Molecular characterization of Israel’s spider mites
(Acari: Tetranychidae)

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By

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ABSTRACT

The spider mite family, Tetranychidae, (Acari: Prostigmata), includes ca. 1200 species globally, amongst which are key pests of many vegetables, field crops, fruit trees, cut flowers and ornamentals. Until 2004, only a dozen species, of which most are agricultural pests, were recorded from Israel.

The identification of tetranychid species is problematic due to their minute size, the limited number of available diagnostic characters and the intraspecific variation. Both sexes of many species, especially in the large genera Oligonychus and Tetranychus, are often needed in order to arrive at precise determination. Expert taxonomists, microscopic slide preparations and comprehensive keys are essential for the task. As a result, molecular methods are increasingly being applied for taxonomic purposes.

Nuclear ribosomal DNA (rDNA) provides one of the most complete tools for a multitude of molecular tasks. In eukaryotes, the rDNA region consists of three highly conserved regions (18S rDNA, 5.8S rDNA and 28S rDNA) and two rapidly evolving regions, the internal transcribed spacer regions (ITS1 is located between the 18S and the 5.8S, whereas ITS2 is between the 5.8S and the 28S). Previous studies have indicated that the ITS2 sequence divergence can provide species-level resolution and may be used to differentiate between closely related species, complexes of species and even populations.

In this study I used ITS2 sequences to develop a barcoding system for effective identification of spider mite species. I also addressed two ecological questions that arose from analyses of the diversity between and within important spider mite pest species.
The specific objectives of this work were:

1. To collect and morphologically identify the pestiferous spider mite species existing in Israel and to establish a database for their ITS2 sequences. To record the magnitude of ITS2 sequence divergence within and between species and to determine whether this molecular marker would provide sufficient resolution and could be used as a barcode for identifying specimens from this family.

2. Under the assumption that high diversity within a species may reflect geographic adaptation and perhaps isolation of populations or the presence of unrecognized close species, the second objective of this research was to study the genetic variability of the polyphagous species *E. orientalis*, collected in the three climatic zones of Israel: Mediterranean, Semi-arid and Arid.

3. Under the assumption that low diversity between two species may indicate their incomplete separation, which can result in negative reproductive interactions, the third goal of my research was to study the reproductive interactions between *T. turkestani* and *T. urticae* RF, two important polyphagous pest species commonly present in Israeli agro-ecosystems.

Twenty spider mite species were collected from 21 sites (32 different combinations of host/site) throughout all climatic regions of Israel during 2004-2006. Ribosomal DNA-ITS2 sequences were obtained for 16 species, among them sequences of 12 tetranychid species which were new to the GenBank. The barcode sequences of each species were unambiguously distinguishable in base composition and length (360 to 540 bp) from all other species. Sequence divergences were generally much greater between species than within them. Using a 0.02 (2%) threshold for species diagnosis in the data set, 14 out of 16 species were easily
identified. The only exception was the low divergence, 0.011–0.015 (1.1–1.5%), between \textit{Tetranychus urticae} and \textit{T. turkestanii}. Nonetheless, the two species have fixed alternative rDNA-ITS2 variants, with five diagnostic nucleotide substitutions.

Three species that were identified by molecular and morphological criteria were recorded in Israel for the first time: \textit{Aplonobia histricina} (Berlese), \textit{Tetranychus evansi} Baker and Pritchard and \textit{T. ludeni} Zache. Four additional new records were not included in the molecular catalogue because of insufficient specimens in their collections. These are: \textit{Bryobia graminum} (Schrank), \textit{Eotetranychus hirsti} Pritchard and Baker, \textit{Oligonychus coniferarum} McGregor and \textit{T. neocaledonicus} Andre. These species may become agricultural and forest pests in the future. Their identification and recording in Israel are very important for the import and export of plant material to and from Israel.

The barcoded species formed distinct, nonoverlapping monophyletic groups in the maximum-parsimony phylogenetic tree, in congruence with the morphological classification. Representatives of the two subfamilies: Bryobiinae and Teranichinae are located in different main branches, which are further branched into tribes, as shown in the subfamily Tetranicininae that includes representatives of the tribes Eurytetranychini and Tetranychini. Claded together are species in genera that contain more than one species, namely \textit{Eutetranychus}, \textit{Panonychus}, \textit{Petrobia} and \textit{Tetranychus}. One exception is the genus \textit{Oligonychus} that was not monophyletic in the ITS2 tree. \textit{Oligonychus afrasiaticus} (McGregor) claded next to the \textit{Tetranychus} species, away from the species \textit{O. perseaee} Tuttle, Baker and Abbatiello and \textit{O. mangiferus} (Rahman and Sapra). Another molecular marker, the COI gene (mitochondrial Cytochrom Oxidase sub unit I), was sequenced and revealed a smaller
genetic distance between \textit{O. afrasiaticus} and \textit{T. urticae} whereas \textit{O. perseae} and \textit{O. mangiferus} were close to each other according to both DNA markers. Morphologically, the shape of aedeagi of \textit{O. afrasiaticus} males resembles the tetranychid type of aedeagi and is totally different than the aedeagi type of the two other \textit{Oligonychus} species mentioned. Subdivisions of the genus \textit{Oligonychus} were attempted in the past, but eventually rejected. Herein is additional evidence that this large genus is polyphyletic based on various characteristics.

Only one ITS2 sequence was isolated from each of the following seven species: \textit{A. histricina}, \textit{B. praetiosa}, \textit{P. harti}, \textit{P. tunisiae}, \textit{Schizotetranychus asparagi}, \textit{Oligonychus afrasiaticus} and \textit{T. evansi}. In the other species analyzed, the number of sequences (per species) fluctuated between two and nine.

The most polymorphic species was the indigenous polyphagous species \textit{Eutetranychus orientalis} that had nine different ITS2 sequences. This high variation suggested the possibility of a structured population or the existence of cryptic species. Nevertheless one common sequence was present in all seven populations analyzed and the majority of individual females were heterozygotes, indicating gene flow between these subpopulations. No genetic structure or pattern of the populations could be related to the expansion of the geographic range of this species since the 1930's and its adaptation to the Mediterranean region. No indication of isolation of subpopulations was shown nor the existence of mixtures of closely related species.

Two pest species, \textit{Tetranychus turkestani} and \textit{T. urticae} RF were found in separate agricultural habitats and geographical regions in Israel, in collections made through 2004-2005. The former was collected in the north of Israel from apple and peach trees whereas the latter was collected from vegetables in the center and south of
the country. In additional collections that were undertaken during 2006-2007 (50 combinations of location/host), I found *T. turkestani* in the southern part of Israel as well as in mixed populations with *T. urticae* RF on watermelon in the north. The overlapping niches of these closely related species and their morphological and molecular resemblance raised the possibility that reproductive interactions and interference occur between the two species when they share the same host.

Interspecific crosses showed that the two species are capable of producing viable F\textsubscript{1} females when the mothers were *T. urticae* RF (20% of the tested couples) whereas only a single incidence of F\textsubscript{1} female was obtained in the reciprocal crosses. All F\textsubscript{1} females from both interspecific crosses were sterile indicating a post-zygotic reproductive barrier between the two species. 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* ♀ X *T. urticae* RF ♂) as compared to the intraspecific crosses, a phenomenon not observed in *T. urticae* RF crosses. 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 because additional reproductive factors, such as higher oviposition rate and progeny survival to adulthood in *T. urticae* RF, may reduce the probability of its demographic displacement by *T. turkestani* when sharing a host.
In Summary, my research showed that the rDNA-ITS2 sequence barcodes may serve as an effective tool for the identification of spider mite species and can be applicable as a diagnostic tool for quarantine and other pest management goals. I have contributed to the international ITS2 database the sequences of 12 species and recorded the genetic variation within the different representatives of the Tetranychidae family common in Israel. This may provide the platform for a uniform, accurate, practical and easy-to-use routine of worldwide spider mite species identification in the future. I have updated and added seven species to the Israeli Tetranychidae check list. An understanding of the reproductive interactions between two closely related important pest species in Israel *Tetranychus turkestani* and *T. urticae* RF was also achieved.
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1. Introduction

1.1 Spider mites as plant pests

The family Tetranychidae (Acari: Prostigmata), also known as the spider mites, includes the most injurious plant-feeding mites (Helle and Sabelis, 1985; Meyer, 1987; Zhang, 2003). Some infest a wide range of host plants, whereas others are highly specific (Bolland et al., 1998). Pestiferus spider mite biology is characterized by a short generation time, high fecundity, rapid dispersal, effective exploitation of new feeding sites and rapid development of resistance to acaricides (Helle and Sabelis, 1985). Pestiferus spider mites damage agricultural and horticultural crops and cause severe economic losses. The importance of spider mites in agriculture has greatly increased during the past 60 years; the two-spotted spider mite, Tetranychus urticae Koch, and the European red mite, Panonychus ulmi (Koch), are examples of species that have been given much attention worldwide (Helle and Sabelis, 1985; Jeppson et al., 1975). A few pests are considered invasive species that have recently established in the mediterranean regions, such as Oligonychus perseae Tuttle, Baker and Abbatiello from America (Swirski, 2002), and Tetranychus evansi Baker and Pritchard from America via Africa (Escudero and Ferragut, 2005; Ferragut and Escudero, 1999; Ben-David et al., 2007; Tsagkarakou et al., 2007).

Spider mites have needle like piercing-sucking mouthparts that penetrate the plant tissue, suck out its content and cause the collapse of mesophyll cells, which then appear as small chlorotic spots. Leaf area for photosynthesis decreases. In a severe attack, leaves and fruit show necrotic surface and defoliation, growth cease, plants wilt, the quality of the yield is damaged and even a death of the attacked plant can occur (Jeppson et al., 1975). Some spider mites spin fine webbing on plant parts that
may cover them, increasing the direct damage (Jeppson et al., 1975). Plant pathogens are able to penetrate plant tissue via feeding wounds (Helle and Sabelis, 1985). 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).

1.2 Spider mite taxonomy

The family Tetranychidae comprises ca. 1200 species worldwide (Bolland et al., 1998). It is subdivided into two subfamilies, Bryobiinae and Tetranychinae, each being further separated into tribes. The Bryobiinae encompass the tribes Bryobiini, Hystrichonychini and Petrobiini, whereas the Tetranychinae includes the tribes Eurytetranychini, Tenuipalpoidini and Tetranychini. Systematists consider the genus Bryobia of the subfamily Bryobiinae to be the most primitive genus in the family, with no silk webbing and with many parthenogenetic species, whereas Tetranychus is at the other end of the evolutionary tree, with species that are all biparental and that use extensive webbing (Lindquist, 1985).

1.3 Spider mites in Israel

Although the spider mites of Israel are poorly known, members of most tribes (except the Tenuipalpoidini) were recorded. Until the present study, they numbered about a dozen species, of which most are agricultural pests (presented here according to the date of report): *Eutetranychus orientalis* (Klein) (Klein, 1936a), *Tetranychus cinnabarinus* (Klein, 1936b), *Panonychus ulmi* (Plaut, 1963), *Tetranychus urticae* green-form (herein *T. urticae* GF) (Plaut and Feldman, 1966), *Bryobia praetiosa* Koch (Avidov and Harpaz, 1969), *Eutetranychus palmatus* Attia (Gerson et al., 1983), *Oligonychus afrasiaticus* (McGregor) (Gerson et al., 1983), *Oligonychus*
mangiferus (Rahman & Sapra)(Gerson, 1986), Panonychus citri (McGregor) (Swirski et al., 1986), Schizotetranychus asparagi (oudemans) (Bolland et al., 1998), Oligonychus perseae (Swirski et al., 2002) and Oligonychus senegalensis Gutierrez and Etienne (Palevsky et al., 2003). Two species were recorded on wild flora: Petrobia harti (Ewing) and Petrobia tunisiae Manson (Dubitzki, 1981; Dubitzki and Gerson, 1987). Two additional species are listed in the world catalogue (from 1998) as present in Israel: 1) Oligonychus tylus Baker and Pritchard, on date palm, a misidentification of O. senegalensis (the late Dr. MKP Smith-Meyer in correspondence with U Gerson).

2) Tetranychus turkestani Ugarov and Nikolskii with no reference, as personal knowledge of the authors (Bolland et al., 1998).

Most of the above pest species are non-indigenous. There are first records of new pests in Israel from 1963 till 2002, leaving only two species, namely T. cinnabarinus = T. urticae red-form (herein T. urticae RF) and E. orientalis as long-time inhabitants of the region. Knowledge about the invading or native species' intraspecific variation is not available. In addition, exotic pest species are continuously introduced as a result of the ever-increasing international transfer of commercial plants and their products (Helle and Sabelis, 1985). I believe that the list of tetranychid species in Israel is incomplete.

1.4 Spider mites identification

Species identification is the basis for understanding species diversity, phylogenetic patterns, and evolutionary processes. Only correct identifications allow for comparisons between studies and the repetition or expansion of earlier experiments. In pest species, accurate identification is of paramount importance for
quarantine and management purposes: the development of biological and other control strategies.

The identification of tetranychid species is very problematic due to their minute size, the limited number of available diagnostic characters, the intraspecific variation in some morphological criteria and the similarity between some close species. In addition, both sexes of many species, especially in the large and economically-important genera *Oligonychus* and *Tetranychus*, are often needed in order to arrive at precise determination (Helle and Sabelis, 1985; Jeppson *et al*., 1975). Expert taxonomists, microscopic slide preparations and comprehensive keys are also essential for the task of species identification. Even though the accepted morphological method of classification and identification of species is indispensable, molecular methods are increasingly being applied for accurate taxonomic purposes as a result of the above reasons (Navajas and Fenton, 2000). The two most commonly used DNA markers are some parts of the coding region of the mitochondrial Cytochrom Oxidase sub unit I (COI) gene and the Internal Transcribed Spacer 2 (ITS2), a nuclear ribosomal DNA fragment. Examples include the transfer of *T. viennensis* Zacher and related species to the restored genus *Amphitetranychus* Oudemans (Navajas *et al*., 1997), separating *T. pueraricola* Ehara and Gotoh from *T. urticae* (Gotoh *et al*., 1998), and synonymizing *T. hydrangeae* Pritchard and Baker with *T. kanzawai* Kishida (Navajas *et al*., 2001). Restriction of ITS2 fragments by 2 restriction enzymes results in a distinct RFLP pattern for 4 important tetranychid pest species occurring in Japan, namely *T. ludeni* Zacher, *T. neocaledonicus* Andre, *T. pueraricola*, and *T. urticae* RF and GF (Osakabe *et al*., 2002). This is a fast and relatively inexpensive technique for spider mite identification. The same approach
was utilized in Africa for the discrimination between the invasive pest *T. evansi* and the local closely related species *T. urticae* RF (Knapp et al., 2003). Molecular methods may also be used to determine the origin of invading spider mites. It was shown that *Mononychellus progresivus* Doreste of Africa has originated in Colombia rather than in Brazil, by similarities of base composition of COI and ITS2 fragments of specimens from two locations in Africa and from the two South American locations (Navajas et al., 1994). This information can be used to search for efficient natural enemies of invasive pests in their area of origin.

COI sequence variation within tetranychid species is relatively high, not linked to geographical location nor associated with the host plant (Navajas, 1998; Navajas et al., 1998). The use of mitochondrial genes for delineating and classifying biological species should be done cautiously (Ros and Breeuwer, 2007).

1.5 The ITS2 fragment

Nuclear ribosomal DNA (rDNA) provides one of the most complete tools for a multitude of molecular tasks (Navajas and Fenton, 2000). In eukaryotes, the rDNA region consists of three highly conserved regions (18S rDNA, 5.8S rDNA and 28S rDNA) and two rapidly evolving regions, the internal transcribed spacer regions: ITS1 is located between the 18S rDNA and the 5.8S rDNA, whereas ITS2 is between the 5.8S rDNA and the 28S rDNA) (Hillis and Dixon, 1991). Previous studies on several acarine families, such as Phytoseiidae, Tetranychidae, Listrophoridae and the subfamily Rhizoglyphinae, have indicated that the ITS2 sequence divergence can provide species-level resolution and may be used to differentiate between closely related species, complexes of species and even populations (Navajas, 1998; Navajas et al., 1998; Navajas et al., 1999; Navajas et al., 2000; Navajas et al., 2001; Noge et
al., 2005; Vargas et al., 2005). ITS2 of *T. urticae* GF and RF populations from various hosts in 18 locations in the northern hemisphere was found to be identical (Navajas et al., 1998). ITS2 sequences of 15 *Tetranychus* species have been previously submitted to the international GenBank, including three that have not been published as part of a journal paper (Table 1).

