urticae* strains in presence of methyl-parathion.

Significant ($P < 0.05$) deviations from Hardy–Weinberg expectations were observed at the *Pgm* locus in the AGR and SAMB strains; they were always associated with heterozygote deficits. We observed a significant ($P < 0.05$) nonrandom association between $Est\text{-}1^P$ phenotype and Pgm^{120} allele in the AGR strain. Each strain was significantly ($P < 0.05$) different from the 3 others (ROSAT was excluded from this comparison because only 2 out of 4 loci were tested). As shown by the cluster analysis (Fig. 2), the 3 Greek strains (AGR, LABOAT, and ROSEG) are well differentiated from the SAMB strain originating from Holland. Strains AGR and ROSEG which derived from mites collected in the same village, but on different plants (citrus and rose trees, respectively), are more different than are ROSEG and LABOAT, which derived from mites collected in different localities.

Discussion

Our study revealed that the Greek *T. urticae* strains studied here are more resistant to the 3 insecticides tested compared with the SAMB susceptible reference strain. The shape of the parathion mortality curves (with a clear plateau at ≈20% mortality) (Fig. 1) suggests that the same major gene confers resistance to this insecticide in the 3 strains in which it has approximately the same frequency (≈80%). This conclusion agrees

Table 2. Allelic frequencies observed in mite samples

	AGR	LABOAT	ROSAT	ROSEG	SAMB
Est-1[a]					
(*n*)	162	45	8	85	174
A	0.975	0.933	0.125	0.988	0.276
P	0.025	0.067	0.875	0.012	0.724
Est-2					
(*n*)	162	41	8	83	173
140	0	0	0	0	0
115	0	0.098	0	0.012	0.038
100	1	0.207	0.813	0.133	0.665
95	0	0.024	0	0.012	0
90	0	0.110	0.188	0.319	0.298
80	0	0.085	0	0.301	0
70	0	0.268	0	0.133	0
60	0	0.207	0	0.090	0
HW[b]	—	NS	NS	NS	NS
Gpi					
(*n*)	125	45	—	77	118
100	1	0.978	—	1	0.847
110	0	0.022	—	0	0.153
HW	—	NS	—	—	NS
Pgm					
(*n*)	106	39	—	52	106
120	0.033	0	—	0	0
100	0.816	0.987	—	0.856	0.854
90	0	0	—	0	0.142
80	0.151	0.013	—	0.144	0.005
HW	<0.05	—	—	NS	<0.05

n, Number of tested mites. NS, nonsignificant deviation.
[a] Two phenotypes observed at *Est-1* locus: (A) corresponds to absence of isozyme, (P) to its presence. Phenotype frequencies are given here.
[b] Deviations from Hardy–Weinberg equilibrium.

with preliminary data obtained after 5 generations of selection with methyl-parathion on the LABOAT strain, in which decreased mortality at concentrations within the plateau has been observed (unpublished data).

Although resistance ratios with methidathion and methomyl varied widely between strains (5- to 63- and 6- to 26-fold, respectively), the correlation of LC_{50}s observed with these 2 insecticides suggests that the same resistance mechanism may be involved. Because mortality curves are very different from that of methyl-parathion, the major mechanisms of resistance to these 2 groups of insecticides probably are different. Decreased acetylcholinesterase sensitivity has been reported in *T. urticae* strains that are resistant to both organophosphates and carbamates (Smissaert 1964, Smissaert et al. 1970, Kuwahara 1984). The correlation of LC_{50}s for methidathion (an organophosphate) and methomyl (a carbamate) may reflect the involvement of an insensitive acetylcholinesterase. Increased detoxification by esterases that also hydrolyze large quantities of naphthyl acetate substrate (Kuwahara et al. 1981, Oppenoorth 1985, Osakabe 1991) does not seem to be involved. In our study, differences in esterase staining intensity was not observed at the *Est-2* locus, and the presence of an active isozyme at the *Est-1* locus seemed independent of resistance. The *Est-1*^{P} phenotype was more common in the susceptible SAMB strain (frequency 0.72) than in AGR, LABOAT, and ROSEG strains (frequency <0.07).

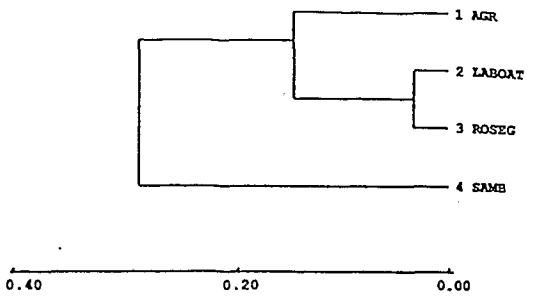

Fig. 2. Genetic relationships of tetranychid strains estimated by Nei genetic distances.

The isoelectrofocusing method on acetate cellulose membranes described by Kazmer (1991) appears to be a suitable technique for studies of population genetics in mites because it permits us to analyze electrophoretic variations of 4 enzymes on each mite. Although our investigation was restricted to a few loci, all were polymorphic, and a strong differentiation was revealed between strains. One of the most striking features of Greek mites was the large difference between the AGR and ROSEG strains derived from mites collected in the same locality. This polymorphism is very low in AGR, especially at the *Est-2* locus where an allele is fixed, and extremely high in ROSEG, with 7 alleles at the *Est-2* locus. Such a difference is not likely caused by genetic drift during laboratory maintenance (12 generations) because SAMB still segregates for 5 alleles after >50 generations of laboratory rearing. Thus, the differences in polymorphism observed between AGR and ROSEG are most likely to exist in field populations. These differences indicate that gene flow between the rose house (where ROSEG was collected) and the citrus grove (where AGR was collected) is likely to be very low despite the proximity of these 2 sites. At present, we cannot determine whether this restricted gene flow is associated with the colonization of different host plant species or with the habitat (open field versus greenhouse).

In conclusion, our study showed that resistance to organophosphates in *T. urticae* populations from Greece is probably due to 2 different mechanisms, one conferring resistance to the organophosphate methyl-parathion and the other to the organophosphate methidathion and the carbamate methomyl. The first mechanism seems to have a uniform frequency in Greek populations, whereas the second mechanism shows large variations. In addition, populations of *T. urticae* are highly polymorphic, and strong differences between local populations may exist. Further studies on the precise nature of resistance mechanisms and population structure in relation to various environmental factors are needed to understand the dynamics of

resistance in this species, and to estimate the effect of gene flow on the dispersal of resistant genotypes between populations.

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