California Avocado Commission
Research Grant Final Report: December 9, 2014

Duration of Project: *12/2012-12/2014*

Project title: Identification of Predatory Mites Associated with Persea Mite in Mexico and Genetic Analyses of Native and Invasive Persea Mite Populations

Research Area: Control of Avocado Pests

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Total funds approved: $ *45,000*

Project contract number: UCR-13020109

Executive Summary:

Molecular analyses of field-collected persea mite (*Oligonychus perseae*) specimens established that (1) ITS-2 provides reliable *Oligonchyus* species identification and (2) COI analyses were able to discern that California's persea mite population originated from central Mexico. Persea mite COI DNA sequences from California also matched populations from Baja California, but central Mexico is the economic center and evolutionary origin of avocado and for these reasons it is the likely source of invasive persea mite populations that became established in California. A comparison of predatory mite species (Phytoseiidae) from Mexico and California as originally intended was limited by challenges in amplifying COI/ITS-2 DNA from individual specimens but significant progress was made in optimizing non-destructive DNA extraction protocols for this taxonomic group.

Background:

The persea mite, *Oligonychus perseae* (Acari: Tetranychidae), is a foliar feeding spider mite pest of avocados (**Fig. 1A**) and it is presumed that its native home range is Mexico, from where persea mite was first described on intercepted avocado plant material examined by Tuttle, Baker, and Abbatiello (1976). Invasive populations of persea mite in California were detected in 1990 from San Diego County (Bender 1993) and quickly spread throughout the avocado growing region (e.g., backyard trees and commercial orchards) extending from San Luis Obispo to San Diego County. Persea mite feeding damage greater than 10% of the total undersurface area of avocado leaves promotes premature leaf drop and this increases the risk for sunburn of young fruit and yield reduction (Bender 1993).

The California biological control program targeting persea mite has been limited to the use of commercially available predatory mites such as *Neoseiulus californicus* (**Fig. 1B**), but these predators are not able to establish in commercial avocado orchards and effective persea mite

control requires seasonal releases of 2,000 predators per tree (Hoddle et al. 2000). *N. californicus* is native to California but it did no co-evolve with persea mite within the native home range of avocado in Mexico. This biological factor may explain the limited efficacy of *N. californicus* to control persea mite in the California avocado system. Consequently, the motivation for this work was to clarify the invasive origin of persea mite found in California and to find novel natural enemies, especially predatory phytoseiid mites, from the home-range of persea mite in Mexico that could be used to design a future classical biological control program. The premise of this biological control strategy is that natural enemies that have co-evolved with persea mite on avocado are likely to be better suited for establishing and controlling persea mite in the California avocado system.

Research Objectives:

1. Sample populations of persea mite and predatory mites on avocados from California and Mexico.

2. Use select DNA markers to find a genetic match for California's persea mite population among sampled Mexican populations. The physical location for these genetic matches in Mexico could represent probable areas from where persea mite originated and was accidentally introduced into California. Determining this source area may also point to strategic locations where co-evolved natural enemies might be recovered for use in a biological control program targeting this pest in California.

3. Use DNA markers to guide molecular species identification of novel mite predators found in target locations from Mexico (determined from objective 2) with specific intent of identifying species associated with persea mite that are lacking in California.

Materials & Methods:

1. Specimen collections:

The primary focus of sampling efforts was to obtain persea mite specimens from a large geographic range that would facilitate assessing the genetic diversity among and between geographic populations. Consequently, persea mite specimens and associated predators (phytoseiids) were previously collected from avocado foliage in California (Lara) and Mexico (Hoddle and Lara) and preserved in 95% ethanol. In California, targeted sampling areas included the commercial range of avocado production where persea mite is found, including San Luis Obispo, Santa Barbara, Ventura and San Diego County.

Similarly, targeted sampling in Mexico included the historical and major avocado production state of Michoacán and four neighboring states: México, Guanajuato, Puebla, and Morelos. Additional persea mite specimens were collected from San Jose, Costa Rica where persea mite established during the 1970s and from Baja California where persea has not been previously been documented (Lara and Hoddle, in press). These additional geographic locations were included in molecular analyses to provide more information on the genetic diversity of persea mite.

2. Persea Mite Genetic Analyses:

Complete DNA was extracted from a subsample of 91 individual persea mite female specimens originating from California, Mexico, and Costa Rica. DNA extractions were conducted using the EDNA extraction kit to facilitate the processing of individual mites specimens; alternative techniques require specimens to be pooled to extract enough genetic material but this was not

necessary for persea mite. Therefore, DNA extractions for each mite specimen were carried out in a 25uL volume consisting of solution 1A (16uL), solution 1B (4uL) and solution 2 (5uL).

Subsequently, DNA extraction samples were subjected to custom PCR conditions to amplify two specific molecular markers, ITS-2 and COI, using spider mite primer sets developed by Navajas (1998). In general, the ITS-2 marker is considered useful for species identification of specimens and the COI marker is useful for determining the origin of invasive populations from their home range. Amplification reactions were carried in a 25uL volume containing standard reagents, i.e., ddH20 (ion-free water), forward/reverse primers, 10x buffer, Taq polymerase, BSA (increases PCR yield), $MgCl_2$, and nucleotides (DNTPs). Following an initial denaturation step at 95°C (4 minutes), PCR cycles (approximately 35) involved DNA template denaturing at 92°C (1 minute), primer annealing at 45°C (1 minute) and primer extension at 72°C (1.5 minutes).