Table 1. Tertanychid species submitted to the GenBank, their Accession numbers and references. (* Submitted to GenBank but not published as a journal paper)

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amphitetranychus quercivorus</em></td>
<td>X99880</td>
<td>Navajas et al., 1998</td>
</tr>
<tr>
<td><em>A. vienensis</em></td>
<td>X99882-3</td>
<td>Navajas et al., 1998</td>
</tr>
<tr>
<td><em>Mononychellus progressivus</em></td>
<td>X77902</td>
<td>Navajas et al., 1994</td>
</tr>
<tr>
<td><em>Panonychus citri</em></td>
<td>AY750707</td>
<td><em>Hsu, K. et al.</em>, 2004</td>
</tr>
<tr>
<td><em>P. ulmi</em></td>
<td>AY750708</td>
<td>Taiwan, 2004</td>
</tr>
<tr>
<td><em>Petrobia harti</em></td>
<td>AY750705</td>
<td></td>
</tr>
<tr>
<td><em>T. evansi</em></td>
<td>AJ419833.1</td>
<td>Knapp et al., 2003</td>
</tr>
<tr>
<td><em>T. kanzawai</em></td>
<td>X99876</td>
<td>Navajas et al., 1998</td>
</tr>
<tr>
<td><em>T. ludeni</em></td>
<td>ABO76370</td>
<td>Osakabe et al., 2002</td>
</tr>
<tr>
<td><em>T. mcdanieli</em></td>
<td>ABO76371</td>
<td>Osakabe et al., 2002</td>
</tr>
<tr>
<td><em>T. neocaledonicus</em></td>
<td>X99877</td>
<td>Navajas et al., 1998</td>
</tr>
<tr>
<td><em>T. pacificus</em></td>
<td>X99879</td>
<td>Navajas et al., 1998</td>
</tr>
<tr>
<td><em>T. pueraricola</em></td>
<td>ABO76372</td>
<td>Osakabe et al., 2002</td>
</tr>
<tr>
<td><em>T. turkestani</em></td>
<td>AJ295611</td>
<td>Navajas et al., 2003</td>
</tr>
<tr>
<td><em>T. urticae</em> RF and GF</td>
<td>X99881</td>
<td>Navajas, 1998</td>
</tr>
<tr>
<td><em>T. urticae</em> RF and GF</td>
<td>ABO76369</td>
<td>Osakabe et al., 2002</td>
</tr>
</tbody>
</table>
Accumulating large number of spider mites sequences, especially from genes such as ITS1 and ITS2 may serve as the scaffold of a molecular method that could simplify spider mites identification. This would facilitate the use of species-specific sequences as DNA “biological barcodes”. Comparing barcode sequences from an unidentified sample can tell the identifier if there are similar DNA sequences in the database, which were previously obtained from vouched specimens, therefore leading to species identification.

1.6 Phylogeny

According to Futuyama (1997) a phylogenetic tree is the estimate of species divergence from a common ancestor. Data collected by systematists, especially morphological characters, comparative embryology, fossil records and comparative anatomy are the data used to suggest how similar organisms are. According to species' similarities they can be classified into taxonomic groups. The classification may change with the discovery of new species and/or identification of new diagnostic characters in existing species. Cladograms are branched diagrams that illustrate patterns of relatedness where the branch lengths are not necessarily proportional to the evolutionary time between related organisms. A phylogenetic tree is sometimes presented as a specific type of cladogram where the branch lengths are proportional to the predicted or hypothetical evolutionary time between organisms. Bioinformaticians produce cladograms representing relationships between sequences of DNA or amino acids of organisms. The cladograms generated by bioinformatics tools are based on sequence data alone. Each position in a sequence is a character with 4 states (nucleotides): A, T, C or G, which are clearly defined; this is an advantage over morphological traits such as dimensions, which are sometimes variable. Hence, DNA
sequence relatedness is considered to be a very powerful predictor of the relatedness of species (Futuyama, 1997). Barrett and Hebert (2005) suggested using a 0.02 (2%) divergence as the threshold for species diagnosis with mtDNA-COI sequences.

Three methods are commonly used for constructing phylogenetic trees:

Maximum Parsimony (MP) – The assumption at the basis of parsimony is that the most likely tree is the one that requires the fewest number of unproven assumptions (such as evolutionary changes / point mutations). The simplest hypotheses that explain the data in the alignment is the most parsimonious and will be chosen.

Maximum Likelihood (ML) – inferring an evolutionary tree by finding the tree that maximizes the probability of observing the data. Use is made of the evolutionary model of each character (nucleotide) with many parameters, such as substitution rate of a nucleotide into the other 3 nucleotides. Probabilities are small and expressed as log likelihoods. The tree with the largest log likelihood is chosen (Felsenstein, 1981).

Neighbor-Joining (NJ) – The shortest tree that can be constructed based on the distance between sequences, expressed as the fraction of sites that differ between them. It uses a specific series of calculations, manipulating a distance matrix (a matrix of distances for pairs of sequences in all combinations) derived from a multiple alignment. Construction of trees is done step by step, using algorithms that deal with large sets of data, which the former methods are unable to, as a result of long computation time. NJ does not assume that all lineages evolve at the same rate and that all taxa are equally distant from the root. It does not estimate which taxon preceded the others in the tree, but rather which sequences are closer to each other, according to the minimum evolution criterion (Hall, 2004; Saitou and Nei, 1987).
In this work I used the Maximum Parsimony (MP) methods for phylogenetic tree construction based on DNA base composition of the complete ITS2 fragment and also for partial mtDNA-COI.

1.7 Gene flow and Speciation

Gene flow between different populations of the same species is important for all biological species. The exchange of genes determines the homogeneity of the species. When analyzing different populations of a species, different alleles of a certain gene are identified and the frequency of each allele in a certain population can be calculated and compared to their frequency in the meta-population. When gene flow exists, no differences between these values are expected. Nevertheless, loss of some alleles or fixation of others, are signs of random genetic drift or adaptation to new hosts or conditions, processes that can occur as a result of isolation. In time, natural selection forces combined with isolation and random genetic drift can cause speciation – the development of a separate biological entity. Speciation is mostly a result of coincidental, non adaptive genetic changes in populations that can cause reproductive isolation. Factors that favor a high rate of speciation include topographic barriers that allow alloptric divergence; low dispersal rates; ecological specialization; ecological opportunities ("vacant niches"); strong sexual selection; bottlenecks in population size. The rate of speciation is highly variable. Aside from instantaneous speciation by polyploidy, the time required for speciation ranges from a few thousands to more than 20 million years, perhaps about 3 million years in evarage (Futuyma, 1997).
1.8 speciation and molecular divergence

DNA sequences evolve at roughly constant rates over time, and might thereby provide internal biological timepieces for dating past evolutionary events. The concept of molecular clocks fits well with neutrality theory because the rate of neutral evolution in genetic sequences is, in principle, equal to the mutation rate to neutral alleles. Nevertheless, different DNA sequences evolve at different rates e.g. introns and pseudogenes evolve faster than non-degenerate sites in protein coding genes, and mtDNA in many vertebrate animals evolve 5-10 times faster than single-copy nuclear DNA. Data from fossils and biogeography evidence gives the conventional mtDNA clock calibration of 2% sequence divergence per million years between recently separated lineages in mammals whereas evolutionary rate of 1% per 50 million years is estimated in 16S rDNA of eubacteria.

The branching orders in phylogenies can be inferred directly from distributions of qualitative character states, using cladistic, parsimony or maximum likelihood analyses, which are valid irrespective of whether molecules evolve in strictly time-dependent fashion.

The correlation between speciation rates and genetic divergence is not universal. If genetic divergence is proportional to time, than mean genetic distance among extent species should be similar in rapidly speciating (species-rich) and slowly speciating (species-poor) clades of similar evolutionary age, whereas if genetic divergence is a function of the number of speciation events, mean genetic distance among extent forms should be obviously greater in a species-rich clade. Empirically this link was rejected when mtDNA sequences were studied in pairs of temperate and tropical avian taxa, but was accepted from studies of allozyme genetic distances within 111
vertebrate genera. At least 50 percent of phylogenetic trees showed no significant correlation between rates of molecular evolution and apparent rate of speciation (Avise, 2004).

1.9 *Eutetranychus orientalis* (Klein)

Two phenomena regarding the ITS2 base composition of tetranychid species in Israel were chosen for extended investigation. The first was the high variation found in the ITS2 sequences of the indigenous polyphagous species *Eutetranychus orientalis*. The "oriental red spider" in "Palestine" was discussed in details by Klein, (1936a). It was mentioned in that paper as having been observed by Bodenheimer in 1922 and that it was spreading from the Jordan Valley – Sea of Galilee area westwards and southwards to citrus groves since 1926 (Ein Harod). In 1930 it was first reported in the coastal plain (Hadera), a year later in Petach Tikvah and Rehovot. (Figure 1: locations 6, 10, 12, 16, 17 respectively) *Eutetranychus orientalis* is a pest of citrus, almond, persimmon, carambola and avocado in the arid and semi-arid regions of Israel (Swirski, 2002). It has been found also on *Melia azedarach, Salix* sp., *Plumeria alba, Carica papaya, Morus alba, Ficus macrophylla* and *Ricinus communis* in regions with a Mediterranean climate, including the coastal strip of Israel (personal collections). Its host list includes 217 hosts from 37 countries in Africa, Asia and Australia (Bolland et al., 1998).

A few similar species were reported/described from Egypt: *Eutetranychus africanus* (Tucker) on *Dalbergia sissa, Morus alba, Psidium guajava* and *Eucaliptus globules; E. pyri* Attiah on *Ricinus communis, Cynodon dactylon, Citrus aurantium, Solanum melongena* and *Dalbergia sissa* (Zaher, 1982). Other morphologically close
species of *Eutetranychus* were described from Yemen by Smiley and Baker (1995): *E. pruni* and *E. sanaae* on *Prunus amygdalus* and *E. ricinus* on *Ricinus communis*.

### 1.10 *Tetranychus turkestani* and *T. urticae*

The second issue chosen for further study was the small genetic distance between two of the most abundant and pestiferous species in Israel. The most common spider mite pest in Israel is *T. urticae* Koch - RF (synonym = *T. cinnabarinus* Boisduval).

Although listed from Israel (Bolland *et al*., 1998), the closely related species *T. turkestani* Ugarov and Nikolskii was overlooked and received no attention by farmers, quarantine and extension-service experts (farm advisors). This could have been due to past misidentifications and confusion with the green morph of *T. urticae*, first reported as a pest of deciduous fruit trees in Israel in 1965 (Plaut and Feldman, 1966). *Tetranychus urticae* (GF as well as RF) and *T. turkestani* are hard to discriminate morphologically as well as molecularly (Navajas and Boursot, 2003). Microscopic examination of the male genitalia is needed for positive identification, because no other morphological trait separates the two species. The genetic distance between *T. urticae* and *T. turkestani*, based on the sequences of ITS2 as reported by Navajas and Boursot (2003) and Ben-david et al. (2007) is only ca. 1%, too small to be considered as two different species (Barrett and Hebert, 2005). In addition, the species can not be discriminated by their mtDNA COI sequences. Navajas and Boursot (2003) showed that intraspecific divergences in COI ranged from 0.9% to 5.3% in *T. turkestani*, and from 2.1% to 6.2% in *T. urticae* and that interspecific divergences were of the same order, from 2.4% to 6.1%. Moreover, COI of *T. turkestani* from The Netherlands and from the USA lie on the same branch as *T. urticae* samples from Greece and Spain, whereas COI of French and Polish *T. turkestani* samples were closer to *T. urticae*.
collected in Italy, Egypt, Tunisia and The Netherlands. It was therefore suggested that the two species may have only recently diverged. This observation was reinforced by Ros and Breeuwer (2007) using a large data set of 165 COI sequences from all over the world. 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.

Both species are polyphagous and globally distributed (Bolland *et al.*, 1998), but whereas *T. urticae* RF is probably indigenous (Klein, 1936b), *T. turkestani* was not introduced to Israel before the 1960's. The oldest mounted male specimen I found was in Gerson's collection, dated 08/Feb/1980 (location: Revivim, host: artichoke – *Cynara cardunculus*). The formerly-reported host list of *T. urticae* RF in Israel included as many as 80 wild and ornamental plant species (of which 10% were trees) from 32 botanical families including 17 agricultural crops, mostly herbaceous, such as vegetables in the families Cucurbitaceae and Solanaceae (Klein, 1936b; Avidov and Harpaz, 1969). Nevertheless, until the 1960's *T. urticae* RF was considered a serious pest in apple and pear orchards as well (Klein, 1936b; Plaut and Feldman, 1966). *Tetranychus urticae* RF overwinters in greenhouses, in polyethylene covered low tunnels and on some perennials. In the spring and early summer, when temperatures increase, the mites move onto crops in open fields mainly by aerial dispersion (Dubitzki, 1981).

*Tetranychus turkestani* was reported as an agricultural pest of cotton in Western USA (Carey and Bradley, 1982; Colfer *et al.*, 2004; Simons, 1964); of strawberry in Russia (Popov, 2003), Turkey (Cakmak *et al.*, 2003) and USA (Jeppson *et al.*, 1975); of field grown roses in California (Karlik *et al.*, 1995); of eggplant in Iran
(Soleimannejadian et al., 2006); of corn and sunflower in France (Bailly et al., 2004). The host list of *T. turkestani* includes 207 host plants from 59 botanical families, amongst which are deciduous perennials (Rosaceae) as well as many low growing plants from the Asteraceae, Malvaceae, Fabaceae etc, from 35 countries (Bolland et al., 1998). Jeppson et al. (1975) reported that *T. turkestani* is a serious pest of low growing crops and is also found on apple, peach, pear, plum and walnut, in which it is not a serious pest. Females of *T. turkestani* may undergo winter diapause, like *T. urticae* GF (Jeppson et al., 1975).

1.11 Reproductive interference

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 interspecific matings were seen in spider mites of the genus *Tetranychus* (Tetranychidae) (Helle and Sabelis, 1985). Most of these were totally ineffective and had no effect on the success of subsequent fertilizations by conspecific males (Helle and Van De Bund, 1962; Boudreaux, 1963). However, a few interspecific matings did produce infertile hybrid (*F*$_1$) female offspring or eggs that did not hatch (Boudreaux, 1963). In these cases, subsequent conspecific matings were not effective, indicating that a reproductive interference has occurred in these heterospecific matings. The mechanism of reproductive interference is known from interactions between closely related species that have incomplete isolated recognition and mating systems (Reitz and Trumble, 2002). Allopatric species often demonstrate greater similarity in mating signals than sympatric species, even if they have been diverging for a similar length of time (Butlin, 1995). As a consequence of biological
Invasions, previously allopatric species are brought together and their partially similar mate recognition systems may promote mating interactions (Liu et al., 2007). Interaction and competition between related/sibling species may adversely affect population dynamics, abundance, habitat choice and the distribution of these species (Gröning et al., 2007). One possible result is a displacement of one competitor by the other in a certain ecological niche. Reproductive interference between two tetranychid pests, namely Panonychus mori (Koch) and P. citri (genetically incompatible congeners), is an important process underlying the habitat segregation under laboratory and field conditions and explains the exclusion of P. mori from peach orchards where the two species may coexist (Takafuji et al., 1997). This mechanism was also considered of importance in annual cropping systems. For example, the relatively fast displacements of the local strains of the whitefly Bemisia tabaci (Gennadius) by the B biotype of this insect in the USA, China and Australia (Reitz and Trumble, 2002). In the southwestern USA the takeover was partly due to the aggressiveness of B biotype males when courting the local B. tabaci females, resulting in a lower reproduction of the latter in mixed populations (Perring et al. 1991). In east China and in Queensland (Australia), asymmetric mating interactions between local biotypes of B. tabaci and the invader B biotype resulted in an increased frequency of copulations. This lead to increased production of female progeny among the invader, as well as reduced copulations and female production in the indigenous biotype, a process that played a critical role in determining the capacity of the invading B biotype to displace the local populations (Liu et al., 2007).
At this time, there is no way to predict, without close examination, whether the behavior previously observed in *T. turkestani* and *T. urticae* crosses, has an effect on the reproductive success of either in the habitats where they coexist.

