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Asymmetric reproductive interference between two closely related spider mites: *Tetranychus urticae* and *T. turkestani* (Acari: Tetranychidae)

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Abstract *Tetranychus turkestani* Ugarov and Nikolskii and *Tetranychus urticae* Koch RF (red form) (Acari: Tetranychidae) are closely related species. Previously, the two species were found in separate agricultural habitats in Israel. Here, additional collections were undertaken and mixed populations of the two species were found. Manipulation experiments were conducted in order to test whether sexual interactions occur when *T. turkestani* and *T. urticae* RF share the same host. Interspecific crosses showed that the two species are capable of producing viable F_1 females, but that these females are sterile as their F_2 eggs failed to hatch. This indicates a post-zygotic reproductive barrier, supporting the current placement of *T. turkestani* as a separate taxon. Mating behavior parameters revealed that males of both species courted virgin conspecific and heterospecific females at the same rate and readily tried to copulate with them. Female mate recognition seemed to be more reliable in *T. turkestani* than in *T. urticae* RF as the number of copulations was significantly higher and their duration significantly shorter in the *T. turkestani* interspecific (*T. turkestani* ♀ × *T. urticae* RF ♂) as compared to the intraspecific crosses, a phenomenon not observed in *T. urticae* RF. In mixed cultures, a significant reduction in female production was observed for *T. urticae* RF but not for *T. turkestani*, suggesting an asymmetric reproductive interference effect in favor of *T. turkestani*. The long term outcome of this effect is yet to be determined since additional reproductive factors such as oviposition rate and progeny survival to adulthood may reduce the probability of demographic displacement of one species by the other in overlapping niches.

Keywords Host preference · Interspecific interaction · Mating behavior · Reproductive interference · Agricultural pests

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Introduction

Tetranychus urticae Koch—red form (herein *T. urticae* RF) and *Tetranychus turkestani* Ugarov and Nikolskii are two common spider mite pests in Israel. Both are polyphagous and globally distributed (Bolland et al. 1998). *T. urticae* RF is probably indigenous to the Israeli region (Klein 1936), where *T. turkestani* was only recently found (Ben-David et al. 2007). This is probably due to past misidentifications and confusion with the green form (GF) of *T. urticae*, first reported as a pest of deciduous fruit trees in Israel in 1965 (Plaut and Feldman 1966). *T. urticae* RF and *T. turkestani* are closely related and hard to discriminate, morphologically as well as molecularly (Navajas and Boursot 2003; Ros and Breeuwer 2007). Microscopic examinations of the shape of the male genitalia are needed for positive identification, because no other morphological trait separates the two species. In addition, the genetic distance between *T. urticae* RF and *T. turkestani*, based on the sequences of the second internal transcribed spacer of nuclear ribosomal DNA (rDNA—ITS2) is less than 1.5% (Navajas and Boursot 2003; Ben-David et al. 2007) and the species cannot be discriminated by their mitochondrial cytochrome oxidase I (COI) sequences (Navajas and Boursot 2003). This observation was reinforced by Ros and Breeuwer (2007) using a large data set of 165 COI sequences. Their phylogenetic analyses revealed that *T. urticae* and *T. turkestani* COI sequences fall into the same taxonomic clade (clade 2), and do not form separate monophyletic groups.

Most spider mites are arrhenotokous, where males are haploid and develop from unfertilized eggs, whereas females are diploid and develop from fertilized eggs (Helle and Sabelis 1985). Previous attempts to cross *T. turkestani* and *T. urticae* RF did not produce female offspring, although mating occurred (Migeon and Navajas unpublished data; reported without accompanying data in Navajas and Boursot 2003). These findings are not exceptional, because closely related species often have incompletely isolated recognition and mating systems (Reitz and Trumble 2002). Interspecific matings were previously reported in spider mites of the genus *Tetranychus* (Helle and Sabelis 1985). These attempts are usually totally ineffective and the mated females do not produce female offspring (Helle and van de Bund 1962). However, a few interspecific matings produce infertile hybrid (F_1) female offspring or eggs that do not hatch (Boudreaux 1963).

Interspecific matings between closely related species may result in reproductive interference, a term defining negative interactions between species that are associated with their mating systems (Gröning et al. 2007). Reproductive interference can adversely affect the population dynamics, abundance, habitat choice and spatial distribution of the species involved (Gröning et al. 2007; Hochkirch et al. 2007; Konuma and Chiba 2007; Liu et al. 2007; Reyer 2008; Thum 2007). In some cases, the intensity of reproductive interference reduces population size in an asymmetric manner. For example, Takafuji et al. (1997) showed that interspecific matings can occur between the two closely related spider mite species, *Panonychus citri* (McGregor) and *Panonychus mori* Yokoyama. In laboratory experiments, *P. mori* males showed a strong preference for guarding and copulating with conspecific quiescent deutonymph females, whereas *P. citri* males did not show any guarding and mating preference. In orchards where the two species co-exist, the proportion of females that did not produce female offspring was higher in *P. mori* than in *P. citri*, indicating a stronger deleterious effect of reproductive interference on *P. mori* (Takafuji et al. 1997).

Previously (Ben-David et al. 2007) we found that *T. turkestani* and *T. urticae* RF occur in separate agricultural habitats in Israel. The latter was collected from low growing plants, such as weeds and vegetables in greenhouses and in open fields, whereas *T. turkestani* was

obtained from deciduous fruit trees. Here, additional collections were undertaken and mixed populations of the two species were found. We then conducted manipulated experiments in order to test whether sexual interactions occur between *T. turkestani* and *T. urticae* RF when they co-exist on the same host and examined the reproductive consequences of these interactions.