To determine if these markers had been properly amplified, resulting PCR products were screened using standard gel electrophoresis protocols. In general, a strong band revealed under UV light indicates that PCR conditions and the starting genetic material were suitable for marker amplification. PCR products, determined to be properly amplified, were cleaned using the Wizard® genomic DNA purification kit from Promega and submitted for DNA sequencing at the UCR Genomics Facility.

DNA sequences for individual mites were manually aligned in the program BioEdit to produce consensus COI and ITS-2 sequences. COI consensus sequences were used to compare the genetic diversity among samples from each geographic area. For ITS-2, additional spider mite species sequences from Genbank were imported into BioEdit and compared to the sequences produced from this study to assess the species identification reliability of this marker for persea mite.

3. Phytoseiid Genetic Analyses:

Before unidentified specimens collected in Mexico could be included in genetic analyses, DNA extraction and amplification (PCR) protocols needed to be optimized. For this purpose, colonies of predatory mite species associated with persea mite in California were reared in the laboratory exclusively on ice plant pollen. These species included *Agistemus longisetus* (originally collected from an avocado orchard in Santa Paula, CA), *Euseius* sp. (also collected from an avocado orchard in Santa Paula) and *N. californicus* (purchased from Biotactics). Using these colonies, a combination of DNA extraction methods, PCR primer sets and PCR cycle settings were evaluated.

Results:

1. Specimen Collection:

Overall, the sampled geographic terrain covered more than 4,700 kilometers in distance extending from San Luis Obispo (USA) to San Jose (Costa Rica) (**Fig. 2**). Of great importance is the state of Michoacán (central Mexico) because this territory represents a major commercial avocado production center and a likely source of persea mite to other areas where avocado and persea mite were introduced (e.g., California, Costa Rica, Israel and Spain). **Table 1** provides an overview of specimen collections from central Mexico.

2. Persea Mite Genetic Analyses:

Persea mite ITS-2 sequences obtained in this study (N=91) and additional conspecific sequences imported from Genbank (N=3 from Israel, N=1 from Spain, and N=24 from Mexico) revelead low *intraspecific* variation between geographic areas (**Fig 3.**). In addition, a

comparison of ITS-2 sequences obtained from GenBank (N=92) revelaed higher *interspecific* variation between *Oligonychus* species (**Fig. 4**). This supports the potential use of ITS-2 as a reliable and easy-to-use molecular marker for species identification of persea mite from unidentified samples.

COI sequences for persea mite specimens (N=75, ~350 base pairs) provided evidence that supports the origin of California's invasive population being Mexico (**Fig. 5**). Low genetic diversity was detected in California's population for the COI marker (1 unique DNA sequence or haplotype) while relatively higher genetic diversity was detected from specimens collected in Mexico, with at least 8 haplotypes recovered. The persea mite COI haplotype from California matched populations sampled from Baja California and four municipalities from the state of Michoacán, including Uruapan, Charo, Morelia and Tarímbaro. Additional sequences incorporated into this study revealed that (1) the Israeli haplotype, for which there was only 1 sequence available from GenBank (Accession no. DQ656485.1), matched the haplotype detected in California, Baja California and Michoacán and (2) the haplotype from Costa Rica did not match any of the 8 haplotypes recovered from Mexico.

3. Predatory Mite Genetic Analyses:

The next step in this project was to identify predatory mite species collected from central Mexico belonging to families Phytoseiidae and Stigmaeidae (**Fig. 6A-E**). In contrast to generalist insect natural enemies detected in Mexico (**Fig. 6F-I**), some members of the Phytoseiidae and Stigmaeidae can provide effect control for spider mites and have been observed on avocado foliage at low persea mite densities in California (Lara and Hoddle, in press). For these reasons these natural enemies were of primary interest. Unfortunately, amplifying both ITS-2 and COI DNA from individual phytoseiid and stigmaeid specimens in a non-destructive manner (preserving carcasses of unidentified specimens is necessary for morphology-based identification) proved to be challenging. Consequently, the DNA amplification protocols for this project were revised using modifications suggested by previous studies (Okassa et al. 2009, Tixier et al. 2011). Destructive, pooled DNA extractions were performed with lab reared specimens and Qiagen kit protocols but follow up electrophoresis results for screening successfully amplified PCR products (not shown) were inconsistent for individual mites.

Ultimately, a new set of ITS (Navajas et al. 1999) and LCO/HCO COI primers (Folmer et al. 1994) in combination with the EDNA kit (used for persea mite) were employed for processing individual predatory mites; methods for stigmaeids still require optimization. Fortunately, the ITS primer includes ribosomal DNA for ITS-1, ITS-2 and the 5.8S gene. Strong bands seen in gel electrophoresis results revealed that this entire region was successfully amplified in a small subset of individual specimens sourced from lab colonies of phytoseiids associated with avocado in California, but not in *O. peseae* (**Fig. 7A**). On the other hand, the LCO-HCO COI primers were not useful for amplifying this region in individual phytoseiid specimens (**Fig. 7B**) and this limitation may need to be addressed in future studies attempting to compare the genetic population structure of phytoseiid species present in both Mexico's and California's avocado system (e.g. *Euseius hibisci*).