**1.12 The objectives of this work were:**

1. To collect and morphologically identify the pestiferous spider mite species existing in Israel and to establish a database for their ITS2 sequences. To record the magnitude of ITS2 sequence divergence within and between species and to determine whether this molecular marker would provide sufficient resolution and could be used as a barcode for identifying specimens from this family.

2. Under the assumption that high diversity within a species may reflect geographic adaptation and perhaps isolation of populations or the presence of unrecognized close species, the second objective of this research was to study the genetic variability of the polyphagous species *E. orientalis*, collected in the three climatic zones of Israel: Mediterranean, Semi-arid and Arid.

3. Under the assumption that low diversity between two species may indicate their incomplete separation, which can result in negative reproductive interactions, the third goal of my research was to study the reproductive interactions between *T. turkestani* and *T. urticae RF*, two important polyphagous pest species commonly present in Israeli agro-ecosystems.
2. Materials and Methods

2.1 Collection of spider mites from Israeli agricultural habitats

Spider mites were collected from 32 mainly agricultural habitats, orchards and open fields, as well as urban and wild ecosystems in different regions of Israel during the years 2004-2005; sites, host plants, habitat and region are listed in Figure 1 and Table 3. All mites, along with their host foliage, were placed in cooled polyethylene bags (Ca. 1 liter of foliage) and brought to the laboratory. Each sample was checked for the presence of adults, live females were used for genomic DNA extraction (see below), and five to ten males and females were preserved in 70% ethanol for identification purposes. These mites were cleared with lactic acid, mounted on slides in Hoyer's solution (Gutierrez, 1985) and identified by using the keys in Bolland et al. (1998) and Meyer (1987).

Additional collections of E. orientalis were obtained in 2005 in order to study the genetic variation between populations of this highly divergent species. Details of all E. orientalis populations are given in Table 2.

In order to obtain more information on the distribution of T. turkestani and T. urticae (red form and green form) in Israeli agricultural systems, 44 additional collections were undertaken during 2006-2007. Mites from these collections were identified mostly by morphological criteria, although some specimens were also identified by ITS2 sequencing and/or by ITS2 PCR-restriction fragment length polymorphism (RFLP) (see below). Details of these collections are included in Table 8. Locations are as numbered in Figure 6.
Table 2. Collection data of *E. orientalis* (2004-2005) with reference to host plant, ecosystem and climatic region:

<table>
<thead>
<tr>
<th>Location Symbol (numbers refer to figure 1)</th>
<th>Host plant</th>
<th>Ecosystem</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td><em>C. paradisi</em></td>
<td>Orchard</td>
<td>Mediterranean, sea shore</td>
</tr>
<tr>
<td>N</td>
<td><em>C. reticulata</em></td>
<td>Orchard</td>
<td>Mediterranean</td>
</tr>
<tr>
<td>T</td>
<td><em>Salix sp.</em></td>
<td>Rural, ornament</td>
<td>Semi-arid</td>
</tr>
<tr>
<td>A</td>
<td><em>Ricinus communis</em></td>
<td>Rural, weed</td>
<td>Mediterranean, sea shore</td>
</tr>
<tr>
<td>Z</td>
<td><em>C. paradisi</em></td>
<td>Urban, ornament</td>
<td>Mediterranean</td>
</tr>
<tr>
<td>S</td>
<td><em>Prunus dulcis</em></td>
<td>Orchard</td>
<td>Semi-arid</td>
</tr>
<tr>
<td>Y</td>
<td><em>Citrus paradisi</em></td>
<td>Orchard</td>
<td>Arid</td>
</tr>
</tbody>
</table>

2.2 Genomic DNA extraction

Genomic DNA was individually extracted from 10 live females per population (species at a certain site and host). Each mite was placed on a section of parafilm stretched over a hard surface and hand ground in cold 40µl lysis buffer (5mM Tris-HCl, pH 8.0 containing 0.5 mM EDTA, 0.5% Nonidet P-40, and 3 mg/ml Proteinase K) with a sterilized plastic microtube. The extracts were incubated at 65°C for 15 min, then at 95°C for 10 min and kept on ice before PCR amplification. Ethanol- preserved mites were placed on filter paper to evaporate excess alcohol for 10 min at room temperature prior to grinding. Genomic DNA from the larger mites, *Petrobia hartii* and *Aplonobia histricina* (Berlese), was extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's recommendations.
2.3 ITS2 and COI amplification, cloning and sequencing

I used the specific PCR primer ITS2-forward 5’- GTCACATCTGTCTGAGAGTTGAGA- 3’ and the degenerate PCR primer ITS2-reverse 5’- GTARCCCTACCTRMTCTGAGATC- 3’ for amplification of the ITS2 region from all 16 spider mite species analyzed. The ITS2-forward primer starts at the flanking 5.8S rDNA sequence and ends seven nucleotides within the ITS2 sequence. The ITS2-reverse primer is completely within the 28S rDNA flanking sequence and ends one base downstream of the ITS2 sequence. I designed these primers from an alignment of 7 tetranychid species presented in Navajas et al., (1998). My primers are internally positioned in comparison with those used in that paper. For COI amplification I used the primers: forward 5’-TGATTTTTTGGTCACCCAGAAG-3’ and reverse 5’-TACAGCTCCTATAGATAAAAC-3’ from Navajas et al., (1998).

Amplification reactions were performed in 30µl volume containing: 5µl of the genomic DNA, 0.5µM of each of the above primers, 0.2mM dNTP's, 3µl of 10 X buffer and 2U Taq DNA polymerase (Promega). Reaction conditions were: denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 30 sec, 51°C for 40 sec and 72°C for 40 sec, with final extension at 72°C for 10 min. PCR products of the expected size (~370-540 bp in case of ITS2 and ~410 bp in case of COI), were extracted from 1% agarose gel using QIAquick gel extraction kit (Qiagen) and cloned using the InsT/A clone PCR product cloning kit (Fermentas). After transformation, plasmids were purified by Wizard Plus SV miniprep kit (Promega). Two-three plasmids from each individual were unidirectionally sequenced by automated DNA sequencer ABI model 3700, using the SEQ forward primer 5’-GAGCTCGGTACCTCGCGAATG -3’, located 10 bp upstream of the cloning site of the plasmid vector.
pTZ57R/T. Sequences were aligned and analyzed using Vector NTI (Invitrogen Corp). In order to determine if the sequences obtained are ITS2 sequences, I searched for the presence of three motives: motive I: 5'- TATATCCTGTTCTT -3', at position 80-120; motive II: 5' - AGTAAGGAGA -3', at position 147-200; and motive III: 5' - AAAGTCGTA -3', at position 250-330. These motives were originally identified in the alignment presented by Navajas et al. (1998), and were found to be a powerful tool for recognizing ITS2 sequences within the Tetranychidae.

I used the following approach to collect polymorphic ITS2 sequences and to correct for PCR errors caused by occasional mis-incorporation of an oligonucleotide by the Taq DNA polymerase used (estimated mis-incorporation rate between 1 X 10^-4 – 1 X 10^-5 per bp). I subjected DNA extracted from 5 individual females from each population to separate PCR reactions, and sequenced 2-3 plasmids from each individual. Polymorphism occurring in only one plasmid was considered as PCR, cloning or sequencing errors. Polymorphism found in two or more plasmids from more than one individual (different PCR reactions) was considered "true" polymorphism.

2.4 Phylogenetic analyses

DNA sequences were aligned using the multiple alignment program ClustalX (1.81) (Thompson et al., 1997) using the default 15 and 6.66 values for the gap opening and the gap extension penalty, respectively. No manual adjustments to the CLUSTAL X alignment were done. I also studied the effect of decreased gap penalties values on the final phylogeny but observed no changes in the tree structure. Differences between sequences of a given species (distance within species) and between species of the same genus (distance within genus) were calculated using the
program PAUP* 4.0b10 (Swofford, 2002), as uncorrected ($p$) distance, a measure of
the number of aligned sequence positions containing non-identical nucleotides
divided by the total number of positions compared. Estimation of $\pi$, the number of
nucleotide differences per nucleotide site between 2 nucleomorphs, was calculated for
each species using the program DnaSP version 4.10.9 (Rozas and Rozas, 1999).

In phylogenetic analyses I also used the program PAUP* 4.0b10 (Swofford 2002).
Applying the heuristic search option, maximum-parsimony (MP) trees were generated
independently for a data set containing 46 different ITS2 sequences; a reduced set of
16 sequences representing the most frequent ITS2 sequence from each species. This
subset manages to represent all major monophyletic groups present in the full tree and
had the advantage of reduced computational time. Two additional MP phylogenetic
trees were generated, for nine COI sequences of Tetranychus and Oligonychus species
and for nine E. orientalis ITS2 sequences.

In the full data set, forty-three of the ITS2 sequences were obtained from the 16
Israeli tetranychid species analyzed in this study. In addition, three acarine ITS2
sequences were obtained from the GenBank (summing to a total of 46 sequences): the
ITS2 sequence (GenBank no. AJ297573) from Phyllocopites gracilis (Nalepa) (Acari:
Eriophyidae) was used as outgroup; the ITS2 sequences from Amphitetranychus
quercivorus Ehara and Gotoh, and Oligonychus coffeae (Nietner) (Tetranychidae)
(GenBank nos. X99880 and AY750706, respectively) were inserted into the
phylogenetic analysis to simulate ITS2 sequences obtained from unidentified samples.

Heuristic searches were run for 1000 random additions. Tree length and
statistical indices were related to informative sites only. Gaps were treated as missing
data, and the tree bisection-reconnection (TBR) branch-swapping algorithm with
collapsing zero branch length option was employed. All character state transitions were equally weighted and unordered. A strict consensus tree for each data set was constructed. Robustness of the inferred trees was evaluated using bootstrap analyses with 1000 replicates.

2.5 Laboratory mite strains

Populations of *Tetranychus turkestani* were collected from outdoors, off fig (*Ficus caricae*), *Malva* sp. and *Cucurbita pepo* (within close vicinity), in the center shore 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 two years (~35 generations) on 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: i) visual observations of three sampled heavily damaged bean leaves under a dissecting microscope for female body color (green = *T. turkestani*, red = *T. urticae RF*); ii) 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).
2.6 Host preference of individual *T. 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) and peach (*Prunus persica*). All four hosts were free of pesticide treatments. Discs of 3-4 cm² in area were placed lower side up on a piece of moist cleaning mat (2 cm² in area each) in a 9 cm diameter plastic Petri dish. Dishes were stacked in groups of 10 and transferred to a constant 25 °C and 14:10 h light: dark chamber 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 1-13 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 0.5:0.5 ratio (random choice) by log-likelihood ratio (G) test, using JMP statistical software version 7.0.1 (SAS Institute, USA).

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

Female deutonymphs were isolated from each laboratory strain. After emerging as adult, each female was individually transferred to the lower side of a bean leaf disc (ca. 2 cm in diameter), placed on 1% agar. After an hour, each female was checked under a stereoscopic microscope and supplemented with a male from the same or the other species if seen feeding normally. The courtship and copulation behavior of each couple was recorded continuously for 20 minutes, using a stereoscopic microscope.
Male courtship behavior was indicated by a physical contact between the male and the female. A pair "with copulation" was determined when copulation position was observed continuously for 30 s or more. Observations were repeated 26-41 times for each of the four combinations: \(T. \text{turkestani} \, \checkmark X T. \text{urticae \, RF} \, \checkmark\), \(T. \text{urticae \, RF} \, \checkmark X T. \text{turkestani} \, \checkmark\), \(T. \text{urticae \, RF} \, \checkmark X T. \text{urticae \, RF} \, \checkmark\), \(T. \text{turkestani} \, \checkmark X T. \text{turkestani} \, \checkmark\).

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 among treatments by log-likelihood ratio (G) test, using JMP statistical software version 7.0.1 (SAS Institute, USA). 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 in which copulation had occurred for 30 s or more. All parameters were compared among treatments by analysis of variance (ANOVA) using JMP statistical software version 7.0.1 (SAS Institute, USA). Means were separated by the Tukey-Kramer honestly significant difference (HSD) test. Statistical significance was assumed at \(P \leq 0.05\).

2.8 Crossing experiments between \(T. \text{turkestani}\) and \(T. \text{urticae \, RF}\)

Single virgin females (see above) were transferred to a bean leaf in a 9 cm diameter Petri dish. Each female was supplemented with a male from the same or the other species. Females were allowed to lay eggs for 5-10 days under constant 25 °C and 14:10 h light: dark conditions. The numbers of oviposited \(F_1\) eggs, developing nymphs and emerged adults were 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 seven days. F₁ 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* ♀ X *T. urticae* RF ♂ and 16 times for *T. urticae* RF ♀ X *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 F₁ females / number of F₁ females + number of F₁ 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* ♀ X *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 the Kruskal-Wallis non-parametric tests, using JMP statistical software version 7.0.1 (SAS Institute, USA). Statistical significance was assumed at *P* ≤ 0.05.

### 2.9 ITS2 PCR-RFLP analysis

In order to verify that progeny females from interspecific crosses, and mixed cultures, are hybrids of *T. urticae* RF X *T. turkestani*, their ITS2 sequences were analyzed. 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 instruction. RFLP was conducted
with the restriction enzyme *Hpa*I (=KspAI, Fermentas) in volume of 30 µl containing: 0.5 µl enzyme (5 units), 3 µl buffer B (X 10), 11.5 µl H₂O 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 ethidium 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.

2.10 Screening for bacteria possibly associated with *T. turkestani* and *T. urticae* RF laboratory strains

To search my laboratory 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 were ground individually in lysis buffer, as described above. The 16S rRNA gene fragment (~550 bp) was amplified by PCR from the lysate using the primer combination of 341F with a GC clamp (40-nucleotide, GC-rich sequence; 5' CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCC GCCCGCCGCTAC GGGAGGCAGCAG 3') and 907R (5' CCGTCAATTCMTTTGAGTTT 3'), which targets most known bacteria. The PCR conditions used permit the amplification of the 16S rRNA gene fragment from most known bacteria (Muyzer *et al.*, 1996). Reactions were performed in a 50-µl volume containing 5 µl of the template DNA lysate, 0.4 µM concentrations of each primer, 5 µl of 0.2 mM deoxynucleoside triphosphate, 1x ExTaq buffer, and 1 unit of ExTaq (TaKara Bio, Inc. Japan). PCR conditions were: denaturation for 2 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C; followed by a 5-min final extension at 72 °C. Five µl of the PCR mix was tested using agarose gel electrophoresis, and the
remaining 45 µl was subjected to denaturing gradient gel electrophoresis (DGGE) analysis as described in Gottlieb et al. (2006).

To detect the presence of *Rickettsia*, the sampled mite lysates were subjected to PCR using the *Rickettsia*-specific primers Rb-F (5' GCTCAGAACGAACGCTATC 3') and Rb-R (5' GAAGGAAAGCATCTCTGC 3') as described in Gottlieb et al. (2006). The presence of *Wolbachia* was tested with primers Wsp-F (5' GGTCCAATA AGTGATGA AGAAAC 3') and Wsp-R (5' AAAAAATTAAACGCTACTCCA 3') as described in Enigl et al. (2005). The presence of *Cardinium* was tested with primers Clo-F (5' GCGGTGTAAAAATGAGCGTG 3') and Clo-R1 (5' ACCTMTTCTTAACT CAAGCCT 3') as described in Weeks et al. (2003). PCR conditions in all analyses were: denaturation for 2 min at 95 °C; 35 cycles of 30 s at 92 °C, 30 s at: 60 °C (*Rickettsia*), 56 °C (*Wolbachia*) or 57 °C (*Cardinium*), and 30 s at 72 °C; followed by a 5-min final extension at 72 °C. 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*. DNA quality was assessed using the specific PCR primer ITS2-forward (5' GTCACATCTGTCTGAGAGTTGAGA 3') and the degenerate PCR primer ITS2-reverse (5' GTARCCCTACCT RMTCTGAGATC 3') as described above. PCR products were visualized by staining with Envision™ (Amresco, USA) using 1.2% agarose gel electrophoresis.