Material and methods

Collection of *Tetranychus turkestani* and *T. urticae* RF from Israeli agricultural habitats

Spider mites were collected from 50 agricultural habitats, mainly orchards and open fields, in different regions of Israel during the years 2005–2007. Details of host plant, habitat and region are given in Table 1. All mites, along with their host foliage, were placed in cooled polyethylene bags (Ca. 1 l of foliage) and brought to the laboratory. Five to ten males and females were cleared with lactic acid, mounted in Hoyer's solution (Gutierrez 1985) and identified by using the key in Smith-Meyer (1987). All voucher specimens are currently maintained in the collection of the Plant Protection and Inspection Services, Ministry of Agriculture and Rural Development, Israel. When mixed populations of red and green colored females were obtained, five green females were individually analyzed molecularly (see below), to rule out the possibility that *T. urticae* GF individuals were collected.

Table 1 *Tetranychus urticae* RF and *T. turkestani* collection data (2005–2007), with reference to host plants, agro-ecosystems and geographic regions

Mite species	No. of samples	Host plant	Agro-ecosystem	Region in Israel
Tetranychus urticae RF	1	*Prunus persica*	Orchard screen house	Center
	6	*Citrullus lanatus*	Open-field	North, South
	3	*Solanum lycopersicon*	Greenhouse	Center, South
	2	*Convulvulus* sp.	Open-field	Center
	1	*Ricinus cummunis*	Open-field	North
	4	*Solanum nigrum*	Weed-rural	North, Center, South
	3	*Solanum melongena*	Greenhouse, Open-field	North, Center
	2	*Gossypium hirsutum*	Open-field	North, Center
	1	*Prunus persica*	Orchard	Center
	1	*Pyrus communis*	Sprayed orchard	North
	1	*Capsicum annum*	Greenhouse	Center, South
Tetranychus turkestani	1	*Gladiolus* sp.	Greenhouse	Center
	5	*Ficus carica*	Orchard	North, Center
	5	*Malus domestica*	Orchard	North
	1	*Musa acuminata*	Orchard screen house	Center
	1	*Tribulus terrestris*	In *P. persica* orchard	
	1	*Malva* sp.	Weed in *C. lanatus* field	North
	2		Weed in *M. domestica* orchard	
	3	*Prunus persica*	Orchard	North, Center
	1	*Prunus dulcis*	Orchard	South
T. turkestani and *T. urticae* RF	2	*Citrullus lanatus*	Open-field	North
	1	*Prunus persica*	Orchard screen house	Center

Laboratory mite strains

Populations of *T. turkestani* were collected from outdoors fig (*Ficus caricae*), *Malva* sp. and *Cucurbita peppo*, in the central coastal plain of Israel. *T. urticae* RF populations were collected from tomato (*Solanum lycopersicon*), watermelon (*Citrullus lanatus*) and strawberry (*Fragaria* × *ananassa*) plants grown in greenhouses in southern Israel. The laboratory strains of the two species were maintained for 2 years (~35 generations) on caged potted bean plants (*P. vulgaris* var. Palati) in separate greenhouses, at 25 ± 5°C and natural day length of 12–14 h light. Strain purity was assessed using two methods: (1) visual observations of three sampled heavily damaged bean leaves under a dissecting microscope for female body color (green = *T. turkestani*, red = *T. urticae* RF); (2) microscope preparation of the genitalia of 10 males randomly picked from the sampled leaves. Males with the knob of their aedeagi larger than 2 μm were considered *T. turkestani*. Prior to experiments with mites, moderately damaged bean leaves were picked from the caged laboratory strains and put in a marked 9 cm plastic Petri dish sealed with parafilm. The dishes were brought into the laboratory separately, e.g., one strain (species) at a time, to prevent transfer of mites between greenhouses (strains).

Host preference of individual *Tetranychus turkestani* and *T. urticae* RF females

Individual gravid females were collected from the laboratory strains, described above. Each female was transferred with a fine brush to a "bridge" made of a wooden toothpick lying across two leaf discs freshly picked from watermelon (*C. lanatus* var. Trophy) and one of three alternative hosts: cotton (*Gossypium hirsutum* var. Siv-on), apple (*Malus domestica* var. Anna) or peach (*Prunus persica*). All four hosts were free of pesticide treatments. Discs (3–4 cm^2) were placed with their lower side up on a piece of moist cleaning mat (2 cm^2 each) in a 9 cm diam plastic Petri dish. Dishes were stacked in groups of 10 and transferred to a chamber with constant 25°C and 14:10 L:D regimens for 24 h, after which host selection of individual females was recorded (once for each female). Most females were found feeding on the selected host and had founded a colony with few eggs. Females that did not select a host within 24 h were excluded from the statistical analyses. The proportion of females that chose watermelon in each experiment was tested against the extrinsic hypothesis of a 0.5:0.5 ratio (random choice) by log-likelihood ratio test (*G*-test). All statistical analyses conducted in this paper (see below) used JMP statistical software version 7.0.1 (SAS Institute, USA). Statistical significance was assumed at $P \leq 0.05$.