Discussion:

Molecular based identification of persea mite, relative to other *Oligonychus* spp., was established using the ribosomal ITS-2 region. This means that signature ITS-2 sequences for spider mites could be readily used to confirm the identity of persea mite found on interdicted avocado host plant material from US-MX border or port inspection stations. *O. coffeae* and *O. punicae* have been reported on avocado but a basic comparison of persea mite sequences with

GenBank sequences reported for the former two mite species and *Oligonychus* mites found on other host plants show distinct base substitutions and gaps (**Fig. 4**).

Equally important is the finding of a single persea mite COI haplotype among California samples from an extensive geographic area (e.g., San Luis Obispo, Ventura and San Diego). This result suggests that the establishment of California's persea mite population on avocado could have been the product of just one single successful introduction event of this mite pest from its home range in Mexico where, as expected, more genetic diversity exists (at least eight COI haplotypes were detected). Pinpointing this origin in the home range requires careful interpretation of the molecular results.

The California haplotype matched the haplotype from Baja California, but this Mexican state is not known for its commercial or historic avocado production. It is more likely that persea mite specimens introduced into California's Hass-dominated avocado system in the early 1990s (Bender 1993) originated from populations found in Michoacán, but the exact invasion pathway remains unclear. Michoacán is in close proximity to the center of origin for Mexican avocados, and over recent decades this state has become a major commercial Hass avocado fruit producer in the world market. Therefore, a parsimonious explanation is that persea mite-infested host plant material (e.g., avocado foliage used in traditional cooking, whole young plants or budwood used for propagation) from Michoacán was transported to the northern portion of the country (i.e., Tijuana) and from there was introduced into San Diego, California (the initial detection site) either through further human activities or natural wind-mediated dispersal (persea mite produces strands of webbing that allow it to be transported by wind currents to new patches of host plant material).

Interestingly, the single COI sequence recovered from avocado in Israel, matched the haplotype found in Michoacán, Baja California and California. This information does not resolve the invasion pathway for persea mite in Israel but it does show that COI can be used to uncover potential genetic relationships between well-separated geographic populations; additional molecular markers (e.g., microsatellites) or techniques may provide the necessary resolution to determine the precise origin of the Israeli persea mite population. Another interesting result is the lack of a matching COI haplotype for the Costa Rica population of persea mite. Briefly, persea mite is not native to Costa Rica so this result implies that the Costa Rica haplotype exists in another area of (southern) Mexico that was not sampled during the course of this project.

With regards to the second objective of this study, the persea mite genetic analyses narrowed the search area for natural enemies that co-evolved with the CA-genotype to Michoacán. Identifying collected specimens to species level was not possible due the difficulty in streamlining DNA amplification methods for small, individual specimens. Still, significant progress was made. The ITS region was amplified in a subset of individual phytoseiid specimens and not in persea mite samples (**Fig. 7A**). This result has important practical implications as it indicates that ITS primers reported by Navajas et al. (1999) may be used for selectively amplifying phytoseiid-specific DNA from field-collected predators that may have consumed *O. perseae*. Consequently, assuming that species-wide homogeneity exists for ITS-2 at the species level for phytoseiids found in the avocado system, it may be possible to screen for new species among field-collected samples from Mexico. Identifying new species with biocontrol potential that are relevant to the avocado system will require future attention but essential protocols have been established in this study.

Summary and Conclusions

Persea mite and associated predatory mites were collected from California and Mexico. Molecular results support the native origin of persea mite from Michoacán, Mexico and Michoacán is a potential area where natural enemies that have co-evolved with persea mite may be found and subsequently used to develop a classical biological control program targeting persea mite in California. Follow-up molecular analyses for phytoseiids, using non-destructive protocols established in this project, will be necessary to compare the predatory mite fauna between California and Mexico. The prospect for establishing native predators from Mexico in the California avocado system has been reviewed extensively by Lara and Hoddle (in press).

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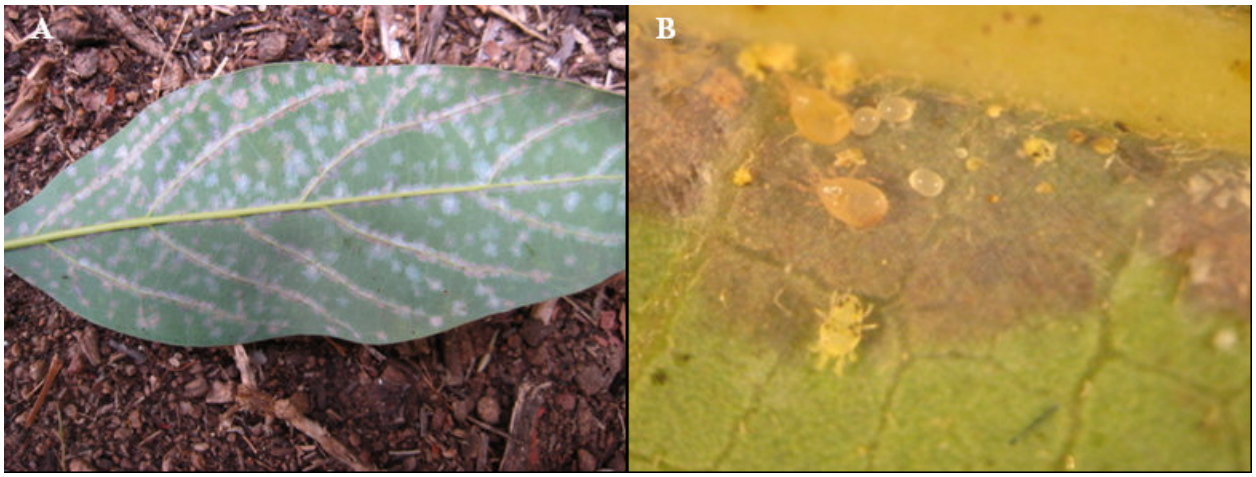

Fig. 1. A) Highly infested avocado leaf with persea mite in Southern California, B) *Neoseiulus californicus* feeding inside persea mite nest.