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

Two virgin females, 1-2 days old, of the same species (either *T. turkestani* or *T. urticae* RF) or one virgin female of each species, were transferred to the lower side of
a bean leaf disc 2 cm in diameter, placed on 1% water agar. If normal feeding behavior was observed after an hour, leaf discs harboring two females of the same species were provisioned with two males of the same species as the females (pure culture), whereas leaf discs harboring one female of each species were provisioned with one male of each 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 in diameter) 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 previously described in the crossing experiments. Female progeny with orange pigmentation were considered interspecific hybrid suspects. To distinguish between normal and hybrid females, the suspects were genotyped by ITS2 PCR-RFLP (see chapter 2.9). The experiment was replicated 15-18 times for each combination. Comparisons were conducted between the two pure cultures and between pure and mixed cultures within each species (total of three independent comparisons). Proportion data were arcsine transformed prior to analysis. The data did not meet the assumptions of ANOVA (homogeneity of variances among treatments) and were analyzed by the non-parametric Wilcoxon two sample test, using JMP statistical software version 7.0.1 (SAS Institute, USA). 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 (G) test, using JMP statistical software version 7.0.1 (SAS Institute, USA). Statistical significance was assumed at $P \leq 0.05$. 
2.12 Reproductive interference between *T. turkestani* and *T. urticae* RF on whole bean plants

I simulated the field interaction 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 one month old potted bean plants (30 cm high, bearing 3 true leaves, two of which were fully expanded). The experiments were conducted for two weeks (one generation for the earliest-born individuals). Females of *T. turkestani* and *T. urticae* RF from the aforementioned strains were isolated as deutonymphs, four days before initiating the experiment, whereas males of each strain were collected from the rearing cages one day prior to initiating the experiment and kept separated from the females to prevent pre-experiment mating. Mixed cultures were initiated with 10 males and 10 virgin females of each species (n=40). Pure cultures, containing one species only, were initiated with 20 virgin females and 20 males (n=40). The potted plants were wrapped with unwoven fleece in order to prevent mites from moving between plants. Plants were kept in a greenhouse under 25 °C (night) - 30 °C (day) and 14:10 h light: dark conditions. Number of female progeny and their body color were recorded from all parts of each plant after two weeks. Female progeny with orange pigmentation were considered interspecific hybrid suspects and were genotyped by ITS2 PCR-RFLP (see chapter 2.9). The number of female progeny per founder female was compared between pure and mixed cultures within each species by ANOVA, using JMP statistical software version 7.0.1 (SAS Institute, USA).
3. Results

Results from this study were published in Ben-David et al., 2007 and Ben-David et al., 2009 (attached).

3.1 Tetranychid species in Israel

In this work I collected and identified, by morphological criteria, 20 species of the family Tetranychidae (Acari: Prostigmata), adding seven species to the list of the Israeli spider mite fauna. These are: *Aplonobia histricina*, *Bryobia graminum* (Schrank), *Eotetranychus hirsti* Pritchard and Baker, *Oligonychus coniferarum* (McGregor), *Tetranychus evansi*, *T. ludeni* and *T. neocaledonicus*. One species, *Oligonychus senegalensis* Gutierrez and Etienne, formerly reported to infest date palms (*Phoenix dactylifera*) in Israel was not found in my collections, perhaps due to its displacement by *O. afrasiaticus* in that habitat (Palevsky et al., 2003). *Bryobia graminum*, *E. hirsti*, *O. coniferarum* and *T. neocaledonicus* were only found in low numbers on *Calicotome villosa*, fig (*Ficus carica*), cypress (*Cupressus sempervirens*) and *Acalipha amentacea*, respectively, and hence they did not undergo DNA extraction.
Figure 1. Spider mite collection sites in Israel. Map colors represent the three main Mediterranean climate regimes: arid (dark gray), semi-arid (light gray) and Mediterranean (white). Numbers on the map correspond to location numbers in Table 3.

List of collection sites:

1. Alonei Habashan
2. Aco
3. Newe Yaar - Tzipori
4. Sha'ar Hagolan
5. Tirat Tzvi
6. Binyamina
7. Avihayil
8. Ra'anana
9. Shfayim - Yakum
10. Rehovot
11. Kfar Adumim
12. Tzova
13. Lachish
14. Migdal Oz
15. Yad Mordechai
16. Sa'ad
17. Gilat
18. Beit Hagadi
19. Kalia
20. Hatzeva
21. Yotvata
Table 3. Spider mite species collected in Israel, their location (numbers as in Figure 1), host plant, ITS2 fragment size (bp), number of sequences per species, species nucleotide diversity index (π) and accession numbers in GenBank.

<table>
<thead>
<tr>
<th>Mite species</th>
<th>Location</th>
<th>Host plant</th>
<th>ITS2 Fragment size (bp)</th>
<th>Nuc Diversity Index (π)</th>
<th>Number of sequences</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aplonobia histricina</em></td>
<td>9</td>
<td><em>O. pes-caprae</em></td>
<td>455</td>
<td>-</td>
<td>1</td>
<td>DQ656450</td>
</tr>
<tr>
<td><em>Bryobia praetiosa</em></td>
<td>10</td>
<td><em>Malva sp.</em></td>
<td>483</td>
<td>-</td>
<td>1</td>
<td>DQ656439</td>
</tr>
<tr>
<td><em>Petrobia harti</em></td>
<td>10</td>
<td><em>Oxalis corniculata</em></td>
<td>368</td>
<td>-</td>
<td>1</td>
<td>DQ656449</td>
</tr>
<tr>
<td><em>Petrobia Tunisiae</em></td>
<td>9, 17</td>
<td><em>Avena sterilis</em></td>
<td>372</td>
<td>-</td>
<td>1</td>
<td>DQ656459</td>
</tr>
<tr>
<td><em>Eutetranychus orientalis</em></td>
<td>21</td>
<td><em>Citrus paradisi</em></td>
<td>538-542</td>
<td>0.00582</td>
<td>9</td>
<td>DQ656470 - DQ656479</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td><em>C. reticulata</em></td>
<td></td>
<td></td>
<td></td>
<td>DQ656454 - DQ656455</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td><em>Prunus dulcis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td><em>Salix sp.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Eutetranychus palmatus</em></td>
<td>11</td>
<td><em>Phoenix canariensis</em></td>
<td>532</td>
<td>0.00088</td>
<td>2</td>
<td>DQ656454 - DQ656455</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td><em>P. loureirii (=roebelini</em>)</td>
<td></td>
<td></td>
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<tr>
<td><em>Oligonychus perseae</em></td>
<td>2, 9</td>
<td><em>Persea americana</em></td>
<td>467</td>
<td>0.00207</td>
<td>3</td>
<td>DQ656456 - DQ656458</td>
</tr>
<tr>
<td><em>Oligonychus afrasiaticus</em></td>
<td>19, 21</td>
<td><em>Phoenix dactylifera</em></td>
<td>517</td>
<td>-</td>
<td>1</td>
<td>DQ656437</td>
</tr>
<tr>
<td><em>Oligonychus mangiferus</em></td>
<td>20</td>
<td><em>Mangifera indica</em></td>
<td>497</td>
<td>0.00121</td>
<td>2</td>
<td>DQ656486</td>
</tr>
</tbody>
</table>

*Sequences of species that are published for the first time; B Subfamily Bryobiinae, all others belong to subfamily Tetranychinae (See table 4)
Table continues in the next page
Table 3. (Continue)

<table>
<thead>
<tr>
<th>Mite species</th>
<th>Location</th>
<th>Host plant</th>
<th>ITS2 Fragment size (bp)</th>
<th>Nuc Diversity Index ($\pi$) DnaSP</th>
<th>Number of sequences</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Panonychus citri</em></td>
<td>9, 15</td>
<td><em>C. sinensis</em></td>
<td>538-539</td>
<td>0.00441</td>
<td>5</td>
<td>DQ656443 - DQ656447</td>
</tr>
<tr>
<td><em>Panonychus ulmi</em></td>
<td>1, 12, 14</td>
<td><em>Malus sylvestris</em></td>
<td>523-524</td>
<td>0.00124</td>
<td>7</td>
<td>DQ656460 - DQ656466</td>
</tr>
<tr>
<td><em>Schizotetranychus asparagi</em></td>
<td>8</td>
<td><em>Asparagus virgatus</em></td>
<td>536</td>
<td>-</td>
<td>1</td>
<td>DQ656438</td>
</tr>
<tr>
<td><em>Tetranychus urticae</em></td>
<td>18</td>
<td><em>Citrullus lanatus</em></td>
<td>473-4</td>
<td>0.00046</td>
<td>3</td>
<td>DQ656440 - DQ656442</td>
</tr>
<tr>
<td>Green and Red forms</td>
<td>13</td>
<td><em>Solanum lycopersicon</em></td>
<td>473-4</td>
<td>0.00046</td>
<td>3</td>
<td>DQ656440 - DQ656442</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td><em>S. nigrum</em></td>
<td>473-4</td>
<td>0.00046</td>
<td>3</td>
<td>DQ656440 - DQ656442</td>
</tr>
<tr>
<td><em>Tetranychus turkestani</em></td>
<td>7</td>
<td><em>Ficus carica</em></td>
<td>474</td>
<td>0.00066</td>
<td>2</td>
<td>DQ656467 - DQ656468</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Malus sylvestris</em></td>
<td>474</td>
<td>0.00066</td>
<td>2</td>
<td>DQ656467 - DQ656468</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td><em>Prunus persica</em></td>
<td>474</td>
<td>0.00066</td>
<td>2</td>
<td>DQ656467 - DQ656468</td>
</tr>
<tr>
<td><em>Tetranychus evansi</em></td>
<td>4</td>
<td><em>Vithania somnifera</em></td>
<td>490</td>
<td>-</td>
<td>1</td>
<td>DQ656448</td>
</tr>
<tr>
<td></td>
<td>10, 21</td>
<td><em>S. nigrum</em></td>
<td>490</td>
<td>-</td>
<td>1</td>
<td>DQ656448</td>
</tr>
<tr>
<td><em>Tetranychus ludeni</em></td>
<td>7</td>
<td><em>Lavatera sp.</em></td>
<td>479</td>
<td>0.00397</td>
<td>3</td>
<td>DQ656451 - DQ656453</td>
</tr>
</tbody>
</table>

*Sequences of species that are published for the first time
3.2 Sequence variation

Forty-three complete ITS2 sequences were PCR amplified, cloned and sequenced from 32 collections, representing 16 spider mite species found in large populations in Israel. GenBank accession numbers for ITS2 sequences are listed in Table 3. Four of the 16 species are new records from Israel (Ben-David et al., 2007). ITS2 sequences of twelve species are reported in this work for the first time (Table 3). The length of the ITS2 sequences ranged from 368–483 bp for species of the sub family Bryobiinae and 474-542 bp for species of the sub family Tetranychinae (Table 3).

Only one ITS2 sequence was isolated from each of the following seven species: A. histricina, B. praetiosa, P. hartii, P. tunisiae, Schizotetranychus asparagi, Oligonychus afrasiaticus and T. evansi. In the other species analyzed, the number of sequences (per species) fluctuated between two and nine (the number of sequences is given in breakets, see Table 3): Eutetranychus palmatus Attiah (2), E. orientalis (9) O. Perseae (3), O. mangiferus (2), P. citri (5), P. ulmi (7), T. ludeni (3), T. turkestani (2), T. urticae R/G F (3). The nucleotide diversity index (\(\pi\)) values ranged between 0.00046 and 0.00587 (Table 3). Within the Tetranychinae, Three species, E. orientalis, P. citri and T. ludeni showed relatively high nucleotide diversity in their ITS2 base composition (\(\pi\) values of 0.00587, 0.00441 and 0.00397, respectively).

Intraspecific genetic distances ranged from 0.002 (seven species) to 0.017 in E. orientalis (Table 4). Within the Tetranychinae, the intra-genus genetic distances in the Tetranychini were ~10-fold higher than the intra-species distance for Tetranychus (0.012–0.093) and Panonychus (0.099–0.105), but ~100-fold higher for Oligonychus (0.347–0.548); the Eurytetranychini contained only one genus, Eutetranychus, with an intra-genus distance ranging between 0.044 and 0.051. This was ~20-fold higher than the intra-species distance observed for E. palmatus but only ~2–3-fold higher than that observed for E.
orientalis (Table 4). In the Bryobiinae, only one genus, Petrobia, contained more than one species. However, since a single ITS2 sequence was obtained from both P. harti and P. tunisiae, I could not compare the intra-species with the intra-genus sequence distance. Nevertheless, the intra-genus distance in Petrobia, ca. 0.20) is intermediate between that of Tetranychus, Panonychus, Eutetranychus (ca 0.05-0.10) and Oligonychus (ca. 0.35-0.55).

Table 4. Genetic distances within species and between species of the same genus based on differences of 43 ITS2 nucleotide sequences (full set). NA, not applicable because the species had only one sequence or the genus had only one species.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Tribe</th>
<th>Genus</th>
<th>Distance within genus</th>
<th>Species</th>
<th>Distance within species</th>
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</thead>
<tbody>
<tr>
<td>Bryobiinae</td>
<td>Bryobiini</td>
<td>Bryobia</td>
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<td>praetiosa</td>
<td>NA</td>
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<tr>
<td></td>
<td>Hystrichonichini</td>
<td>Aplonobia</td>
<td>NA</td>
<td>histricina</td>
<td>NA</td>
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<tr>
<td></td>
<td>Petrobiini</td>
<td>Petrobia</td>
<td>0.213</td>
<td>harti</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>tunisiae</td>
<td>NA</td>
</tr>
<tr>
<td>Tetranichinae</td>
<td>Eurytetranychini</td>
<td>Eutetranychus</td>
<td>0.044 - 0.05</td>
<td>orientalis</td>
<td>0.002 - 0.017</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>palmatus</td>
<td>0.002</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>afrasiaticus</td>
<td>NA</td>
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<tr>
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<td></td>
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<td>mangiferus</td>
<td>0.002</td>
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<td>perseae</td>
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<td>ulmi</td>
<td>0.002 - 0.006</td>
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<tr>
<td>Tetranichinae</td>
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<td>citri</td>
<td>0.002 - 0.009</td>
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<td></td>
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<td>asparagi</td>
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<td></td>
<td></td>
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<td>evansi</td>
<td>NA</td>
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<td>ludenti</td>
<td>0.002 - 0.010</td>
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<td>turkestani</td>
<td>0.002</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>urticae</td>
<td>0.002 – 0.004</td>
</tr>
</tbody>
</table>

*Low divergence, 0.011–0.015, was observed between T. urticae RF and GF and T. turkestani. All other comparisons ranged between 0.058 and 0.093.
Table 5. Pairwise genetic distances between sixteen Tetranychidae species in Israel based on differences of the ITS2 nucleotide sequences. A reduced set of 16 sequences representing the most frequent ITS2 sequence from each species was used.

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>B. praetiosa</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A. histricina</td>
<td>0.477</td>
<td>-</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>P. harti</td>
<td>0.443</td>
<td>0.213</td>
<td>-</td>
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<td>0.432</td>
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<td>-</td>
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<tr>
<td>O. afrasiaticus</td>
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<td>0.477</td>
<td>0.481</td>
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<tr>
<td>T. urticae</td>
<td>0.544</td>
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<td>0.507</td>
<td>0.478</td>
<td>0.21</td>
<td>-</td>
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<tr>
<td>T. turkestani</td>
<td>0.543</td>
<td>0.475</td>
<td>0.489</td>
<td>0.468</td>
<td>0.211</td>
<td>0.011</td>
<td>-</td>
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<tr>
<td>T. ludeni</td>
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<td>0.483</td>
<td>0.223</td>
<td>0.087</td>
<td>0.089</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. evansi</td>
<td>0.497</td>
<td>0.461</td>
<td>0.494</td>
<td>0.467</td>
<td>0.273</td>
<td>0.076</td>
<td>0.076</td>
<td>0.06</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. asparagi</td>
<td>0.535</td>
<td>0.537</td>
<td>0.539</td>
<td>0.512</td>
<td>0.52</td>
<td>0.43</td>
<td>0.433</td>
<td>0.412</td>
<td>0.434</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. persea</td>
<td>0.568</td>
<td>0.564</td>
<td>0.491</td>
<td>0.459</td>
<td>0.513</td>
<td>0.528</td>
<td>0.473</td>
<td>0.46</td>
<td>0.429</td>
<td>0.418</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. mangiferus</td>
<td>0.502</td>
<td>0.438</td>
<td>0.509</td>
<td>0.489</td>
<td>0.466</td>
<td>0.435</td>
<td>0.431</td>
<td>0.440</td>
<td>0.456</td>
<td>0.390</td>
<td>0.350</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. citri</td>
<td>0.538</td>
<td>0.509</td>
<td>0.532</td>
<td>0.463</td>
<td>0.448</td>
<td>0.462</td>
<td>0.452</td>
<td>0.442</td>
<td>0.441</td>
<td>0.366</td>
<td>0.421</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. ulmi</td>
<td>0.492</td>
<td>0.521</td>
<td>0.505</td>
<td>0.526</td>
<td>0.454</td>
<td>0.473</td>
<td>0.457</td>
<td>0.461</td>
<td>0.516</td>
<td>0.389</td>
<td>0.353</td>
<td>0.410</td>
<td>0.094</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. palmatus</td>
<td>0.504</td>
<td>0.508</td>
<td>0.449</td>
<td>0.515</td>
<td>0.487</td>
<td>0.471</td>
<td>0.462</td>
<td>0.443</td>
<td>0.501</td>
<td>0.49</td>
<td>0.491</td>
<td>0.456</td>
<td>0.482</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E. orientalis</td>
<td>0.519</td>
<td>0.498</td>
<td>0.5</td>
<td>0.492</td>
<td>0.485</td>
<td>0.445</td>
<td>0.454</td>
<td>0.454</td>
<td>0.494</td>
<td>0.49</td>
<td>0.452</td>
<td>0.465</td>
<td>0.484</td>
<td>0.038</td>
<td></td>
</tr>
</tbody>
</table>

42
Pairwise nucleotide divergence among the 16 spider mite species was also calculated for the most frequent sequences (one sequence for each species) and the distance matrix is presented in Table 5. In the subfamily Tetranychinae, genetic distances amongst species ranged between 0.011 (T. turkestani and T. urticae RF and GF) to more than 0.5 (between O. perseae and O. afrasiaticus; between O. perseae and T. urticae RF/GF; between P. ulmi and T. evansi) (Table 5). In the Bryobiinae, B. praetiosa is highly divergent from the other bryobiinae species (genetic distances of 0.432-0.477). This is in agreement with the classification by morphological criteria in which Petrobia and Aplonobia belong to the two other tribes of this subfamily, although the distance between them (ca. 0.2) is much smaller (Table 4, 5).