Mating behavior in interspecific and intraspecific crosses of *Tetranychus turkestani* and *T. urticae* RF

Female deutonymphs were isolated from each laboratory strain. After emergence each female was individually transferred to the lower side of a bean leaf disc (ca. 2 cm diam), placed on 1% agar. After an hour, each female was checked under a stereoscopic microscope and, if seen feeding normally, supplemented with a male from the same or the other species. The courtship and copulation behavior of each couple was recorded continuously for 20 min, using a stereoscopic microscope. Male courtship behavior was indicated by a physical contact between the male and the female. A pair was determined to be *in copula* when copulation position was observed continuously for 30 s or more. Observations were repeated 26–41 times for each of the four combinations: *T. turkestani* ♀ × *T. urticae* RF ♂, *T. urticae* RF

♀ × *T. turkestani* ♂, *T. urticae* RF ♀ × *T. urticae* RF ♂, *T. turkestani* ♀ × *T. turkestani* ♂. The proportion of pairs that showed male courtship (the number of pairs showing male courtship/the total number of pairs), and the proportion of pairs with copulation (the number of copulated pairs/the number of pairs with male courtship) were compared amongst treatments by log-likelihood ratio test (*G*-test). The time to first contact analysis used data of pairs showing male courtship. The analysis of time to first copulation, the number of copulations and the mean copulation time (accumulated time of pair copulation/number of copulations) used data of pairs whose copulation had lasted for 30 s or more. All parameters were compared amongst treatments by ANOVA. Means were separated by the Tukey-Kramer honestly significant difference (HSD) test.

Crossing experiments between *Tetranychus turkestani* and *T. urticae* RF

Single virgin females (see above) were transferred to bean leaves in 9 cm diam Petri dishes. Each female was supplemented with a male from the same or the other species. Females were allowed to lay eggs for 5–10 days at constant 25°C and 14:10 h L:D regime. The number of oviposited F_1 eggs, developing nymphs and emerged adults was recorded every 72 h for 21 days. Nymph progeny were transferred to fresh leaves once a week, until their sex could be determined. F_1 females of interspecific crosses were allowed to lay eggs for 7 days. F_1 females that did not lay eggs during this period, or females that laid eggs that did not hatch in 20 days, were considered sterile. The experiment was repeated 11 times for *T. turkestani* ♀ × *T. urticae* RF ♂ and 16 times for *T. urticae* RF ♀ × *T. turkestani* ♂. Control intraspecific crosses were repeated 9 times for *T. turkestani* and 10 times for *T. urticae* RF. The proportion of hatchability (number of live nymphs/number of eggs oviposited), the proportion of progeny survival to adulthood (number of adults/number of live nymphs) and the progeny sex ratio (number of F_1 females/number of F_1 females + number of F_1 males) were calculated. In order to check for possible post-mating, pre-zygotic reproductive barriers, comparisons were made within each species, between intraspecific families, interspecific families that produced only males and interspecific families that produced males and females. The one family from the *T. turkestani* ♀ × *T. urticae* RF ♂ cross that produced males and one female offspring was excluded from the analysis due to lack of statistical power. The data did not meet the assumptions of ANOVA (homogeneity of variances among treatments) and was analyzed by the Wilcoxon two sample or Kruskal–Wallis nonparametric tests.

ITS2 analyses

In order to verify that F_1 females from interspecific crosses are hybrids of *T. turkestani* × *T. urticae* RF, their ITS2 sequences were analyzed. Genomic DNA extraction, ITS2 amplification, cloning, sequencing and sequence analyses were as previously described (Ben-David et al. 2007). For ITS2 PCR-RFLP identification, amplified ITS2 fragments were extracted from 1% agarose gel using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA) according to the manufacturer's instructions. RFLP was conducted with the restriction enzyme *Hpa*I (=*Ksp*AI, Fermentas) in volume of 30 µl containing: 0.5 µl enzyme (5 units), 3 µl buffer B (10×), 11.5 µl H_2O and 15 µl cleaned PCR product. Restriction reactions were kept overnight at 37°C. Products were separated on 1.5% agarose gel, and then stained with ethidum bromide. *Hpa*I digests the ITS2 fragment of *T. urticae* RF into two fragments of 302 and 172 bp, whereas the ITS2 fragment of *T. turkestani* remains uncut.

Screening for bacteria associated with *Tetranychus turkestani* and *T. urticae* RF populations

To search our mite strains for the presence of bacteria associated with reproductive manipulation in spider mites (Breeuwer 1997; Weeks et al. 2003) and other arthropods (Perlman et al. 2006), four females of each laboratory strain were ground individually in lysis buffer, as in Ben-David et al. (2007). The 16S rRNA gene fragment (~550 bp) was amplified by PCR from the lysate using primers which target most known bacteria (for more details see Muyzer et al. 1996 and Gottlieb et al. 2006). To specifically detect the presence of *Rickettsia*, *Wolbachia* and *Cardinium*, the sampled mite lysates were subjected to PCR reactions that used primers described in Gottlieb et al. (2006), Enigl et al. (2005) and Weeks et al. (2003), respectively. Infected *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae) served as positive controls for *Rickettsia* and *Cardinium.* Infected *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) served as a positive control for *Wolbachia*.