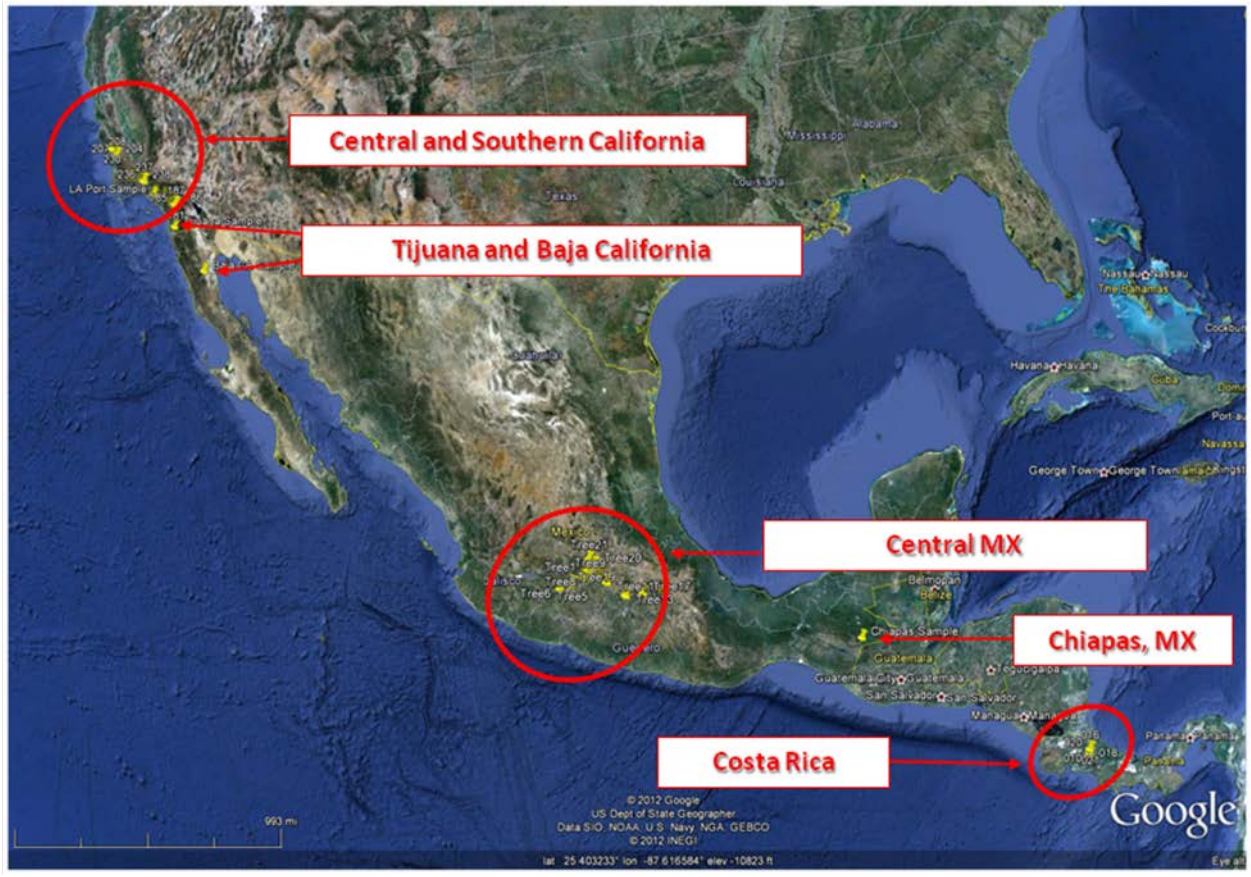

Fig. 2. Overview of areas sampled for persea mite in California (USA), Mexico, and Costa Rica.

