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TERELLIA SERRATULAE IN LEBANON: HOST ADAPTATION IN *PICNOMON ACARNA*

by
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AN ABSTRACT OF THE THESIS OF

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Title: *Terellia serratulae* in Lebanon: host adaptation in *Picnomon acarna*.

*Terellia serratula*e is a flower head infesting tephritid fly, which is commonly found in Lebanon from April to October at all elevations. It is a polyphagous species that infests flower heads of six thistles belonging to *Cirsium*, *Carduu*s, and *Picnomon* genera. In a previous study, molecular and morphometric variations were detected between flies associated with the different host plants and showed that flies associated with *P. acarna* may be distinct. In this study, the extent of genetic divergence between host populations of *T. serratulae* was investigated using both mitochondrial and nuclear loci. The results showed that the *Terellia serratulae* populations associated with *Cirsium lappaceum* most likely constitute a genetically distinct host race compared to the other host populations and that the majority of flies associated with *P. acarna* clustered alone in a clade but also grouped with flies of the other host plants. The ultrastructure of the ovipositor of *T. serratulae* females was studied using scanning electron microscopy. Differences in the arrangement of central sensilla found at the very tip of the ovipositor were found between females of the different host populations. Further sampling is needed to shed light on the dynamics of gene flow between the different host populations in Lebanon.

CONTENTS

ILLUSTRATIONS

TABLES

To My

Beloved Family

CHAPTER I

INTRODUCTION

Host race formation has been studied in numerous insects; the most notable cases are observed in the *Tephritidae* family. The fruit flies of this family adapt and shift to different host plant species. Host shifts are mainly observed in sympatry (Bolnick and Fitzpatrick 2007). Sympatric speciation is the bifurcation of an evolutionary lineage without the existence of a geographical barrier (Berlocher and Feder 2002). In many instances, sympatric speciation is difficult to prove; however, many studies in population genetics have shed light on the subject (Bird, Fernandez-Silva, Skillings and Toonen 2012). Studying host races is similar to witnessing population divergence first hand rather than trying to study organisms that have already speciated (Bird *et al.* 2012). There are many factors that induce host shifting in tephritids, some of which are temporal such as host phenology and differences in insect eclosion times, other factors include spatial separations such as host- plant fidelity, in addition to morphological factors such as varieties in immature stages and ovipositional limitations. Also, competition can play a role in the expansion of hosts (Knio, Goeden and Headrick 1996). Moreover, breakthroughs in the branch of genetic sequencing make it possible to realize some previously overlooked differences in morphological features of flies.

The fruit fly, *Terellia serratulae*, has a large host range of thistles belonging to the *Asteraceae* family (Knio, Kalash and White 2002). This study revolves around those found in the unique host *Picnomon acarna* previously categorized as *Cirsium acarna*. The immature and the life cycle of *T. serratulae* on the *Picnomon acarna* host have

never been described, on the other hand ovipositor morphology and mitochondrial DNA sequencing, but, none from nuclear DNA, have yielded results that implicates that *T. serratulae* populations on *P. acarna* might be distinct from the rest of the populations on the other hosts.

Besides adding a host race model and contributing in the completion of missing pieces in the biodiversity and evolution of non- frugivorous tephritids, this study focuses also on the possibility of utilizing them as important biological control agents, similar to past experiences in controlling the thistles that have reached weed status (Headrick and Goeden 1998).

A. Tephritid Fruit Flies

Fruit flies are found in tropical, subtropical and temperate areas of the world. They belong to the family *Tephritidae*, which is one of the largest families in the order *Diptera* and comprises 4,200 species (Headrick and Goeden 1998; Foote, Blanc and Norrbom 1993).

Tephritids are mainly distinguished by the venation and pattern of their wings. Their wings can be hyaline or can have spots, bands or star-shaped patterns (Freidberg and Kugler 1989)

Tephritids employ two different food intake approaches and consequently can be divided into frugivorous and non-frugivorous species. Flies in the first group are regarded as economically important since they are agricultural threats (Freidberg and Kugler 1989), while those of the second group feed only on the vegetative structures and they are less studied. However, the non-frugivorous flies cause significant damage to their hosts by feeding on the seeds and hence reducing their competitive power (Zwölfer 1983; Harris 1989).

Non- frugivorous species mainly are associated with thistles of the *Asteraceae* family. They feed on their florets, achenes or receptacles. The *Asteraceae* are structurally diverse and denoted by their flower heads (Headrick and Goeden 1998). Several thistles such as the *Cirsium* and *Carduus* genera are aggressive weeds and necessitate the search for natural means to control their expansion as is the case in biological control projects (Zwölfer 1988).