Reproductive interference between *Tetranychus turkestani* and *T. urticae* RF on bean leaf discs

Two virgin females (of either *T. turkestani* or *T. urticae* RF) or one virgin female of each species, all 1–2 days old, were transferred to the lower side of a 2 cm diam bean leaf disc, placed on 1% water agar. After normal feeding behavior had been observed for an hour, leaf discs harboring two females of the same species were supplied with two conspecific males (pure culture), and leaf discs harboring one female of each species were provisioned with one male of either species (mixed culture). All males were removed after 18 h and the females were transferred individually to a fresh bean leaf disc (ca. 4 cm diam) for oviposition. Leaf discs were checked every 48 h and nymph progeny were transferred to fresh discs once a week. Number of eggs laid, hatchability, progeny survival to adulthood and F_1 sex ratio were determined as described in the crossing experiments. The experiment was replicated 15–18 times for each combination. Comparisons were made between the two pure cultures and between pure and mixed cultures within each species (a total of three independent comparisons). Proportionate data were arcsine transformed prior to analysis. As the data did not meet the assumptions of ANOVA (homogeneity of variances among treatments), they were analyzed by a Non-parametric Wilcoxon two sample test. The proportion of fertilizations (number of females with female progeny/total number of females) was compared between pure and mixed cultures of each species by log-likelihood ratio test (*G*-test).

Reproductive interference between *Tetranychus turkestani* and *T. urticae* RF on whole bean plants

We simulated field interactions between *T. turkestani* and *T. urticae* RF by rearing mixed cultures of the two species, as well as control (pure) cultures of either *T. turkestani* or *T. urticae* RF, on 1 month-old potted bean plants (30 cm high, bearing three true leaves, two of which were fully expanded). Experiments were conducted for 2 weeks (one generation for the earliest-born individuals). Females of *T. turkestani* and *T. urticae* RF from the aforementioned laboratory strains were isolated as deutonymphs, 4 days before initiating the experiment. Males of each species were collected from the rearing cages 1 day prior to initiating the experiment and kept separated from the females, in order to prevent pre-experiment mating. Mixed cultures were initiated with 10 males and 10 virgin females of each species (total of 40 individuals). Pure cultures containing one species only were initiated with 20 vir-

gin females and 20 males (total of 40 individuals). The potted plants were wrapped with unwoven fleece to prevent mite movement between plants. Plants were kept in a greenhouse at 25°C (night)—30°C (day) and a 14:10 h L:D regime, with supplemented illumination during the day. Number of progeny females and their body color were recorded from all parts of each plant after 2 weeks. The numbers of female progeny per founder female were compared between pure and mixed cultures within each species by ANOVA.

Results

Distribution of *Tetranychus turkestani* and *T. urticae* RF in Israeli agricultural habitats

Tetranychus turkestani was collected mainly from deciduous trees, including apple, peach, almond, fig, and from weeds in orchards (Table 1). *T. urticae* RF was obtained mostly from herbaceous vegetables, flowers and weeds in open fields and in greenhouses. Mixed populations of *T. urticae* RF and *T. turkestani* were collected from fruit–bearing peach trees grown under netting in a screen house, and from watermelons in open fields in the northern part of Israel. In the laboratory choice assays, solitary gravid females ($n = 48$–74; Table 2) of both *T. turkestani* and *T. urticae* RF (cultured on bean plants for ~35 generations) preferred watermelon over apple, peach and cotton leaf discs. Tested females also showed a propensity for ovipositing on watermelon. These results suggest that *T. urticae* RF and *T. turkestani* overlap in habitat and host plant use in which reproductive interactions are likely to occur.

Mating behavior in interspecific and intraspecific crosses of *Tetranychus turkestani* and *T. urticae* RF

The proportion of males showing courtship behavior did not differ significantly between the four crossing combinations ($G = 3.085$, $df = 3$, $P = 0.38$), and was high: 0.85–0.96, indicating high male affinity to virgin conspecific as well as heterospecific females (Table 3, column 3). Of the pairs showing male courtship, 0.85–0.88 ($G = 0.198$, $df = 3$, $P = 0.98$) achieved copulation (Table 3, column 4). Nevertheless, the number of copulations per copulated pair was significantly higher and their duration significantly shorter in *T. turkestani* when interspecific encounters (*T. turkestani* ♀ × *T. urticae* RF ♂) took place, as compared to intraspecific (*T. turkestani* ♀ × *T. turkestani* ♂) encounters (Table 3, columns 9, 10). A similar phenomenon was not observed in *T. urticae* RF intra- and inter-specific crosses (*T. urticae* RF ♀ × *T. urticae* RF ♂ and *T. urticae* RF ♀ × *T. turkestani* ♂, respectively). The time

Table 2 Host preference of individual *Tetranychus turkestani* and *T. urticae* RF females on three combinations of host plants leaf discs: watermelon/cotton, watermelon/apple and watermelon/peach

Species analyzed	Hosts	No. of individuals on watermelon (eggs)	No. of individuals on other host (eggs)	Proportion females on watermelon (*P*)*
T. turkestani	Watermelon/Cotton	42 (282)	4 (2)	0.913 (<0.0001)
	Watermelon/Apple	28 (48)	11 (6)	0.718 (0.0056)
	Watermelon/Peach	31 (170)	11 (60)	0.738 (0.016)
T. urticae RF	Watermelon/Cotton	34 (131)	4 (2)	0.895 (<0.0001)
	Watermelon/Apple	43 (179)	5 (11)	0.896 (<0.0001)

The total numbers of eggs laid in each treatment is given in parentheses

* Probability of *G*-test for 0.5:0.5 ratio extrinsic hypotheses

Table 3 Mating behavior parameters of *Tetranychus urticae* RF and *T. turkestani* during 20 min observations of non-choice intra- and interspecific crosses