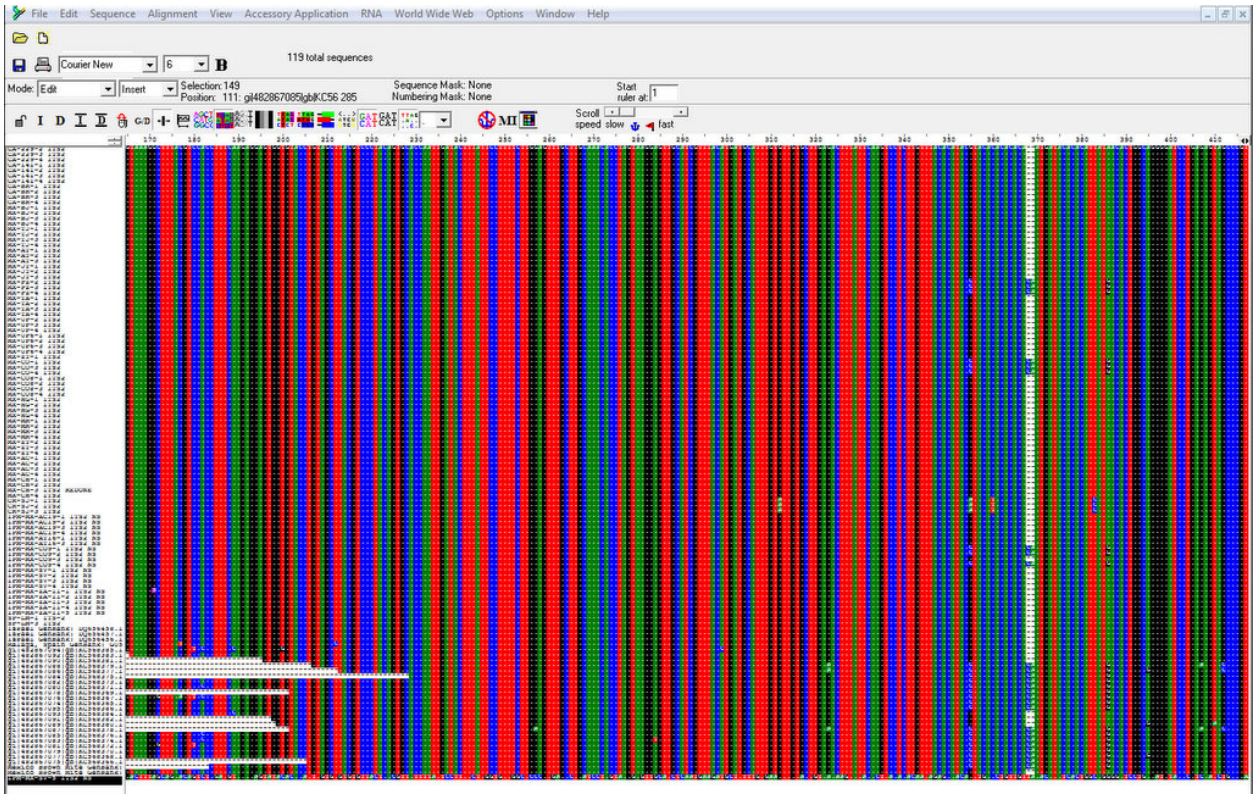

Fig. 3. Sample base-pair comparison among persea mite ITS-2 sequences from MX and CA (obtained in this study) and GenBank. Each row is a separate read for an individual mite specimen. The columns represent nucleotide base position in the read and the color indicates the identity of the nucleotide at each position (A= green, G=black, C=blue, T=red). As shown here, most of the rows share the same color patterns (nucleotide identity) and this indicates low intraspecific variation in the genetic code for ITS-2 among samples.