3.3 Phylogeneic analysis

The parsimony analysis of the aligned matrix included 46 sequences. The bootstrap analysis provided very strong support (100%) for the monophyly of all species (Figure 2), suggesting that ITS2 sequences provide sufficient resolution and could serve as barcodes for species identification in the spider mite family.

Overall, the phylogenetic tree agreed well with the family, subfamily and genus subdivisions previously defined by morphological criteria (Bolland et al., 1998). As expected, the four Bryobiinae (B. praetiosa, A. histricina, P. tunisiae, P. harti) were most distantly related to the Tetranychinae. The Bryobiinae were separated into two groups, one comprising the genus Bryobia and the other the genera Aplonobia and Petrobia. Within the Tetranychinae, the grouping of the Tetranychini, Tetranychus, Oligonychus, Schizotetranychus and Panonychus, leaving Eutetranychus as an external sister group, was moderately supported with a bootstrap value of 71%.

Three of the genera in which more than one species was studied (Tetranychus, Panonychus and Eutetranychus) appeared to be monophyletic.
Figure 2. A maximum parsimony tree based on 46 ITS2 sequences. Forty-three of the ITS2 sequences were obtained from the 16 Israeli tetranychid species analyzed in this study. In addition, three acarine ITS2 sequences were obtained from the GenBank (summing to a total of 46 sequences): the ITS2 sequence (GenBank no. AJ297573) from *P. gracilis* was used as an outgroup (marked with **); the ITS2 sequences from *A. quercivoros* and *O. coffeae* (GenBank nos. X99880 and AY750706, respectively) were inserted into the phylogenetic analysis to simulate ITS2 DNA sequences obtained from unidentified samples (marked with *). Numbers adjacent to branches show the bootstrap values (higher than 50%) of 1000 replicates. The tree was not rooted, as I did not want to make any assumptions.
However, this was not the case for *Oligonychus*, because *O. afrasiaticus* did not group with *O. perseae* and *O. mangiferus* but with *Tetranychus*, although external to it (bootstrap values of 100%). In order to strengthen this finding, I also produced an MP tree for four COI sequences obtained in this study: *Tetranychus urticae* RF (DQ656483), *E. orientalis* (DQ656481), *O. perseae* (DQ656485), *O. afrasiaticus* (DQ656482), and additional five COI sequences obtained in previous studies: *O. ununguis* (Jacobi) (X80865), *O. platani* (McGregor) (X80866), *T. turkestani* (AJ316600), *P. harti* (X80870) and *B. kissophila* van Eyndhoven (X80871) (Navajas *et al.*, 1996; Navajas and Boursot, 2003). The COI phylogenetic analysis strongly supported the findings of the ITS2 analyses (Figure 3).

**Figure 3.**
A maximum parsimony tree of nine species of spider-mites, constructed using partial COI gene sequences. *Petrobia harti* and *B. kissophila* were used as outgroup. Numbers adjacent to branches show the bootstrap values (higher than 50%) of 500 replicates. COI sequences of some taxa (marked by *) have been published previously (Navajas *et al.*, 1996; Navajas and Boursot, 2003).

The phylogenetic analysis of two ITS2 sequences used for simulating unidentified specimens placed correctly the ITS2 barcode obtained from *A. quercivorus* as coming
from a species that is an external sister group to the genus *Tetranychus*. The ITS2 barcode from *O. coffeae* was placed within the genus *Oligonychus*, and again correctly indicated that the DNA is coming from a species that is relatively close to *O. mangiferus* (Figure 2).

3.4 The genetic variability of *Eutetranychus orientalis*

In *E. orientalis*, the number of sequences (alleles), the intraspecific genetic distances and the magnitude of $\pi$ values were standing out as the highest amongst all species. A unique characteristic of *E. orientalis* was the large number (15) of polymorphic sites within the 540 bp fragment, including two insertions-deletions of 2 nucleotides each (Figure 4). This phenomenon was not found in any of the other species analyzed in this study, although some of them were also variable and had similar magnitude of $\pi$ values (Table 1). Data of genetic characteristics of nine *E. orientalis* ITS2 sequences from seven sub-populations are given in Table 6.

Table 6. Frequencies of *Eutetranychus orientalis* ITS2 sequences and their distribution within localities.

<table>
<thead>
<tr>
<th>NCBI Accession No.</th>
<th>Times repeated</th>
<th>Frequency %</th>
<th>Number of repeats in locations (Location No. refer to Figure 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C (2)</td>
</tr>
<tr>
<td>DQ656470</td>
<td>2</td>
<td>3.03</td>
<td>1</td>
</tr>
<tr>
<td>DQ656471</td>
<td>16</td>
<td>24.24</td>
<td>2</td>
</tr>
<tr>
<td>DQ656472</td>
<td>2</td>
<td>3.03</td>
<td>1</td>
</tr>
<tr>
<td>DQ656473</td>
<td>7</td>
<td>10.61</td>
<td>1</td>
</tr>
<tr>
<td>DQ656474</td>
<td>21</td>
<td>31.82</td>
<td>4</td>
</tr>
<tr>
<td>DQ656476</td>
<td>4</td>
<td>6.06</td>
<td>1</td>
</tr>
<tr>
<td>DQ656477</td>
<td>5</td>
<td>7.58</td>
<td>1</td>
</tr>
<tr>
<td>DQ656478</td>
<td>3</td>
<td>4.55</td>
<td>1</td>
</tr>
<tr>
<td>DQ656479</td>
<td>6</td>
<td>9.09</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>66</strong></td>
<td><strong>100.00</strong></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Multiple alignment of nine ITS2 sequences isolated from individual *E. orientalis* mites. Dashes stand for identical nucleotide as the top sequence. Deletions are marked with dots. On the left, the last 3 numbers of the Accession No. DQ656470-479. On the right, position number in the sequence.
Figure 5. A maximum parsimony tree of nine ITS2 sequences of *E. orientalis* (Accession No. DQ656470-479). *Eutetranychus palmatus* was used as outgroup. Bootstrap values next to nodes represent the percentage of 1000 replicate trees that preserved the corresponding clade. Colored letters under Acc. No. represent the locations from which the above sequence was isolated (See Table 6).
Of the above sequences of *E. orientalis* from Israel, DQ656474 was the most frequent (ca. 32% in the meta-population). This sequence was isolated from individual specimens from all seven collections. An additional closely related sequence, DQ656471, had a frequency of 24% and was isolated from six of the seven localities (Figure 5, Table 6). Heterozygote females were found in all locations (Table 7).

The presence of sequences DQ656474 and DQ656471 in nearly all locations and the lack of clear association between ITS2 sequences and host plants ecosystem and climatic region (Table 2) suggest that all analyzed specimens belong to one meta-population of *E. orientalis*. Moreover, genotype freq in the meta-population did not differ significantly from those expected with Hardy-Weinberg equilibrium (HWE) at the observed frequencies (G=31.38, P = 0.923). This suggests that no significant reduction or increase in heterozygocity (and gene flow) is present in the meta-population and that no specific sequences are associated with the heterozygous genotypes. Although the high variability in ITS2 sequences suggested the possible existence of mixtures of closely related species, similar to the situation reported by Hebert *et al.* (2004), my results do not support this hypothesis.
Table 7. The individual genotypes (presented by the last digit of their accession numbers) and the proportion of heterozygote *E. orientalis* females in the different locations, according to ITS2 base composition.

<table>
<thead>
<tr>
<th>Location Symbol</th>
<th>Individual genotypes DQ65647-</th>
<th>Proportion of Heterozygote females</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (2)</td>
<td>2x 1/4, 2x 4/9, 3/8</td>
<td>5/5</td>
</tr>
<tr>
<td>N (3)</td>
<td>2x 1/1, 2x 4/4, 0/4</td>
<td>1/5</td>
</tr>
<tr>
<td>T (5)</td>
<td>1/7, 2/4, 3/9, 4/4</td>
<td>3/4</td>
</tr>
<tr>
<td>A (7)</td>
<td>2x 1/1, 2x 1/4, 0/9</td>
<td>3/5</td>
</tr>
<tr>
<td>Z (10)</td>
<td>1/4, 4/7, 6/7, 9/9</td>
<td>3/4</td>
</tr>
<tr>
<td>S (16)</td>
<td>2x 3/1, 3/2, 3/4, 4/6</td>
<td>5/5</td>
</tr>
<tr>
<td>Y (21)</td>
<td>3/6, 4/6, 4/7, 4/8, 7/8</td>
<td>5/5</td>
</tr>
</tbody>
</table>

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

*Tetranychus turcestani* was collected mainly from deciduous trees, including apple, peach, almond, fig, and from weeds in orchards (Table 8). *Tetranychus 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 (Figure 6).
Table 8. *Tetranychus urticae* RF and *T. turkestani* collection data (2005-2007), with reference to host plants, agro-ecosystems and geographic location (Numbers refer to Figure 6).

<table>
<thead>
<tr>
<th>Mite species</th>
<th>No. of samples</th>
<th>Host plant</th>
<th>Agro-ecosystem</th>
<th>Location (see Figure 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tetranychus urticae RF</strong></td>
<td>1</td>
<td><em>Capsicum annum</em></td>
<td>Greenhouse</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Chrysanthemum indicum</em></td>
<td>Greenhouse</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td><em>Citrullus lanatus</em></td>
<td>Open-field</td>
<td>3, 5, 19, 21, 21</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Convolvulus sp.</em></td>
<td>Open-field</td>
<td>12, 17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Gossypium hirsutum</em></td>
<td>Open-field</td>
<td>9, 12</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Prunus persica</em></td>
<td>Orchard screen house</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Pyrus communis</em></td>
<td>Sprayed orchard</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Ricinus communis</em></td>
<td>Open-field</td>
<td>8, 13</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td><em>Solanum melongena</em></td>
<td>Greenhouse, Open-field</td>
<td>6, 16, 18</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Solanum lycopersicon</em></td>
<td>Greenhouse</td>
<td>18, 19</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td><em>Solanum nigrum</em></td>
<td>Weed-rural</td>
<td>6, 10, 12, 14, 23</td>
</tr>
<tr>
<td><strong>Tetranychus urticae GF</strong></td>
<td>1</td>
<td><em>Citrulus lanatus</em></td>
<td>Open-field</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Phormium sp.</em></td>
<td>Greenhouse</td>
<td>15</td>
</tr>
<tr>
<td><strong>Tetranychus turkestani</strong></td>
<td>1</td>
<td><em>Gladiolus sp.</em></td>
<td>Greenhouse</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td><em>Ficus carica</em></td>
<td>Orchard</td>
<td>8, 13, 16, 17, 20</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td><em>Malus domestica</em></td>
<td>Orchard</td>
<td>1, 1, 2, 3, 5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Malva sp.</em></td>
<td>Weed in <em>C. lanatus</em> field</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>Weed in <em>M. domestica</em> orchard</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Musa acuminata</em></td>
<td>Orchard screen house</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Prunus dulcis</em></td>
<td>Orchard</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td><em>Prunus persica</em></td>
<td>Orchard</td>
<td>3, 12, 17</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Tribulus terrestris</em></td>
<td>In <em>P. persica</em> orchard</td>
<td>12</td>
</tr>
<tr>
<td><strong>T. turkestani and T. urticae RF</strong></td>
<td>2</td>
<td><em>Citrullus lanatus</em></td>
<td>Open-field</td>
<td>3, 5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td><em>Prunus persica</em></td>
<td>Orchard screen house</td>
<td>12</td>
</tr>
</tbody>
</table>
List of collection sites in Figure 6:

1. Alonei Habashan
2. Elrom
3. Yesod Ha'ma'ala
4. Sha'ar Hagolan
5. Eli'ad
6. Tel Katzir
7. Beit Yosef
8. Yas'oor
9. Afek
10. N.Y. - Tzipori
11. Habonim
12. Binyamina
13. Avihayil
14. Tzur Moshe
15. Gan Hayim
16. Givat Hashlosha – Nehalim
17. Na'an
18. Kedma - Lachish
19. Netiv Ha'asara
20. Kfar Mymon
21. Beit Hagadi
22. Ramat Negev
23. Kalia
24. Hatzeva

Figure 6. Tetranychus urticae (RF and GF) and Tetranychus turkestani collection sites in Israel. Colors for climatic regions as in Figure 1. Numbers on the map correspond to location numbers in the list on the right and in Table 8.
3.6 Host preference of individual *T. turkestani* and *T. urticae* RF females

In the laboratory choice assays, solitary gravid females (n = 48-74, Table 9) 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* have overlapping niches in which reproductive interactions are likely to occur.

### Table 9. Host preferences of individual *T. turkestani* and *T. urticae* RF females on three combinations of host plant leaf discs: watermelon/cotton, watermelon/apple and watermelon/peach. The total numbers of eggs laid in each treatment is given in parentheses.

<table>
<thead>
<tr>
<th>Species analyzed</th>
<th>Hosts</th>
<th>No. Individuals on watermelon (eggs)</th>
<th>No. Individuals on other host (eggs)</th>
<th>Proportion females on watermelon (<em>P)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. turkestani</em></td>
<td>Watermelon/Cotton</td>
<td>42 (282)</td>
<td>4 (2)</td>
<td>0.913 (&lt;0.0001)</td>
</tr>
<tr>
<td></td>
<td>Watermelon/Apple</td>
<td>28 (48)</td>
<td>11 (6)</td>
<td>0.718 (0.0056)</td>
</tr>
<tr>
<td></td>
<td>Watermelon/Peach</td>
<td>31 (170)</td>
<td>11 (60)</td>
<td>0.738 (0.016)</td>
</tr>
<tr>
<td><em>T. urticae RF</em></td>
<td>Watermelon/Cotton</td>
<td>34 (131)</td>
<td>4 (2)</td>
<td>0.895 (&lt;0.0001)</td>
</tr>
<tr>
<td></td>
<td>Watermelon/Apple</td>
<td>43 (179)</td>
<td>5 (11)</td>
<td>0.896 (&lt;0.0001)</td>
</tr>
</tbody>
</table>

* Probability of G-test for 0.5:0.5 ratio extrinsic hypothesis
3.7 Mating behavior in interspecific and intraspecific crosses of *T. turkestani* and *T. urticae* RF

The proportion of males showing courtship behavior did not differ significantly between the four crossing combinations ($G = 3.085$, $df = 3$, $P = 0.3788$), and was high: 0.85 to 0.96, indicating high male affinity to virgin conspecific as well as heterospecific females (Table 10, column 3). Of the pairs showing male courtship, 0.85 to 0.88 ($G = 0.198$, $df = 3$, $P = 0.978$) achieved copulation (Table 10, column 4). Nevertheless, the number of copulations was significantly higher and their duration significantly shorter in *T. turkestani* when interspecific encounters took place, as compared to intraspecific encounters (Table 10, columns 9, 10). A similar phenomenon was not observed in *T. urticae* RF intra- and interspecific crosses. The time to first contact and the time to first copulation did not differ significantly amongst the four crossing combinations (Table 10, columns 6, 8).
Table 10. Mating behavior parameters of *Tetranychus turkestani* and *T. urticae* RF during 20 minutes' observations of non-choice intra- and interspecific crosses. Proportions were compared amongst treatments by log-likelihood ratio (G) test. Other parameters were compared by one-way ANOVA. Values within each column followed by different letters are significantly different ($P \leq 0.05$).