Cross	*N*	Proportion of males showing courtship behavior	Proportion of pairs with copulation	*N*	Time to first contact (min) ± SE	*N*	Time to first copulation (min) ± SE	Number of copulations per copulated pair ± SE	Mean duration of copulation (min) ± SE
T. turkestani ♀ × *T. turkestani* ♂	27	0.85a	0.86a	23	3.70 ± 0.68a	19	5.05 ± 0.87a	1.21 ± 0.16b	3.21 ± 0.23a
T. urticae RF ♀ × *T. urticae* RF ♂	32	0.91a	0.86a	29	4.36 ± 0.63a	25	5.88 ± 0.96a	1.24 ± 0.09b	2.39 ± 0.20b
T. turkestani ♀ × *T. urticae* RF ♂	26	0.96a	0.88a	27	3.52 ± 0.76a	24	5.87 ± 0.97a	2.08 ± 0.25a	1.83 ± 0.20b
T. urticae RF ♀ × *T. turkestani* ♂	41	0.95a	0.85a	39	4.05 ± 0.57a	33	4.94 ± 0.93a	1.70 ± 0.17ab	1.96 ± 0.17b

Proportions were compared amongst treatments by log-likelihood ratio test (*G*-test). Other parameters were compared by one-way ANOVA. Values within each column followed by different letters are significantly different ($P \leq 0.05$)

N number of replicates

to first contact and the time to first copulation did not differ significantly amongst the four crossing combinations (Table 3, columns 6, 8).

Crossing experiments between *Tetranychus turkestani* and *T. urticae* RF

A summary of the reciprocal interspecific crosses as well as control intraspecific crosses is given in Table 4. In interspecific crosses, where the female was *T. turkestani* (*T. turkestani* ♀ × *T. urticae* RF ♂), one out of 11 families produced one hybrid F_1 female offspring. In the reciprocal interspecific crosses (*T. urticae* RF ♀ × *T. turkestani* ♂), three out of 16 families produced a total of 18 hybrid F_1 females, of which three died after 2–3 days, and eight laid no eggs and were orange in color (similar to diapausing *T. urticae* GF). The remaining seven F_1 females came from one family and laid 4–25 eggs. Using PCR-RFLP, these F_1 females proved to be hybrids of the *T. urticae* RF × *T. turkestani* cross, carrying rDNA-ITS2 sequences of both species (Fig. 1). However, none of the 84 F_2 eggs laid by these F_1 females hatched, indicating a post-zygotic reproductive barrier between the two species. Within species, comparisons between intraspecific and interspecific crosses indicated no significant differences in hatchability rate, progeny survival rate and female progeny ratio ($0.20 \leq P \leq 0.83$). PCR screening for bacteria did not detect the presence of *Rickettsia*, *Wolbachia*, *Cardinium* or other bacteria in our *T. turkestani* and *T. urticae* RF laboratory strains, suggesting the presence of other post-zygotic mechanism of reproductive isolation between the two species.

Reproductive interference between *Tetranychus turkestani* and *T. urticae* RF on bean leaf discs

In order to study reproductive interference when males and females of both species are present in the same arena, three independent treatments were compared: pure cultures of each of the two species and a mixed culture of both. No differences were found in oviposition rates between the two pure cultures or between the pure and mixed cultures within each species ($0.32 \leq P \leq 0.8$; Fig. 2A), suggesting that it is an intrinsic parameter, unaffected by mating.

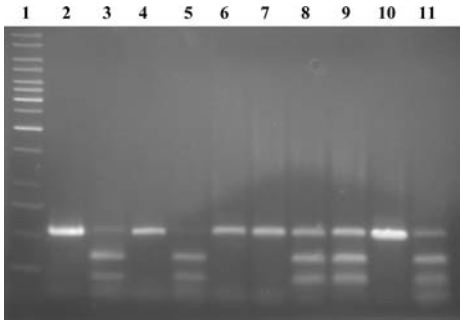


Fig. 1 Gel presentation of ITS2 PCR-RFLP analysis of F_1 female progeny produced by crossing *Tetranychus urticae* RF ♀ × *Tetranychus turkestani* ♂. The restriction enzyme *Hpa*I cuts the 474 bp ITS2 fragment of *T. urticae* RF into 302 and 172 bp fragments while leaving the *T. turkestani* ITS2 fragment uncut. *Lane 1* 1 kb ladder; *lanes 2–5* ITS2 PCR-RFLP analysis of two *T. urticae* RF females: *lanes 2* and *4* uncut fragment, *lanes 3* and *5* ITS2 fragments after digestion with *Hpa*I; *lanes 6–7* ITS2 PCR-RFLP analysis of one *T. turkestani* female: *lane 6* uncut fragment, *lane 7* ITS2 fragment after digestion with *Hpa*I; *lanes 8–11* ITS2 PCR-RFLP analysis of three hybrid F_1 females: *lane 10* uncut fragment, *lanes 8*, *9* and *11* ITS2 fragments after digestion with *Hpa*I

Fig. 2 Oviposition rate (**A**), progeny survival to adulthood (**B**) and proportion of female progeny (**C**) of *Tetranychus urticae* RF and *Tetranychus turkestani* individual females from each of three mating cultures: Pure—*T. turkestani* ♀♀ × *T. turkestani* ♂♂ ($n = 17$); *T. urticae* RF ♀♀ × *T. urticae* RF♂♂ ($n = 15$); Mixed—*T. turkestani* ♀♂ × *T. urticae* RF ♀♂ ($n = 16$ and 18 for *T. turkestani* ♀♀ and *T. urticae* RF ♀♀, respectively). *Error bars* represent standard error of the means. Comparisons were made using the non-parametric Wilcoxon two sample test ►