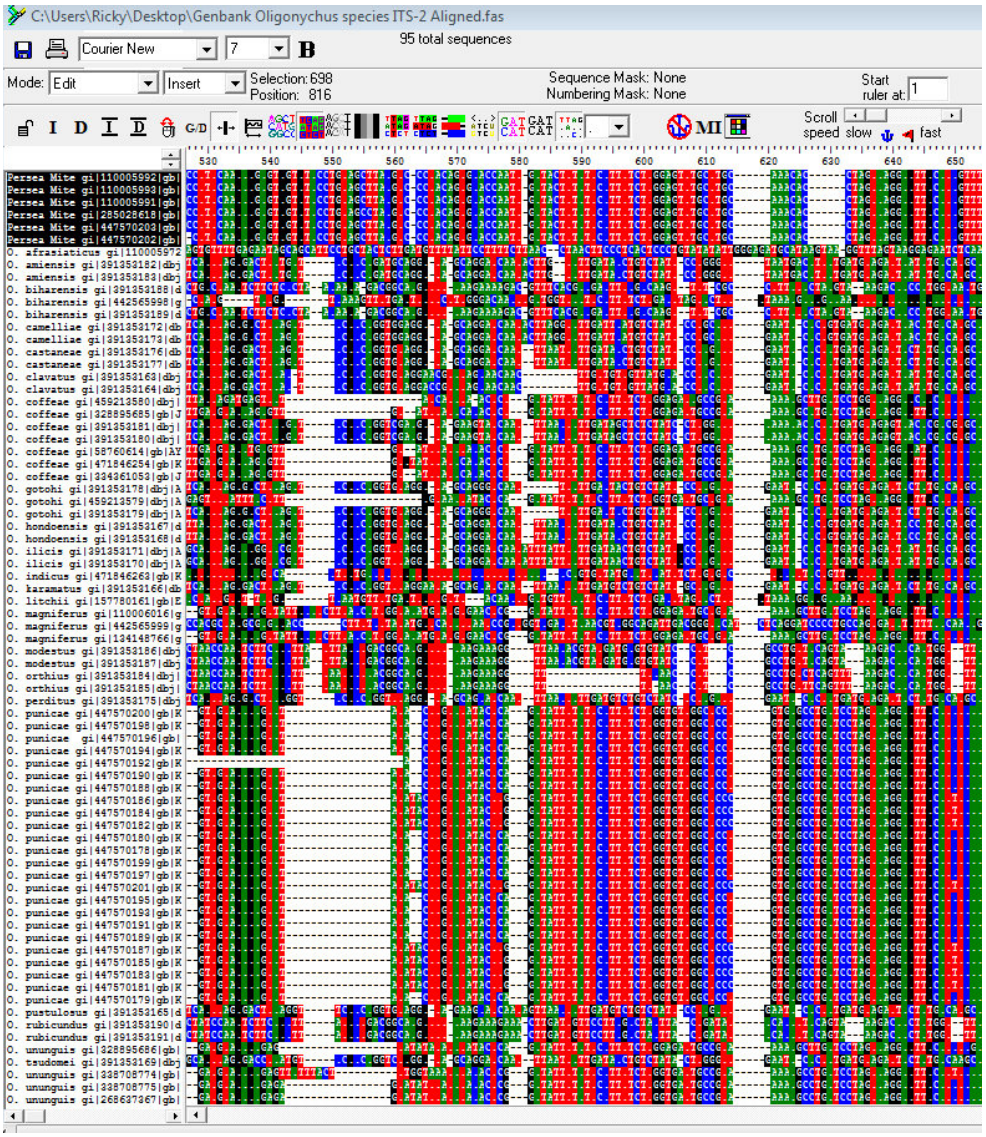

Fig. 4. Sample base-pair comparison between ITS-2 sequences for persea mite and other *Oligonychus* species, including: *O. afrasiaticus*, *O. amiensis*, *O. biharensis*, *O. camelliae*, *O. castaneae*, *O. clavatus*, *O. coffeae*, *O. gotohi*, *O. hondoensis*, *O. ilicis*, *O. indicus*, *O. karamatus*, *O. litchii*, *O. magniferus, O. modestus, O. orthius, O. perditus, O. punicae, O. pustulosus*, *O. rubicundus*, *O. tsudomei* and *O. ununguis*.

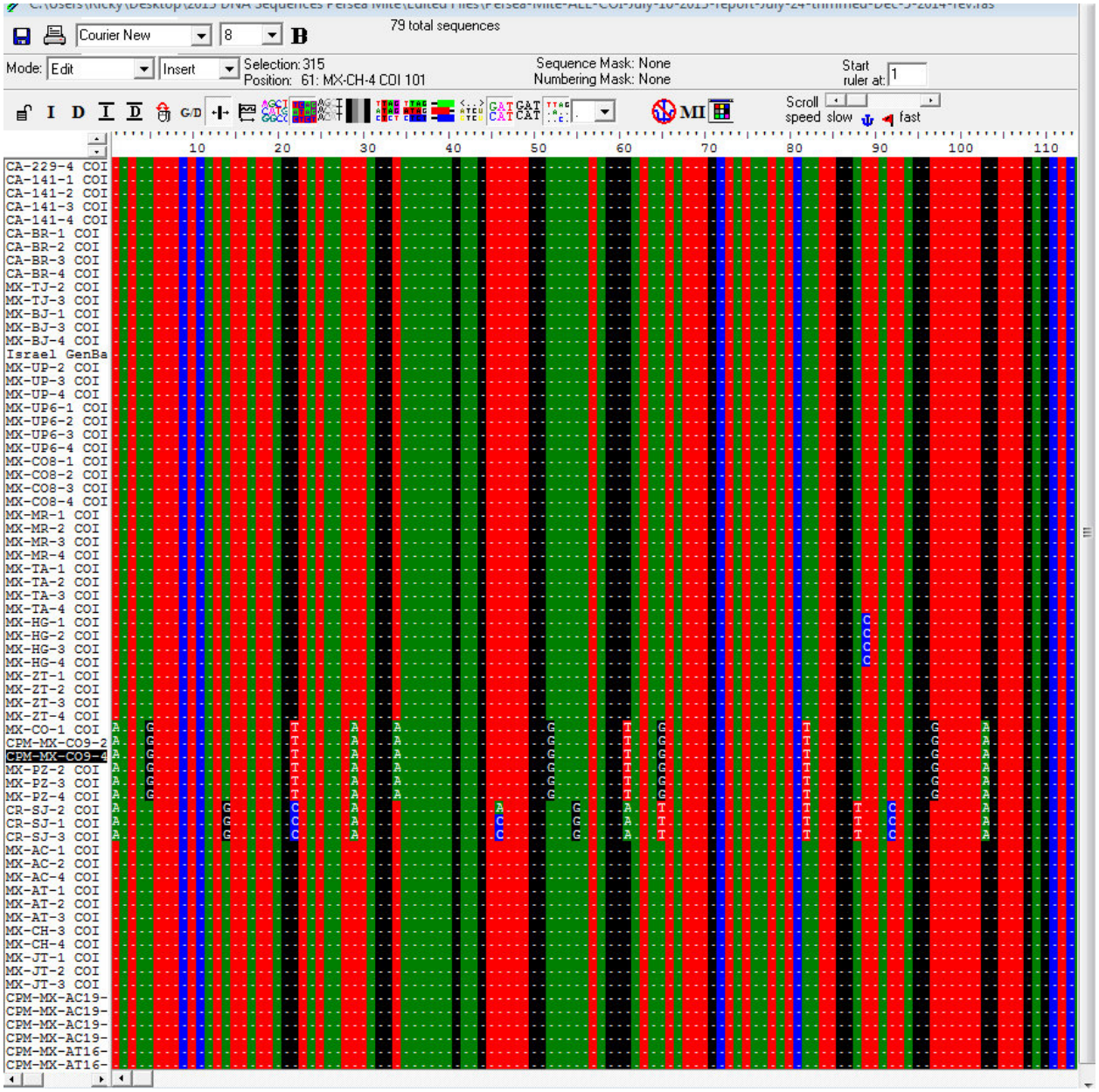

Fig. 5. Sample comparison of COI sequences obtained from persea mite collected in Mexico (MX), Israel, California (CA) and Costa Rica (CR). The Israeli sequence was available from GenBank.