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

N indicates number of replicates.
3.8 Crossing experiments between *T. turkestani* and *T. urticae* RF

A summary of the reciprocal interspecific crosses as well as the control intraspecific crosses is given in Table 11. In interspecific crosses, where the female was *T. turkestani* (*T. turkestani* ♀ X *T. urticae* RF ♂), one out of 11 families produced one hybrid F₁ female offspring. In the reciprocal interspecific crosses (*T. urticae* RF ♀ X *T. turkestani* ♂), three out of 16 families produced a total of 18 hybrid F₁ 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 F₁ females came from one family and laid 4-25 eggs. Using PCR-RFLP, these F₁ females proved to be hybrids of the *T. urticae* RF X *T. turkestani* cross, carrying ITS2 sequences of both species (Figure 7). However, none of the F₂ eggs (n=84) that were oviposited by these F₁ females hatched, suggesting a post-zygotic reproductive barrier between the two species. Within species, comparisons between intraspecific and interspecific crosses showed no significant differences in the hatchability rate, in progeny survival rate or in the female progeny ratio (0.2036 ≤ P ≤ 0.8303). PCR screening for bacteria did not detect the presence of *Rickettsia*, *Wolbachia*, *Cardinium* or any other bacteria in my *T. turkestani* and *T. urticae* RF laboratory strains, suggesting the presence of other post-zygotic mechanisms of reproductive isolation between the two species.
Table 11. Number of F₁ eggs obtained, their hatchability, progeny survival to adulthood and female progeny ratio, in inter- and intraspecific crossing experiments of *Tetranychus turkestani* and *T. urticae* RF.

<table>
<thead>
<tr>
<th>Cross</th>
<th>N*</th>
<th>Sex of progeny</th>
<th>Number of eggs obtained</th>
<th>Hatchability ± S.E.</th>
<th>Survival to adulthood ± S.E.</th>
<th>♀♀ Progeny Ratio ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. turkestani</em> ♀ X <em>T. turkestani</em> ♂</td>
<td>9</td>
<td>♀♂</td>
<td>264</td>
<td>0.82 ± 0.05</td>
<td>0.69 ± 0.09</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td><em>T. urticae</em> RF ♀ X <em>T. urticae</em> RF ♂</td>
<td>10</td>
<td>♀♂</td>
<td>422</td>
<td>0.71 ± 0.05</td>
<td>0.72 ± 0.07</td>
<td>0.64 ± 0.05</td>
</tr>
<tr>
<td><em>T. turkestani</em> ♀ X <em>T. urticae</em> RF ♂</td>
<td>10</td>
<td>♂</td>
<td>245</td>
<td>0.66 ± 0.10</td>
<td>0.59 ± 0.06</td>
<td>NA</td>
</tr>
<tr>
<td><em>T. urticae</em> RF ♀ X <em>T. turkestani</em> ♂</td>
<td>13</td>
<td>♂</td>
<td>335</td>
<td>0.74 ± 0.07</td>
<td>0.72 ± 0.07</td>
<td>NA</td>
</tr>
<tr>
<td><em>T. urticae</em> RF ♀ X <em>T. turkestani</em> ♂</td>
<td>3</td>
<td>♀♂</td>
<td>82</td>
<td>0.74 ± 0.14</td>
<td>0.76 ± 0.10</td>
<td>0.45 ± 0.16</td>
</tr>
</tbody>
</table>

* N* - Number of families

NA – Non Applicable. Progeny ratios were calculated only for interspecific families that produced females.
Figure 7. Gel presentation of ITS2 PCR-RFLP analysis of F₁ female progeny produced by crossing *Tetranychus urticae* RF ♀ X *Tetranychus turkestani* ♂. The restriction enzyme *HpaI* 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– 1kb ladder; lanes 2-5 – ITS2 PCR-RFLP analyses of two *T. urticae* RF females: lanes 2 and 4 - uncut fragment, lanes 3 and 5 – ITS2 fragments after digestion with *HpaI*; lanes 6-7 – ITS2 PCR-RFLP analyses of one *T. turkestani* female: lane 6 - uncut fragment, lane 7 – ITS2 fragment after digestion with *HpaI*; lanes 8-11 – ITS2 PCR-RFLP analyses of three hybrid F₁ females: lane 10 - uncut fragment, lanes 8, 9 and 11 – ITS2 fragments after digestion with *HpaI*. 
3.9 Reproductive interference between *T. turkestani* and *T. urticae* RF on bean leaf discs

In order to study putative 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. In experiments 2.8 and 2.9 I used the pigmentation of next generation's females as the proof of their parental origin. The F₁ hybrid females occurred in the cross-experiments were different in color when all mites were cultured on green bean leaves. The hybrids were light orange vs. the reddish-brown *T. urticae* RF and the greenish-yellow *T. turkestani* females. Their mothers were all red. In the leaf discs mixed cultures, the maternal identity of F₁ females' was known, because mothers were separated after the first day of the mixing, and each had its own culture. Genotyping was not done as there were no hybrids suspects (orange females) among progeny. In the mixed treatment of the "whole been plant experiment" the parental identity of female offspring was unknown. Using the above criteria to distinguish between normal and hybrid females, I found 5 females suspected to be interspecific hybrids, in the mixed treatment. These were genotyped by ITS2 PCR-RFLP (See chapter 2.9 and 3.8). No differences were found in oviposition rates between the two pure cultures or between the pure and mixed cultures within each species (0.3167 ≤ P ≤ 0.8; Figure 8A), suggesting that oviposition rate is an intrinsic parameter, unaffected by mating. The proportion of progeny surviving to adulthood was significantly higher in the *T. urticae* RF pure cultures than in the *T. turkestani* pure cultures (Figure 8B; χ² two-sample test = 3.6441, df = 1, P = 0.056), but there were no significant differences in this parameter between the pure and mixed cultures within each species (Figure 8B; P = 0.772 and P = 0.781, 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
significant reduction in this parameter was observed when pure and mixed cultures of *T. urticae* RF were compared (Figure 8C; \( \chi^2 \) two-sample test = 3.7719; df = 1, \( P = 0.054 \)). The same phenomenon was not seen in *T. turkestani* (Figure 8C; \( P = 0.81 \)). 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; \( P = 0.023 \)). Fertilization rates of *T. turkestani* were similar in mixed and pure cultures (13/16 and 15/17, respectively; \( G = 0.314 \), df = 1, \( P = 0.575 \)). 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*. 
Figure 8. 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* ♀♀♀ X *T. turkestani* ♂♂ (n = 17); *T. urticae* RF ♀♀♀ X *T. urticae* RF♀♀ (n = 15); Mixed - *T. turkestani* ♀♂ X *T. urticae* RF ♀♀♀ (n = 16 and 18 for *T. turkestani* ♀♀♀ and *T. urticae* RF ♀♀♀ respectively). Error bars represent standard error of the means. *P* values indicate nearly significant differences, according to the Wilcoxon non-parametric test.
3. 10 Reproductive interference of *T. 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* (Figure 9). Each founding female of *T. urticae* RF had a mean of 34.9 (±S.E = 2.31) daughters (ca. 700 per plant), whereas each founding female of *T. turkestani* had only 8.1 (±S.E = 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 (reddish-brown in body color) was highly significant (21.5 ±S.E = 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). No hybrid suspects were found. Again, these data indicate 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*. 
Figure 9.
The mean number (+S.E.) of female progeny produced by individual *Tetranychus urticae* RF and *Tetranychus turkestani* females during a two-week period (one generation) on bean plants.

For each species, solid bars represent pure cultures of twenty 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* ≤ 0.05) between the two culture types within the same species.
4. Discussion

Species identification is the basis for understanding species diversity, phylogenetic patterns, and evolutionary processes. In pest species, accurate identification is of paramount importance for quarantine and management purposes: the development of biological and other control strategies. DNA sequences are being used as a tool for delineating and identifying species. In DNA barcoding a short section of DNA sequence, is used to identify species. DNA barcoding can be used to identify and assign unknown specimens to species that have been previously described (Hebert et al., 2003). DNA taxonomy concerns the circumscription and delineation of species using evolutionary species concepts and can serve as a database for DNA barcoding (Ros and Breeuwer, 2007).

In my PhD thesis I show that ribosomal DNA – ITS2 sequences are suitable for the above purposes and, in addition, can serve as a tool for identifying and pointing out important ecological aspects of the family Tetranychidae.

4.1 Spider mites in Israel

Updated information on the spider mite species present in Israel is important for pest management and biodiversity conservation objectives. In this study I collected and identified by morphological characters, 20 species of the family Tetranychidae (Acari: Prostigmata), adding seven species to the list of the Israeli spider mite fauna. One species, *Oligonychus senegalensis*, formerly reported to infest date palms (*Phoenix dactylifera*) was not found, perhaps due to its displacement by *O. afrasiaticus* in that habitat (Palevsky et al., 2003). Another newly recorded species, *Aplonobia histricina*, is specific to a wild and invasive host plant, *Oxalis pes-caprae*. *Bryobia graminum* was found on *Calicotome villosa* but it can also damage agricultural crops (Jeppson et al., 1975). The other species
that I added to the list are well known pests in other countries. For example, *E. hirstii* is a pest of *Ficus carica* (fig) in India and Iran; *O. coniferarum* of conifers in the USA; *T. evansi* of *Solanum lycopersicon* in Africa; *T. ludeni* of *Phaseolos vulgaris* in Australia and *T. neocaledonicus* of *Gossypium hirsutum* and *S. melongena* in India as well as other hosts and countries like Hawai, Brazil and Malagash (Jeppson et al., 1975; Meyer, 1987). Although these species are considered important agricultural pests elsewhere, they have not yet caused economic damage in Israel and should be regarded as potential pests. If they become fully established, changes in ecological factors such as climate or abundance of host plants, or implementation of new cropping technologies such as protected environment, or the use of new pesticides, can result in outbreaks and economic losses. For example, distinguishing the four red colored *Tetranychus* species present in Israel is essential because pest management tactics to control *T. evansi* differ substantially from those taken to control *T. urticae* RF, due to different susceptibilities to miticides and to the specificity of biological control agents (Escudero and Ferragut, 2005).

### 4.2 ITS2-based barcoding of spider mite species

Herein I present the ITS2 sequences of 16 species of spider mites found in large populations in agriculture and rural ecosystems throughout Israel. ITS2 sequences of twelve species were added by me to the GenBank and to the literature (Table 3), a substantial contribution to the international molecular databases that had contained ITS2 sequences of fifteen spider mite species prior to my work (Table 1). Because these sequences were found to be species-specific (Knapp et al., 2003; Navajas and Boursot, 2003; Navajas et al., 2001; Osakabe et al., 2002; Ben-David et al., 2007), they may serve as barcodes for species identification in the spider mite family. The accumulation of such data internationally can provide the platform for a uniform and accurate method of
tetranychid species identification. It will also enhance the characterization of new invading or existing spider mite species in Israel. As the current sequence diversity is well documented, new unrecognized species can be spotted easily from now on, regardless of the life stages intercepted and the availability of an expert taxonomist. For example, my ITS2 analyses facilitated the identification of *T. evansi*, *T. ludeni* and *T. neocaledonicus*, previously unknown to be present in Israel. In addition, a restriction fragment length polymorphism (RFLP) approach, using different restriction enzymes recognition sites in the DNA amplified fragment (Figure 7), can facilitate a sophisticated, quicker and cheaper method of distinguishing between sampled mites than the sequencing procedure. This was already done with four *Tetranychus* species in Japan (Osakabe *et al.*, 2002) and for *T. urticae* and *T. turkestanii* in France (Navajas and Boursot, 2003) with a different restriction enzyme.

Sequence divergences were very low within species. Using a 0.02 (2%) threshold for species diagnosis in my data set, 14 out of 16 species recognized by morphological criteria would be accurately identified. The only exceptions involved the low divergence, 0.011–0.015 (1.1–1.5%), between *T. urticae* and *T. turkestanii* (Table 4). It appears from my data that the speciation of *T. urticae* and *T. turkestanii* is recent enough that significant divergence in their ITS2 sequences has not yet occurred. Still, these species have fixed alternative ITS2 sequences which, despite the intraspecific polymorphism detected, are monophyletic in each of the two species, with five diagnostic nucleotide substitutions separating them. Although *T. urticae* and *T. turkestanii* were not monophyletic in the alignment of COI sequences (Ros and Breeuwer, 2007), they are two separate biological species as attempts to cross *T. turkestanii* and *T. urticae* (RF in this research) did not produce female offspring, although mating occurred (Ben-David *et al.*, 2009; Migeon and Navajas unpublished data; reported without accompanying data in Navajas and Boursot,
Therefore I conclude that there may be circumstances where DNA barcoding might fail to distinguish between sibling species. However, I expect that such cases will be rare (Barrett and Hebert, 2005) and recommend that when ITS2 nucleotide divergence of 1% to 2% from a known species is found, identification will be treated carefully and confirmation by morphological criteria and crossing experiment should be performed.

4.3 ITS2-based phylogeny of spider mites

The ITS2 sequences of the Tetranychidae may serve as molecular markers for phylogenetic studies at the family level. Sequence divergences were generally much greater between species than within them (Tables 2 and 4). *Bryobia praetiosa* was the most distant species in the ITS2 based phylogenetic tree. The genus *Bryobia* is traditionally considered to be the most primitive in the family, as compared to the genus *Tetranychus* (Lindquist, 1985). The phylogenetic relationships found by using the Maximum Parsimony method (Figure 2 and Table 4) are in good agreement with the family, subfamilies, tribes and genera subdivisions as defined by morphological characters (Bolland et al., 1998). This is at odds with the generally accepted claim that ITS2 sequences are appropriate only for studying low-level phylogenetic relationships, such as the separation of closely related species, complexes of species and populations (Gotoh et al., 1998; Porter and Collins, 1991).