Table 4 Number of F_1 eggs obtained, their hatchability, progeny survival to adulthood and female progeny ratio, in inter- and intra-specific crossing experiments of *Tetranychus turkestani* and *T. urticae* RF

Cross	*N**	Sex of progeny	Number of eggs obtained	Hatchability ± SE	Survival to adulthood ± SE	♀♀ Progeny ratio ± SE
T. turkestani ♀ × *T. turkestani* ♂	9	♀♂	264	0.82 ± 0.05	0.69 ± 0.09	0.53 ± 0.08
T. urticae RF ♀ × *T. urticae* RF ♂	10	♀♂	422	0.71 ± 0.05	0.72 ± 0.07	0.64 ± 0.05
T. turkestani ♀ × *T. urticae* RF ♂	10	♂	245	0.66 ± 0.10	0.59 ± 0.06	NA
	1	♀♂	25	0.20	0.60	0.33
T. urticae RF ♀ × *T. turkestani* ♂	13	♂	335	0.74 ± 0.07	0.72 ± 0.07	NA
	3	♀♂	84	0.74 ± 0.14	0.76 ± 0.10	0.45 ± 0.16

Progeny ratios were calculated only for interspecific families that produced females

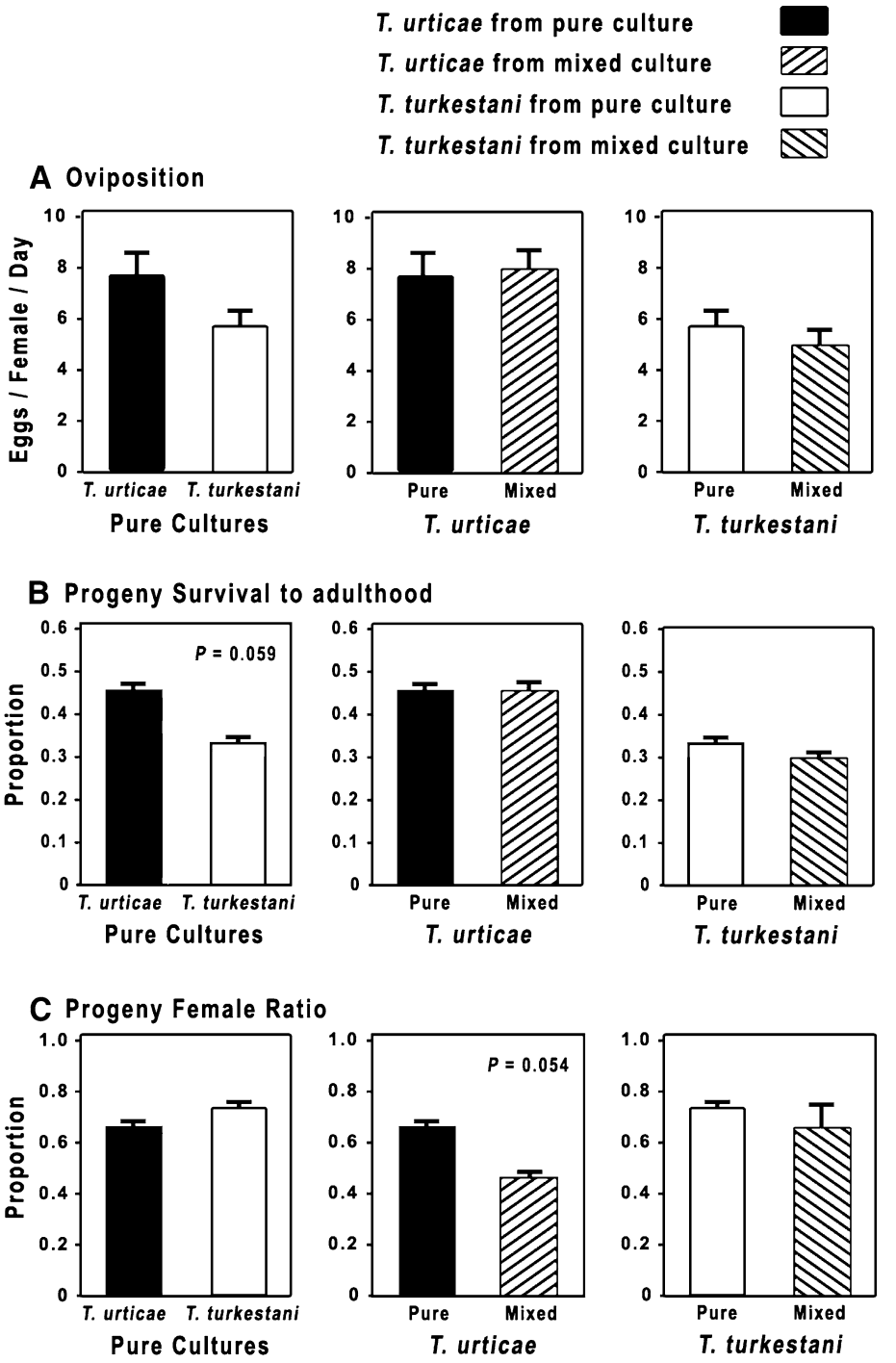
NA Non applicable

* Number of families

The proportion of progeny surviving to adulthood was nearly significantly higher in the *T. urticae* RF pure cultures (0.46 ± 0.05; mean ± SE) than in the *T. turkestani* pure cultures (0.33 ± 0.05) (Fig. 2B; χ^2 two-sample test = 3.6441, $df = 1$, $P = 0.059$), but there were no significant differences in this parameter between the pure and mixed cultures within each species (Fig. 2B; $P = 0.77$ and $P = 0.78$, for *T. urticae* RF and *T. turkestani*, respectively). The proportion of female progeny was not significantly different between *T. turkestani* and *T. urticae* RF pure cultures ($P = 0.24$). Nevertheless, a nearly significant reduction in this parameter was observed when pure (0.67 ± 0.06) and mixed (0.46 ± 0.09) cultures of *T. urtica*e RF were compared (Fig. 2C; χ^2 two-sample test = 3.7719, $df = 1$, $P = 0.054$). The same phenomenon was not seen in *T. turkestani* (Fig. 2C; $P = 0.81$). The proportion of fertilizations was significantly lower in the *T. urticae* RF mixed culture (11/18) when compared to its pure culture (14/15) ($G = 5.15$, $df = 1$, $P = 0.02$). Fertilization rates of *T. turkestani* were similar in mixed and pure cultures (13/16 and 15/17, respectively; $G = 0.314$, $df = 1$, $P = 0.58$). Overall, these experiments suggest that the presence of males and females of both species in the same arena affected the productivity of *T. urticae* RF much more than that of *T. turkestani*.

Reproductive interference of *Tetranychus turkestani* and *T. urticae* RF on potted beans

The production of female progeny by *T. urticae* RF females in pure cultures was significantly higher than that of *T. turkestani* (Fig. 3). Each founding female of *T. urticae* RF had a mean of 34.9 (±2.31) daughters (ca. 700 per plant), whereas each founding female of *T. turkestani* had only 8.1 (±1.42) daughters (ca. 160 per plant) ($t = -10.3221$, $P < 0.0001$). The reduction of almost 40% in the production of female progeny in *T. urticae* RF mixed cultures was highly significant (21.5 ± 1.42 daughters per founder female, $t = 4.3206$, $P = 0.0025$). There was no significant difference in the production of females in *T. turkestani* pure and mixed cultures ($t = -0.3155$, $P = 0.76$). Again, these data indicate