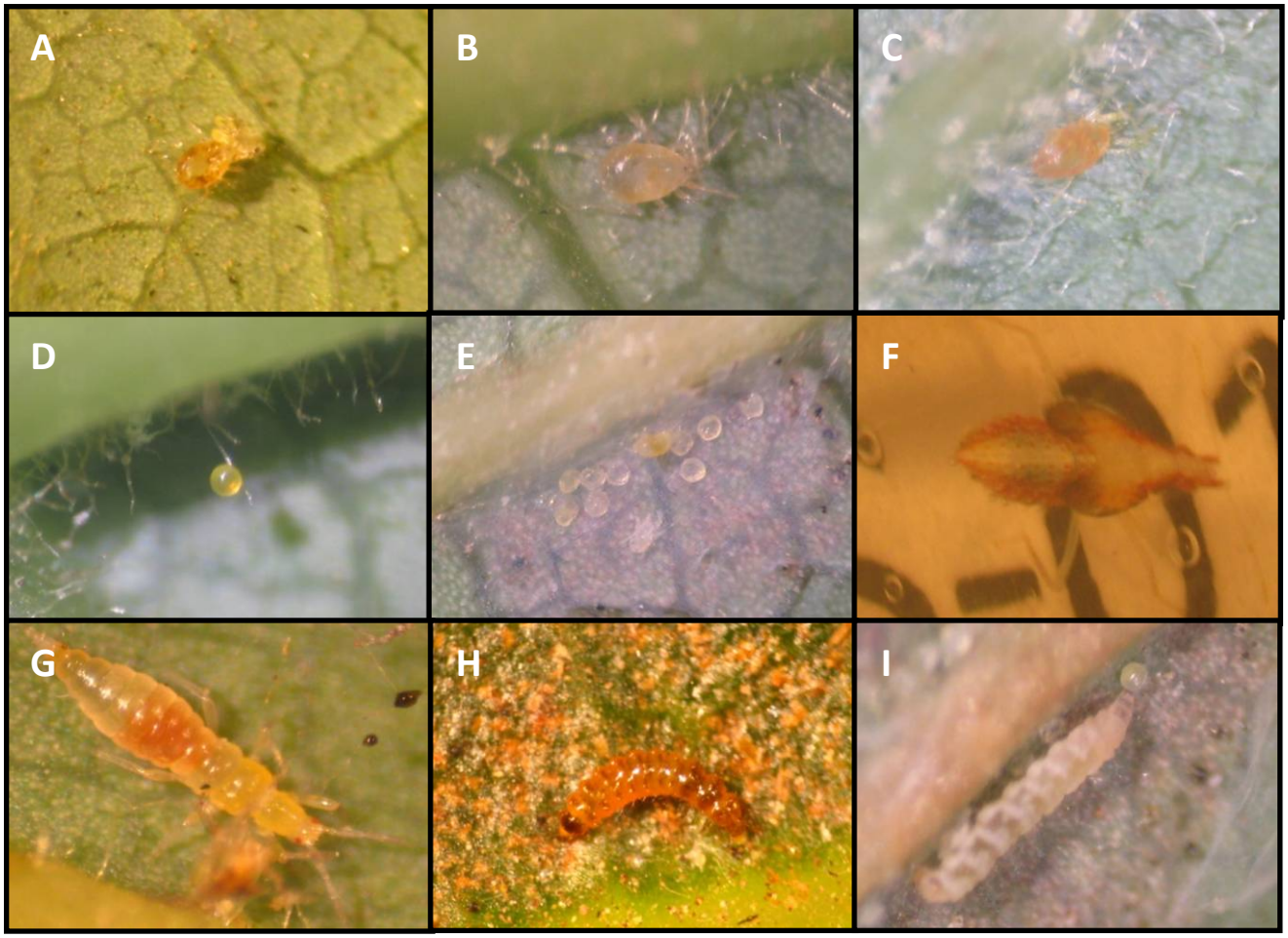

Fig. 6. Some of the natural enemies observed on persea mite infested leaves from central Mexico: A) phytoseiid predator feeding on adult persea mite, B) phytoseiids were commonly found resting along leaf veins, C) female stigmaeid resting along leaf vein with trichomes, D) stigmaeid eggs are round, yellow and can usually be seen attached to leaf hairs E) an immature stigmaeid found feeding on persea mite eggs inside a webbed nest, F) an immature assassin bug (Reduviidae), G) second instar Franklinothrips sp., H) predatory beetle larvae feeding on a tydeid mite, I) cecidomyiid larva found inside a persea mite nest feeding on eggs.