Overall, the present data provide good support for the monophyly of the sub families Tetranychinae and Bryobiinae and for that of their genera, with only two areas of incongruence. These are i) the large genetic distance between the two *Petrobia* species (Table 5), and ii) the polyphyletic nature of the genus *Oligonychus* (Figure 2).

i. The ITS2 sequence analysis revealed a genetic distance of 0.21 between the congeneric species *Petrobia harti* and *P. tunisiae* (sub family Bryobiinae; tribe Petrobiini). It also
showed that *A. histricina*, which belongs to tribe Hystrichonichini, is similarly close to both *Petrobia* species (0.21 – 0.22). These findings need further discussion because the genetic distances within other genera, e.g. *Eutetranychus, Panonychus* and *Tetranychus* (leaving out *T. urticae* and *T. turkestani* to a detailed discussion below) were 0.038, 0.094 and 0.06-0.089, respectively (Table 5), much lower than between *P. harti* and *P. tunisiae*. One possible explanation is the improper assignment of one or both of the *Petrobia* species to this genus. The taxonomic status of both species is controversial. *Petrobia harti* was assigned to *Tetranychina* by Banks (Banks, 1917), but placed in *Petrobia* by Pritchard and Baker (1955) and retained there by Tuttle and Baker (1968). Later, Meyer (1987) as well as Tuttle *et al.* (1976) returned the species to *Tetranychina*. Currently *P. harti* is assigned to *Petrobia*, sub-genus *Tetranychina* (Bolland *et al.*, 1998). *Petrobia tunisiae* has been retained in that genus, but in the sub-genus *Mesotetranychus*. Bolland *et al.* (1998) listed 13 species that were previously placed in *Tetranychina* but are now considered members of *Petrobia*. The sub family Bryobiinae and its Petrobiini tribe are under-represented in the international molecular database. The genus *Petrobia* included one *P. harti* ITS2 sequence (that have not been published as part of a journal paper) and one *P. harti* COI sequence (Navajas, *et al.* 1996) prior to Ben-David *et al.* (2007). The additional data from one more *Petrobia* species from Israel is not sufficient for the analysis of the genus and additional ITS2 data of *Petrobia* species are needed in order to elucidate the taxonomic complexity of this genus.

ii. The monophyly of *Oligonychus* was rejected by ITS2 and COI sequence analyses because *O. afrasiaticus* grouped near the *Tetranychus* species, although external to their branch, and not with its congener species *O. perseae* and *O. mangiferus* (Figure 2). Polyphyly in *Oligonychus* was already reported by Navajas *et al.* (1996), after analyzing the COI sequences of *O. ununguis, O. platani* and *O. gossypii*. The former two were
monophyletic and closer to *Panonychus* species, whereas *O. gossypii* (Zacher) grouped with *Tetranychus*. Using the morphological criteria of the females, especially the placing and proximity of the two pairs of duplex setae on tarsus I, and the shape of the empodia (clawlike, split into proximoventral hairs in *Oligonychus* versus absence of claw but presence of proximoventral hairs in *Tetranychus*), *O. afrasiaticus* belongs to the genus *Oligonychus*. On the other hand, the shape of the aedeagus of *O. afrasiaticus* resembles that of members of the genus *Tetranychus*: it is duck-like, with a dorsally directed head that is rounded anteriorly and pointed posteriorly (the aedeagus of *O. gossypii* is also dorsally directed). Males of the other two species of *Oligonychus*, *O. perseae* and *O. mangiferus*, have their aedeagi directed ventrally and lack a knob, like the aedeagi of *O. ununguis* and *O. platani*. In fact, the genus *Oligonychus* was divided according to the shape of the aedeagus and other characters by more than one author in the past, but the suggested criteria were found to be unstable and the concept of sub-genera/new genera was rejected (Meyer, 1987; Bolland *et al.*, 1998). Although COI and ITS2 sequencing data suggest the division of the *Oligonychus* genus into sub-genera, additional DNA sequences from other *Oligonychus* species are required before a final decision on the validity of the sub-genera concept can be drawn. The COI data (Navajas *et al.*, 1996; Figure 3 in this study) along with ITS2 data (Ben-David *et al.*, 2007) support close relationships between some *Oligonychus* and *Tetranychus* species, questioning the significance of the shape of the empodia and tarsus I duplex setae proximity as major characters for separating these genera.

### 4.4 Genetic variation in *Tetranychus urticae* Koch

The ITS2 sequences obtained from populations of *T. urticae* RF and GF in this study allowed me to contribute new data to the ongoing debate regarding the homogeneity of this
species. It has long been considered as a complex of species because of differences in morphology, pigmentation (red and green), response to photoperiod and temperature, and cross-breeding experiments (e.g. Dupont, 1979; Gotoh, 1986; Gotoh and Tokioka, 1996; Sugasawa et al., 2002; Zhang and Jacobson, 2000). The lack of polymorphism in ITS2 sequences in different populations of *T. urticae* RF/GF collected from several host plants in an extensive geographical area suggested species-wide homogenization and a conspecific status of the red- and green-forms (Navajas, 1998; Navajas et al., 1998). Analysis of mtDNA variations showed as much as 6% divergence for the same mites, with 26 polymorphic positions in the 390bp fragment (whereas interspecific distances of 6.2-11.5% were found between COI sequences of *T. urticae* RF/GF and four other tetranychid species). Nevertheless, no correlations were found between mtDNA divergence and geographic distribution, host plant or physiological characteristics, such as the red or green body color of the summer females (Navajas, 1998; Navajas et al., 1998). There are, however, reports that genetic divergence in *T. urticae* might be caused by host plant effects. Studies of allozyme polymorphism in *T. urticae* populations from open field and greenhouses in continental Greece showed that open field specimens from lemon trees were genetically more similar to other 'lemon' samples collected in different localities than to mites collected in the same locality on other plant species (Tsagkarakou et al., 1998). A different ITS2 sequence was found in populations collected from *Nerium oleander* in the western Mediterranean region, a polymorphism attributed to a reproductive barrier between GF mites carrying the regular ITS2 sequence of *T. urticae* and the *N. oleander* strain (Navajas et al., 2000). However, both reports were treated by their authors as exceptions. They concluded that if host specialization exists in *T. urticae*, it has to be weak, fairly recent and unstable, and associated with the colonization of “marginal plants unsuitable for arthropods” (Navajas et al., 2000; Tsagkarakou et al., 1998). In my study,
three different ITS2 sequences were obtained from *T. urticae*. One sequence (Accession No. DQ656440) was shared between the red and green forms feeding on watermelon and solanaceous weeds, and the other two (Acc. No. DQ656441–DQ656442) were found only in *T. urticae* RF. The sequences differ from each other only by one polymorphic site each and are also different by one additional nucleotide from the previously reported ITS2 sequences of *T. urticae* RF/GF: X99881 (Navajas, 1998; Navajas *et al.*, 1998) and AB076369 (Osakabe *et al.*, 2002). A few heterozygote *T. urticae* RF females (DQ656440/DQ656441 and DQ656440/DQ656442 see Table 1) were found in my collections, indicating that there is gene flow between individuals carrying different sequences and that polymorphism within *T. urticae* RF populations exists. Perhaps such polymorphism was overlooked in the early works on *T. urticae* RF/GF ITS2 due to sample size. For example, Navajas *et al.* (1998) sequenced only 1-3 females from each of the 19 sites in their research. Perhaps no ITS2 variants were isolated in my *T. urticae* GF because I sequenced the ITS2 fragment of 5 females from one location only (due to its scarceness in Israel, see Table 8) that could have all been progenies of one founder female. In light of the data shown here, the suggestion of species-wide homogenization of the ITS2 in *T. urticae* by Navajas *et al.* (1998) is therefore not applicable in our part of the species' world-wide distribution.

The ITS2 sequences produced in my research also provide conclusive evidence for the synonymy of *T. urticae* RF and *T. cinnabarinus* that was not accepted by few acarologists (e.g. Zhang, 2003). The summer female body color and the diapausing ability of *T. urticae* GF differ greatly from those of *T. urticae* RF. On the other hand, DNA markers show that these are all populations of the same species. Moreover, crosses between red-form females and green-form males I performed during preliminary experiments, produce fertile progeny and normal F₂ generation was obtained (data not
shown). I therefore conclude that as long as there is no complete reproductive barrier between the red and the green forms of *T. urticae*, they should be considered as one biological species.

4.5 Genetic variation in other tetranychid species

A large number of ITS2 variants were isolated from populations of three tetranychid Israeli species, *E. orientalis*, *Panonychus ulmi* and *P. citri* (twelve, seven and five ITS2 sequences, respectively). In contrast, eight species had only one ITS2 sequence (Table 3). Previously it was suggested that reduced polymorphism can result from recent colonization and/or utilization of many host species (polyphagy), which facilitates extensive gene flow between populations living on different host plants, thus conserving the unity of the species (Navajas, 1998; Navajas *et al.*, 1998). Of these ecological factors, polyphagy was considered to be dominant. Samples of *T. urticae* RF/GF (which is highly polyphagous) off several host plants obtained at 18 different locations from 12 different countries of the northern hemisphere, showed complete homogeneity of ITS2 sequences, although some were collected in the Mediterranean region, where colonization of *T. urticae* is considered to be very old (Navajas, 1998; Navajas *et al.*, 1998). In contrast, ITS2 sequence polymorphism was detected in four different geographic strains of *Mononychellus progresivus* Doreste, a monophagous tetranychid that feeds only on cassava plants (Navajas *et al.*, 1994). My results do not support the hypothesized close association between ecological factors, such as time of colonization and feeding habits, and the genetic structuring of tetranychid populations. According to Bolland *et al.* (1998), *E. orientalis*, *P. ulmi*, and *P. citri* that showed the highest numbers of ITS2 variants, are polyphagous species. Each of these species infests more than five host plant species, assignable to more than a single botanical family. Moreover, both *P. ulmi* and *P. citri* are
considered recent invaders into Israel (Plaut, 1963; Swirski et al., 1986; Swirski et al., 2002). It is therefore likely that other biotic factors, such as the species' intrinsic potential for genetic plasticity, their relative fitness in different ecological and agro-ecological communities, population dynamics and life history traits (e.g. fecundity, number of annual generations, silk production) or abiotic factors (e.g. climate or human activities) may play a significant role in shaping the genetic structure of spider mites populations.

To conclude this part of my work, it is clear that the DNA-based approach for species identification will not lead to the displacement of morphological taxonomy. The validity of DNA-based species identification systems depends on establishing reference sequences from morpho-taxonomically confirmed specimens, a process requiring the cooperation of a diverse group of scientists and institutions (Barrett and Hebert, 2005). However, more work should be carried out with pestiferous tetranychid species, especially quarantine and invasive species, in order to isolate sufficient barcodes needed for efficient identification of economically important threats to crop production around the world.

Two of the above phenomena regarding the ITS2 base composition of tetranychid species in Israel were chosen for extended investigation. The first was the high variation found in the ITS2 sequences of the indigenous polyphagous species *Eutetranychus orientalis* (Table 3). The second was the small genetic distance between two of the most abundant and pestiferous species in Israel: the invader *Tetranychus turkestani* and the indigenous *T. urticae* RF (Table 5).

### 4.6 The variation in *Eutetranychus orientalis* Klein

Although the sample size of *E. orientalis* mites that underwent DNA analysis had been small (5 single females from each location), I found a considerable amount of polymorphism within this species in all locations. The distribution of *E. orientalis* ITS2
sequences indicates that all seven populations of this mite belong to the same meta-population and that there is gene flow between them (Table 6). No indication for adaptation of *E. orientalis* populations to geographic/climatic regions was found in relation to ITS2 base composition, and the assumption of the isolation of any of these populations could be rejected. Furthermore, I have found no cryptic and/or closely related species, which were previously reported from Egypt or other Middle East countries (Smiley and Baker, 1995; Zaher, 1982), in my collections.

### 4.7 The relationship between *Tetranychus turkestani* and *T. urticae* RF in Israel

Navajas and Boursot (2003) presented molecular evidences suggesting the recent separation of *T. turkestani* and *T. urticae*. In their COI (mtDNA) based phylogenetic tree, *T. turkestani* from The Netherlands and the USA were positioned on the same branch as *T. urticae* from Greece and Spain, whereas the COI data of French- and Polish-originated *T. turkestani* were closer to the *T. urticae* collected in Italy, Egypt, Tunisia and The Netherlands. The small although distinct differences in ITS2 sequences (Navajas and Boursot, 2003; Ben David *et al.* 2007, 2009) and the sole morphological trait that distinguishes *T. urticae* from *T. turkestani* (a minute difference in male aedeagus knob size) constitute more evidence for their close relatedness.

I found mixed populations of *T. urticae* RF and *T. turkestani* on watermelon grown in open fields and on peach grown under netting in a screen house (Table 8). My studies showed that individual gravid females of both species preferred watermelon over cotton, apple and peach leaves (Table 9). 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 I present evidence that T. turkestani and T. urticae RF are capable of producing F₁ females (Table 11), but that the resulting F₂ generation is not viable (hybrid breakdown). Therefore I 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; Meyer, 1987).

Comparisons between intraspecific and interspecific crosses of T. urticae RF (T. urticae RF ♀ X T. urticae RF ♂ and T. urticae RF ♀ X 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 ♀ X T. urticae RF ♂) could not be studied due to lack of statistical power, as only one (sterile) F₁ female was obtained. As my laboratory strains were free of bacteria that could manipulate reproduction, I 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 is 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 (Dobzhansky, 1937; Butlin, 1987; Coyne and Orr, 1989; Noor, 1995; Saetre et al., 1997).

Specific mate recognition systems should enable species to recognize conspecific mates correctly. My data on mating behavior 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 (Table 10). 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* ♀ X *T. urticae* RF ♂) as compared to the intraspecific (*T. turkestani* ♀ X *T. turkestani* ♂) crosses, a phenomenon not observed in the *T. urticae* RF crosses (*T. urticae* RF ♀ X *T. turkestani* ♂ versus *T. urticae* RF ♂ X *T. urticae* RF ♀). The short duration of copulation and the number of male re-mating attempts in the *T. turkestani* ♀ X *T. urticae* RF ♂ crosses (Table 10) 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). My findings suggest the possible existence of different mate recognition mechanism in 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 their reproductive success and survival (Foster et al., 1972). Nevertheless, asymmetric types of reproductive interference are probably more common in nature, as it is unlikely that two related species have completely similar reproductive properties (Hochkirch et al., 2007). My results indicate that asymmetric reproductive interference occurred in mixed populations of *T. turkestani* and *T. urticae* RF. On bean leaf discs, a 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* cultures (Figure 8C). 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 (Figure 9). As suggested above, the reduced mating success of *T. urticae* RF in the mixed treatments may be the consequence of asymmetric mate recognition ability: *Tetranychus urticae* RF females did not discriminate between heterospecific and conspecific males while *T. turkestani* females showed a greater propensity for rejecting heterospecific males, increasing their chances of mating with conspecific males (Table 10). 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 coexist, the deleterious effect of 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 extent, 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 rare 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 rate than *T. turkestani* in leaf disc assays (Figure 8B) and produced more female progeny in whole plant assays (Figure 9). Fewer intraspecific matings will result in excess of male progeny in the second generation, may increase the chance of 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 on annual crops, such as watermelon, without completely excluding each other.

Habitat and/or geographical segregation of *T. urticae* RF and *T. turkestani* as agricultural pests in Israel were suggested from the first set of spider mite collections, in which *T. turkestani* was identified from apple and peach orchards in the northern part of Israel (Table 3). With the addition of populations collected in order to record the distribution of this species, I found that neither geographic nor climatic regions stand out as factors limiting *T. turkestani* distribution in Israeli agriculture. The species was collected from all regions of Israel, as was *T. urticae* RF. Nevertheless, *T. turkestani* was found on fruit trees and weeds in orchards whereas *T. urticae* RF was almost absent in collections from deciduous tree orchards, but was present on low growing herbaceous crops, on wild plants and in protected ecosystems (Table 8).

In the past, *T. urticae* RF was recorded from pear trees (Klein, 1936b) and later, during the 1960's, was considered a pest in apple and pear orchards, especially after pesticide applications such as the carbamate "carbaryl" (Plaut and Feldman, 1966). More recently it was found only in protected (greenhouses, screen houses) and heavily sprayed orchards (personal knowledge). My finding may suggest that *T. turkestani* has displaced *T.
urticae RF from perennial deciduous orchards in Israel. Such orchards are more suitable for T. turkestani because of its ability to undergo female winter diapause on the trees, where its congener, T. urticae RF is unable to survive, due to lack of foliage (Jeppson, et al., 1975). The phenology of T. turkestani, synchronized with its deciduous hosts, provides the post-diapausing females (that had over-wintered in the orchards) the advantage of early colonization in the spring. At this time, T. urticae RF, which overwinters on protected crops and on some perennials, is absent from deciduous tree orchards due to low temperatures in their growing regions. When temperatures increase, T. urticae RF can migrate to open fields mainly by aerial dispersion (Dubitzki, 1981). Tetranychus turkestani is also known as an early season pest of cotton in the USA, as compared to the congener spider mites T. pacificus McGregor and T. urticae GF (Carey and Bradley, 1982). In Israel, the early seasonal advantage of T. turkestani in deciduous fruit orchards, and its possible population buildup by mid-summer, when T. urticae RF is dispersing, suggest that local extinctions of T. urticae RF from deciduous orchards may be due, at least in part, to the intense reproductive interference caused by T. turkestani. The colonization of deciduous orchards by T. urticae RF could also be hindered by other ecological factors, such as the deterioration of host plant quality due to the early attack of T. turkestani or other herbivores (e.g. Harrison et al., 1995) that may also cause the induction of the host's systemic resistance response (English-Loeb et al., 1993). Seasonal changes in the biochemistry and architecture of the host-plant are known to play a role in regulating host-plant mediated interspecific interactions (Bounfour and Tanigoshi, 2001; Reitz and Trumble, 2002).

A displacement of T. urticae RF from pear and apple orchards in Israel by the newly invader at that time, T. urticae GF was proposed by Plaut and Feldman (1966). It is not unlikely that the invader species in the mid 1960's was T. turkestani, erroneously identified
as *T. urticae* GF. Both green colored mites were not recorded in Israel at that time. Regretfully, slide preparations from that period are not available for the reconsideration of the 1965 identification. The earliest *T. turkestani* male I found, dated 1980. If the invasive species that was initially recorded in 1965 from apple and pear orchards in the Izrael valley was *T. turkestani*, then the exclusion of *T. urticae* RF from deciduous orchards by *T. turkestani* happened ca. 40 years ago. Alternatively, if it was *T. urticae* GF back then, another displacement had to take place in this agro-ecosystem, when *T. turkestani* invaded, after the mid 1960's. In this scenario, *T. turkestani* eventually took over apple, as well as fig, almond and peach (Table 8) and displaced *T. urticae* GF from these orchards. *Tetranychus urticae* RF and *T. turkestani* may have consequently outcompeted *T. urticae* GF to near extinction, as nowadays it is rarely found throughout various hosts and growing regions in Israel (Table 8).