T. urticae from pure culture
T. urticae from mixed culture
T. turkestani from pure culture
T. turkestani from mixed culture
A Oviposition
Eggs / Female / Day
T. urticae
T. turkestani
Pure Cultures
Pure
Mixed
T. urticae
T. turkestani
B Progeny Survival to adulthood
Proportion
P = 0.059
C Progeny Female Ratio
P = 0.054

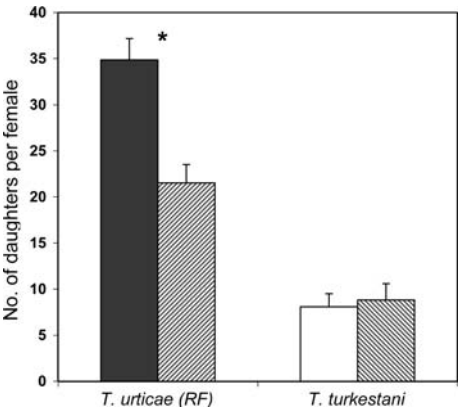


Fig. 3 The mean number (+SE) of female progeny produced by individual *Tetranychus urticae* RF and *Tetranychus turkestani* females during 2 weeks period (one generation) on bean plants. For each species, *solid bars* represent pure cultures of 20 couples (*black* for *T. urticae* RF and *white* for *T. turkestani*) whereas *striped bars* stands for mixed cultures of 10 couples of each species. *Asterisk* indicates significant differences ($P \leq 0.05$) between the two culture types within the same species

that the presence of *T. turkestani* affects the female production by *T. urticae* RF much stronger than the presence of *T. urticae* RF affects female production by *T. turkestani*.

Discussion

In the current study mixed populations of *T. urticae* RF and *T. turkestani* were found on watermelon grown in open fields and on peach grown under netting in a screen house. Our laboratory studies showed that individual gravid females of both species preferred watermelon over cotton, apple and peach leaves. These results suggested that *T. urticae* RF and *T. turkestani* have overlapping niches in which reproductive interactions between the two species are likely to occur naturally.

Previous attempts to cross *T. turkestani* and *T. urticae* RF did not produce female offspring, although mating occurred (Migeon and Navajas unpublished data; reported without accompanying data in Navajas and Boursot 2003). Recently, Ros and Breeuwer (2007) questioned the taxonomic status of *T. turkestani* in light of their finding that *T. turkestani* COI sequences are not monophyletic but scatter within the *T. urticae* clade. Here we present evidence that *T. turkestani* and *T. urticae* RF are capable of producing viable F_1 females, but that the resulting F_2 generation is not viable (hybrid breakdown). We therefore conclude that a post-zygotic reproductive barrier exists between *T. turkestani* and *T. urticae* RF, supporting the current placement of *T. turkestani* as a separate taxon (Bolland et al. 1998; Jeppson et al. 1975; Smith-Meyer 1987). Comparisons between intraspecific and interspecific crosses of *T. urticae* RF (*T. urticae* RF ♀ × *T. urticae* RF ♂ and *T. urticae* RF ♀ × *T. turkestani* ♂, respectively) indicated no significant differences in the hatchability rate, in progeny survival rate or in the female progeny ratio, excluding the possibility of post-mating, pre-zygotic reproductive barriers. The possible existence of such barriers in the *T. turkestani* crosses (*T. turkestani* ♀ × *T. urticae* RF ♂) could not be studied due to lack of statistical power.