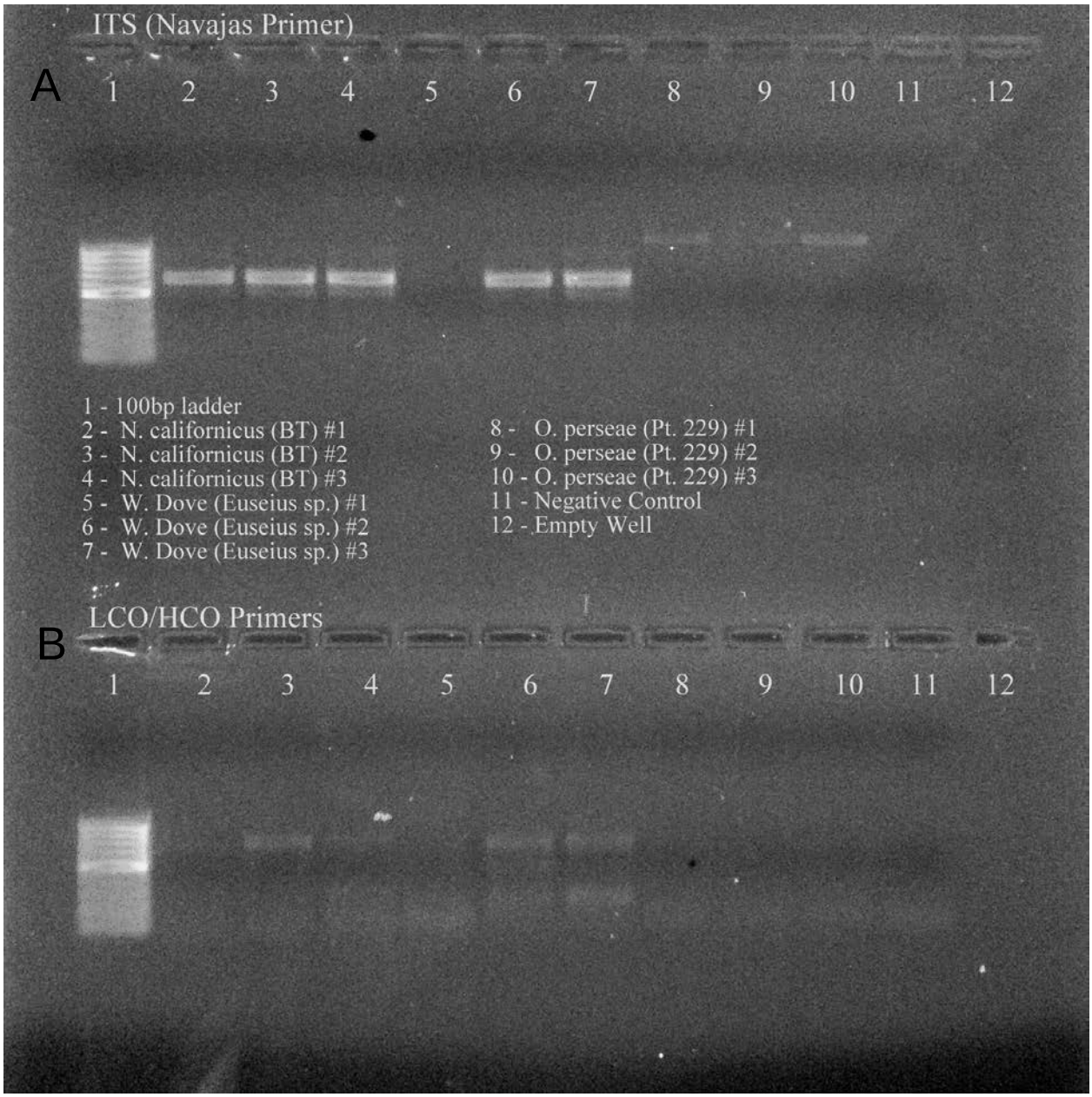

Fig. 7. Gel electrophoresis results for a subsample of predatory mites and persea mites using A) ITS primers and B) LCO/HCO primers.

Table 1. Geographic location, avocado variety, and elevation information for each site sampled in Mexico in 2012 for persea mite and associated predatory mites. Specimens for this DNA study were taken from leaves that were collected from a total of 42 trees (10 non-Hass and 32 Hass trees) in five different states in Mexico.

Site	Sample Date	Trees	State	Municipality	Variety	Elevation (M)
1	April 30	1	Michoacán	Tarímbaro	Non-Hass	1786
2	April 30	1	Michoacán	Tarímbaro	Non-Hass	1786
3	April 30	1	Michoacán	Morelia	Possibly Hass	1019
4	April 30	1	Michoacán	Pátzcuaro	Possibly Hass	2086
5	April 30	1	Michoacán	Pátzcuaro	Possibly Hass	2083
6	May 1	2	Michoacán	Uruapan	Possibly Hass	1631
7	May 1	1	Michoacán	Uruapan	Possibly Hass	1630
8	May 1	1	Michoacán	Charo	Possibly Hass	2021
9	May 1	5	Michoacán	Charo	Possibly Hass	2205
10	May 1	1	Michoacán	Hidalgo	Hass	2092
11	May 2	1	Michoacán	Zitácuaro	Non-hass	1980
12	May 2	2	Michoacán	Zitácuaro	Possibly Hass	2119
13	May 2	6	México	Coatepec-Harinas	Possibly Hass	2282
14	May 3	7	Puebla	Atlixco	Hass	1900
15	May 3	1	Puebla	Atlixco	Non-Hass	1912
16	May 3	4	Puebla	Atlixco	Non-Hass	1906
17	May 4	1	Morelos	Jiutepec	Hass	1389
18	May 4	1	Morelos	Jiutepec	Non-Hass	1392
19	May 5	2	Guanajuato	Acámbaro	Possibly Hass	1900
20	May 5	1	Guanajuato	Acámbaro	Possibly Hass	1864
21	May 5	1	Guanajuato	Salvatierra	Non-Hass	1765