4.8 Prospects

In future studies, I suggest additional collection of spider mites in wild flora and in desert habitats in Israel in order to find more tetranychid species. Receiving mite samples from additional African, Middle East and Mediterranean countries could contribute to the ITS2 database that contains mainly European, Japanese and Chinese data. It might reveal historic cases of misidentifications and/or synonymization of species (Ros and Breeuwer, 2007) and enable a better phylogenetic analysis of large genera that should be revised, which are currently poorly represented in the molecular database. Moreover, it can improve the understanding of colonization patterns, distribution and host selection of invasive and indigenous pests.

The phenomenon of the incomplete reproductive barrier in a portion of *T. urticae* RF females should be further studied. Those females that produce unfertile F₁ daughters when
crossed with *T. turkestani* males, are lacking a mechanism preventing the fertilization by interspecific males, which *T. turkestani* females apparently posses. It will be interesting to test whether a second mating with a conspecific male could "correct" the situation in the females inseminated / fertilized by a male of the other species first. In dense spider mite cultures that are common in agricultural habitats, the odds of second matings are high. If a "correction" can occur in a second mating, it may result in a smaller deleterious effect of *T. turkestani* on *T. urticae* RF, in mixed cultures, than was shown here.

To summarize the main achievement of my research, I should emphasize that utilizing the DNA-based approach enables the efficient identification of spider mite species and the analyses of relationships between the different taxa of family Tetranychidae, for the benefit of plant protection and biodiversity conservation issues.
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Appendices – In separate pdf attachments: Ben-David et al 2007; Ben-David et al 2009

Appendix 1.

**ITS2 sequences as barcodes for identifying and analyzing spider mites (Acari: Tetranychidae)**

By

Ben-David Tselila, Melamed Sarah, Gerson Uri and Morin Shai


Appendix 2.

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

(Acari: Tetranychidae)

By

Ben-David Tselila, Gerson Uri and Morin Shai

מאפיינים מולקולריים של מיני משפחת אקריות הקורים בישראל

ה ancor לشم קבלת תואר דוקטור לפילוסופיה
מאת
עילה בן-דוד

הוגש לסינט האוניברסיטה העברית בירושלים
דצמבר 2008
עבודה זו נעשתה בהדרכת של
פרופסר אויר גרוז
דוקטור שי מורן
הפקולטה לחקר פרות, מון וסביבה עדishment רובי. סמית
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עבודה זו נעשתה בהדרכת של
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שלמי תודה:

לפרופסור אור גורון, דר. שовор ששתלד אט אוניברסיטת חיפה, ghéלphetamine את ארצן הידע של ענינו ביסודות DNA ו��רטוריאזיס עובילנ和服务 היצורים� עינב ביצורים ופרטוריאזיס האֶ الثالים גלוי על תורת התכני ביצורים

לשרוד ממלד שלד. פנו מימיצים של ביצורים ביבשת מחובד

תכלית ספילארוסיɟ על עיבוד נוגבי טיפתם ביצורים ואֶ היצורים

בלפוש אֶל söz עם התכני ביצורים אֶ ביצורים

לנסוג מירון על מתכני ביצורים איורכית

לדר. יוסף בורנר, דר. יואיק פלסקסי ואֶ היצורים שsertו ינותי ביבשת תמיתים אֶ פישר את

במעט תסיי העצפים בנֶה-יעד

לחוחרת התדה התדה, דר. יואיק פלסקסי יוד. עפר עדיבת על עץ assort טעויות פעלת

לאֶל đứa, לעבר מוגלי החירויות הנוגע לשחות ויצורים העיבוד והיצורים ביבשת החילומן פיתחה הכנר, על

הנומיס והאישה ביכעל מים עבירות וארמוכיותMessageBox

לקריקטיר ש"מי" áoבק גוצלֶב, פאֶ ומר, סֶגֳר, אֶרייל, טעמו ביטון, רוארה אוק, פֶלטנה

דוגרין, יואל מיסיקה, אלף סטֶל, דובֶה אֶבֶטתיה, דר. מירם גובֶר, יֶדֶפֶל אֶלברשטיין, דר. שֶמוּאָל נֶט, דר.

שאלו ב-יהדות, יואל כֶס, יואל דרֶרֶשטיין, יואל מֶרֶרי, תמר חֶב, שרֶהֶרֶה גֶנֶסֶר, דר. לובֶסֶק, יואל

שלים שֶמוּאָל, שאלו גרֶפ, פֶתֶח תֶלֶר, אנ רֶנֶגרְפֶי, יֶהֶפֶהֶר, יואל אֶמֶן ביגֶל, לאֶ תֶל, יואל

המשטחי, יואל, אֶמֶנֶאֶל טֶטֶט, דר. רומי רֶלֶפ, שֶרֶפֶה ינותי ביבשת תמיתים על ינותי ביבשת מומי אֶ鲕ו

קוּורֶיס יֶבֶילוּם תֶכֶליס בַּרבֶי אֶהאר

ואֶנֶרוני הֶבֶינֶה, יֶנֶפֶת תֶכֶליס בַּבֶּוּמֶיה בַּבר, שֶאֶמוֹנֶי לֶאֶ סֶני, תֶמֶכֶס והֶהֶאֶר בֶּסֶלֶטֶה

מררךשית עַדְק הֶטֶסֶס הָמַיָּוִיט

תודה
The family Tetranychidae (Red Spiders) includes about 1200 species worldwide, including pests that attack vegetables, flowers, ornamental plants and fruit trees. Despite the economic importance of this family, it has not been studied in Israel in depth until this work. Only 14 species are known to attack agriculture and two unique species have been recorded in Israel until 2004.

The correct identification of living creatures is the basis for understanding the differences between species, family relationships and evolutionary processes. Correct identification is crucial for comparison between studies from different periods, in different parts of the world, and for scientific progress. It also has great importance and economic significance in pest control (preventing disease spread between countries) and chemical and biological pest control.

In the past decade, all taxonomic works have been based solely on morphological characteristics, using keys based on these characteristics. To do this requires mature specimens, males and females, to define species. Recently, new molecular tools have been developed allowing accurate identification of species and orders in all stages of development, using DNA markers. The nuclear rDNA-ITS2 marker can be used to identify closely related species in this family and in other mites as well.

In my work, I dealt in detail with three species of red spiders:

1. The eastern red spider, Eutetranychus orientalis (Klein), a local and ancient species in our region, described by Klein in the 20th century.
2. The most common red spider in Israeli agriculture, Tetranychus urticae Koch (sub-species = T. cinnabarinus) is considered a local species.
3. The Tetranychus turkestani Ugarov and Nikolskii, a species similar and very close to the yellow species (T. urticae sub-species), also morphologically and molecularly.

The species T. urticae and T. turkestani are polyphagous cosmopolitan pests.

The work is dedicated to the memory of Professor Yitzhak Shalitin (1921-2006) who has contributed greatly to the field of entomology.
A. The goals of the molecular catalogue were to identify the existing mites in Israel in comparison to the conventional morphological classification, with a special focus on mites likely to be harmful to agriculture.

B. The aim of the genetic differentiation of the populations of the red mite E. orientalis, which were lost due to the different climatic areas in Israel, is to indicate adaptation or the discovery of other similar species that have not been documented before.

C. The study of the genetic distance between two harmful mite species: Tetranychus turkestani (Ugarov & Nikolskii) and T. urticae Koch, indicates an incomplete differentiation process, which may lead to economic impact when they meet in the same host.

During 2004-2005, 31-32 species of webs and fries were collected from 21 sites throughout all climatic areas of Israel, and sequenced ITS2 (sequences) for 16 species, which are documented as never having been sequenced before in the world's banks.

Besides the two close species: T. urticae and T. turkestani, it was found that each species has a different length (between 360-540 base pairs) of the ITS2 region. Amongst the species that were sequenced, there are 12 species published in the world's banks, and 24 species that were sequenced in the research.

It was found that the species T. urticae and T. turkestani have identical sequences in length and similarity, but there is a unique and specific DNA sequence for each of them (5 base mutations), which enables their differentiation.

During the collection, 3 species were found and documented molecularly and morphologically, which were not previously documented in Israel: Aplonobia histricina (Berlese), Tetranychus ludeni Zacher, T. evansi Baker and Pritchard.

Furthermore, 4 species were found in Israel, which were not documented in the world's banks: Eotetranychus hirsti Pritchard and Baker, Bryobia graminum (Schrank), Oligonychus coniferarum McGregor, T. neocaledonicus Andre.

A molecular catalogue of the web mites of Israel is presented, in which is documented for the first time the occurrence of 12 species of mites, which were sequenced in the world's banks.

It is emphasized that these species may become agricultural pests, causing significant economic damage.

In the phylogenetic tree, which was built according to the obtained sequences, all species were located in the expected place compared to the morphological classification. Representatives of the subfamilies Bryobiinae and Teranchininae are indeed placed in separate branches of the tree, and representatives of the tribes within the family Tetranychidae: Eurytetranychini and Tetranychini.

It is exceptional species Oligonychus, as it is not monophyletic in the molecular tree.
The mite *O. afrasiaticus* (McGregor) is closely related to the *Tetranychus* species. It is also closer to the species within the *Oliginychus* genus than to other species of its genus in this tree. A molecular marker, COI (first cytochrome oxidase subunit), was used to investigate the genetic distance between species. This marker showed that the distance between *O. afrasiaticus* and *T. urticae* is smaller compared to the distance between other species of the genus *Oliginychus*: *O. perseae* Tuttle, Baker and Abbatiello and *O. mangiferus* (Rahman and Sapra), which were found to be close to each other based on two molecular markers.

In fact, the male genital apparatus of *O. afrasiaticus* is more similar to *Tetranychus* species than to other species of the genus *Oliginychus*. In the literature, there are several taxonomic divisions of the genus that were not accepted by the scientific community, but they provide additional evidence for the polyphyletic nature of the genus from a morphological perspective.

In some of the species studied - *A. histricina*, *B. praetiosa* Koch, *O. afrasiaticus* Petrobia harti (Ewing), *P. tunisiae* Manson, *Schizotetranychus aspargi* (Oudemans) - a single ITS2 barcode was found. In the other species, there was polymorphism within species, and in them, 2-9 different barcodes were found for each species. The greatest polymorphism was observed in the *E. orientalis* species, a pest species in the *Tetranychidae* family, which was found to have the greatest polymorphism in the ITS2 region. However, in seven sites where it was collected (Tel Aviv, Tzippori, Neve Tzair, Eilat, Nitzanim, CSS), there was one ITS2 barcode that was shared with the heterozygous alleles, along with additional barcodes.

In genetic analysis of these barcodes, there was no structure or pattern, which suggests an adaptation process that sheds light on the expansion process of the species from the eastern rift valley to the coastal plain from the 1930s until now. Within populations of *E. orientalis* in different areas of the country, even in the most remote areas, there was a movement of genes, and it is assumed that this movement is mainly due to human activity, similar to the expansion of the family *Acrididae* to the Negev and the Edom, which contributed to the expansion of the species.

No morphological or molecular evidence of hidden species or additional species of the same genus, which were described in Egypt and Timna, was found. The contribution of the multivorous lifestyle is also discussed in the ITS2 region. It seems to me that the occurrence of different barcodes in the same population is an inner characteristic of the species and is not related to its local, invasive or other factors.
The smallest genetic distance between two species, as mentioned, is found between the species T. urticae and T. turkestani, and it is 1.1–1.5%.

It is customary that a distance (shona) of less than two percent can exist within the same species, and only over that is considered a separate species.

According to COI sequences of these closely related species found in the gene bank, it is not possible to distinguish between them, although it is not yet certain that the process of divergence and speciation of these species has not yet been completed.

In samples from the years 2004-2005 in agricultural fields throughout Israel, it was found that the golden mite, T. urticae, is the pest in dried fields, not the golden T. turkestani, as was previously thought.

The latter does exist in Israel, but is not common, as it was collected from 50 orchards / fields in two years.

In fields and gardens, T. turkestani successfully breeds, while T. urticae does not reproduce in the field environment.

The two species together were collected in the north of the country. As mentioned, these species are close to each other morphologically, ecologically, and molecularly, and the relationships between them are a subject for continued research.

In experiments on mating with females of T. urticae and T. turkestani from the laboratory, it was found that females of both species prefer leaves of vetch over apple, but a unique difference was noted when the females were T. turkestani and the males from the other species.

As a result, vetch is a preferred mite, and inter-specific reproduction is unlikely with the same plant environment.

In inbreeding between species, I obtained offspring from a female in 20% of the cases where the mother was T. urticae red, but only one female in each pair of mating performed in reverse.

These females were not sterile, and the heterozygous in the marker ITS2, which was proven by molecular methods. There were no indications of endosymbionts ("endosymbionts") in my laboratory, which could influence the reproductive success of the populations that I raised.

In experiments on reproduction in the laboratory, I found that in meetings with an adult female of the second species, the number of matings is significantly higher than in meetings of males and females of the same species, but a significant difference was noted only when the females were T. turkestani and the males from the other species.

The number of matings was...
נכתבו ירח ומשה החובה של הגוזה הודות לברך הגוזה הקטן ירח ב vatanda明珠ו - מיני וב חת pigeonsחיים.

דבר המועדף על מפגש החובה האפשרי ירח לש נקבת מי - העד החובה המוחלט של ברך פון

ון-סנה ביני המינים, באומף ולא-ס الرسمي.

הפרעה רב-חוב הלוחה במערכת הגוזים מכים ממקים (ዮון גול hairst, ירוเกม) על כותל של שיער. קבר.

הסתיים והרコピー הא.rectangle הלוחה של נקבת יש הווה, והועשבים מإجراءات נברות חישה וביתות

אור. בלוות וחתות ירידת מורשת חיה מוכנה ב-בזקינativos נבקות ירייה שבиона לשטר

תלונות, בחשון בברך תח - מימי. תלוות ו-לא-תסור הנבזת במופרות, קבר בזקינativos לשטר

בצדכונות משטרות של 30% כתוספת הבקות לקבצה焓 אקולוס דוניה שלית, קבר לשטר

ברקורי, ולא-אפרי על ויידה רחם ירח בברך הרציחתי של

האחותות תמונות ביני המינים.

בניסו המונע של מצאתי כי התשלות הימורדה האבולוציה ובאבולוציה של

תלונות של שילוש, בשילט ו-חזרת הזרם האבולוציה של קבר הב sidelines החיה בברך תח - מימי.

וד untranslated האנונימות של האקרית ת-האחותות המפוגדו, או שילזבייה שירדר

ברקורי, או-אפרי על ויידה רחם ירח בברך הרציחתי של

בסקוראן, נסודות בעיצי הפרי הנשרים, כנראה מעורבים además גם גורמים אקולוגיים כמו חריין באתר,

ב𝕺ון הק篦ים לשנתה, ירח-אקלים, צפיפות-יחסית התחלת ב- matéria ו-יצירת הפרה של המין

ב-ברך תח - מימי, הביל הגרעיני והברך, מתלונות שחרור של ב-ברך תח - מימי.

לסיקון, עבשורי בתוכן竺יתו האקסקולגיה לכל בובות אקקל, ת-השון הנטיכה, 

בתוכ המינים החובה ב(substrum השילזבייה של מיני חברת החירcurity)

vousou השילזבייה של מיני חבר החירcurity, שנbrit, ת-לימות הפילוגוניה של מיני חבר החירcurity

ולפיורקיט שיטתי אחדות, קבר ב-לא-andelier מודויית ירח שירדר-אקקרא מפוגדות,

מלאת וזיחות והידרヶ月 של מיני פוליפטרים ודיגיילר. שירדר המשטหา של מיני-אקקרא קור די-מישראל

ועדכן-新加פ לשבני מים של העציפי קדוש של מישראל, חלב-מוכי חקלאות פוטנציאלים.

המקומם בחדש את האוקולים בתוך מיני-קורבים של מיתו חקלאות, שוטיבים בבקלאץ-ברצליה.