As our laboratory strains were free of bacteria that could manipulate reproduction, we assume that other mechanisms are involved in the observed hybrid breakdown. These may include chromosome reshuffling (translocations and inversions) or other chromosomal changes that can affect meiosis or interfere with normal gametogenesis, as previously proposed for other *Tetranychus* species (Boudreaux 1963; Jordaan 1977). From the evolutionary perspective, the production of unfit offspring can be associated with high fitness costs. It

may involve wastage of energy, time and gametes (Singer 1990) and can negatively affect the reproductive success of the particular spider mite species (Boudreaux 1963; Helle 1967; Helle and van de Bund 1962; Helle and Sabelis 1985; Overmeer 1972; Takafuji et al. 1997). In such cases, the reinforcement model of speciation predicts that natural selection will favor the evolution of pre-mating isolating mechanisms—usually mating behaviors—that will prevent the production of unfit hybrids (Butlin 1987; Coyne and Orr 1989; Dobzhansky 1937; Noor 1995; Saetre et al. 1997).

Specific mate recognition systems should enable species to recognize conspecific mates correctly. Our mating behavior data indicated that males of *T. turkestani* and *T. urticae* RF found and contacted virgin conspecific and heterospecific females at the same rate and readily tried to copulate with them. This male behavior is common among closely related species and probably results from the incomplete species recognition systems (Hochkirch et al. 2007). On the other hand, female mate recognition seemed to be more reliable in *T. turkestani* than in *T. urticae* RF. The number of copulations was significantly higher and their duration significantly shorter in the *T. turkestani* interspecific (*T. turkestani* ♀ × *T. urticae* RF ♂) as compared to the intraspecific (*T. turkestani* ♀ × *T. turkestani* ♂) crosses, a phenomenon not observed in the *T. urticae* RF crosses (*T. urticae* RF ♀ × *T. turkestani* ♂ versus *T. urticae* RF ♀ × *T. urticae* RF ♂). The short duration of copulation and the number of male re-mating attempts in the *T. turkestani* ♀ × *T. urticae* RF ♂ crosses may result from incompetence between *T. turkestani* female and *T. urticae* RF male genitalia (Jordaan 1977), or from different courtship displays of both species (Hochkirch et al. 2006). It could reflect the ability of *T. turkestani* females to recognize and resist the heterospecific males by a variety of signals in communicative behavior, such as acoustic, visual, olfactory, tactile or vibrational signals (Thornhill and Alcock 1983). In any case, these finding indicate the possible existence of an asymmetric mate recognition ability between the two species in which *T. turkestani* females are more selective in their mate choice.

Interspecific mating attempts can reduce fitness and lead to decreased conspecific matings in mixed cultures (McLain and Shure 1987; Singer 1990; Verrel 1994). If both species are equally affected, the initial density should determine the reproductive success and survival (Foster et al. 1972). Nevertheless, asymmetric types of reproductive interference are probably more common in nature, as it is rather unlikely that two related species have completely similar reproductive properties (Hochkirch et al. 2007). Our experiments indicate that asymmetric reproductive interference occurred in mixed populations of *T. turkestani* and *T. urticae* RF. On bean leaf discs, a nearly significant reduction in the F_1 female ratio was observed in *T. urticae* RF when mixed cultures (0.46) were compared to the pure cultures (0.67), a phenomenon that was not reciprocated in *T. turkestani* (Fig. 2C). Similar results were obtained in the whole bean plants experiments, in which a significant (nearly 40%) reduction in the production of female progeny was observed only in *T. urticae* RF mixed cultures (Fig. 3). As suggested above, the reduced mating success of *T. urticae* RF in the mixed treatments may be the consequence of asymmetric mate recognition ability: *T. urticae* RF females did not discriminate between heterospecific and conspecific males while *T. turkestani* females showed a greater propensity for identifying and rejecting heterospecific males, in this way increasing their chances of mating with conspecific males. On the other hand, higher selectivity of spider mites males for conspecific mating was reported to have the opposite effect. *Panonychus mori* males show a strong preference for conspecific females, whereas *P. citri* males did not show any mating preference. When the two species co-exist, the deleterious effect through reproductive interference is more intense for the more selective species (*P. mori*) than for *P. citri* (Takafuji et al. 1997), probably because the former does not interfere with the intraspecific matings of the latter.

The extend to which the asymmetric reproductive interference might affect *T. urticae* RF fitness/reproductive success, when living in mixed populations with *T. turkestani* for a few generations, is yet to be examined. Sexual exclusion is a reasonable effect of reproductive interference (Reitz and Trumble 2002) and might explain the missing coexistence of several closely related species. It might therefore represent a potential threat to the inferior species in this case, *T. urticae* RF. However, in the present study, pure cultures of *T. urticae* RF had higher progeny survival rates than *T. turkestani* in leaf disc assays and produced more progeny in whole plant assays. The excess of male progeny in the second generation may increase the chance of the intraspecific matings and can serve as a compensating factor, restoring *T. urticae* RF fitness in subsequent generations. This may be a mechanism by which *T. urticae* RF and *T. turkestani* coexist in sympatry on annual crops, such as watermelon, without completely excluding each other.

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