B. Life Cycle

The life cycle of a typical fruit fly begins when the female oviposits its eggs in a plant tissue. After the egg hatches, it develops into a larva which sheds its skin and goes through 3 stages called instars; the larva continues feeding throughout its growth. Eventually, the third instar hardens into puparium that houses an inactive fourth instar which eventually becomes pupa (Foote *et al*. 1993). Pupation can occur in the host itself which is the case with the species associated with *Asteraceae*, or it can occur in the soil such as the species infesting fleshy fruits (Friedberg and Kugler 1989). After adults emerge and reach sexual maturity, they mate and a new cycle starts again (Foote *et al*. 1993).

C. Morphology of the *Tephritidae* Family

1. Eggs

The egg is white and ranges 0.5-1 mm in length. It has an anterior and a posterior end. The shape is elongate, cylindrical and it is not completely linear (Friedberg and Kugler 1989). The anterior portion has a pedicel which holds the micropyle and aeropyles. The posterior end of the egg is rounded and does not contain any opening (Headrick and Goeden 1998). The egg is covered with ridges that are

mostly abundant at the anterior.

2. Larvae

Larvae of the tephritids resemble the typical maggots of *Diptera*. There are three larval instars. The larvae have a soft and flexible cuticle and lack body appendages (Friedberg and Kuglar 1989). Their color varies from white to yellow and may have blackening at the caudal end (White 1988). They can be divided into two groups. The first group has a retracted head with a compressed body shape. The second group, that includes species of agricultural pests, has an elongated body with a narrow anterior end that holds the head with the mouth hooks (Christenson and Foote 1960). The difference between first larval and second or third larval instars is noticeable by their cephalopharyngeal skeleton and spiracles. Also, first instars are small and elongated while the shape of second and third instars varies from elongate to short and oval shaped (Friedberg and Kuglar 1989).

3. Pupae

During the development of the pupae inside the puparia, the gas exchange is facilitated through the larval trachea. The puparia use thoracic spiracles for breathing (Headrick and Goeden 1999). They retain some of the larval features such as their body shape, spiracles and mouth parts; they can be of brown, white, yellow, or even black colors (White 1988).

4. Adults

During the development of the pupae inside the puparia, the gas exchange is facilitated through the larval trachea. The puparia use thoracic spiracles for breathing

(Headrick and Goeden 1999). They retain some of the larval features such as their body shape, spiracles and mouth parts; they can be of brown, white, yellow, or even black colors (White 1988).

a. Head

The hypognathous head of tephritids is divided into distinct regions due to the surface sutures that separate them. The eyes are found on the sides of the head; they shine brightly and reflect colors. The ventral part of the head is occupied by the oral aperture where the proboscis is retreated. The maxillary palps are flat and are variable in length (Friedberg and Kugler 1989). Taxonomically important set of setae are divided into two types: acuminate and lanceolate. The acuminate are thin, dark in color and progressively narrowed towards the tip. The lanceolate setae are broader, lighter colored and progressively narrowed toward both ends (Foote *et al*. 1993).

b. Thorax

The thoracic structure is divided into: Prothorax, mesothorax and metathorax. The mesothoracic segment forms the majority of the thorax; it includes the mesonotum, which is separated into scutum and scutellum. Pollinose segments, hairs and setae on the thorax are important in the taxonomy of Tephritidae (Friedberg and Kugler 1989).

c. Abdomen

The fruit fly abdomen is divided into two parts: pre-abdomen and post-abdomen. The pre-abdomen consists of five segments in males and six segments in females. Each of the segments themselves is made of a tergite and a sternite, connected by a pleural membrane (Foot *et al*. 1993). In both males and females, the first two tergal

segments are fused together. Following the pre-abdomen, there are five post-abdominal segments in males and three in females (Friedberg and Kugler 1989). The female's post-abdomen has a reduced segment 6; the degree of reduction of this segment is taxonomically important. This is followed by the segment 7 terminalia, which includes the egg-laying tube (or ovipositor) (Kozanek and Belcari 2002).

d. Ovipositor Morphology and Ultrastructure

The ovipositor consists of 3 parts: oviscape, inversion membrane and aculeus. The ovipositor starts at the 7th post-abdominal segment. The latter has a conical shape, where a pair of spiracles is found. It has a sternite and tergite fused together with a reduced pleural membrane between them. The oviscape is found on this segment, it is a non-retractable and tube-like structure.

A highly specialized section of terminalia starts at segment 8 and continues to its adjacent structures, it is composed of the inversion membrane and the sclerotized aculeus. The inversion membrane, also called eversible membrane, is a retractable structure that facilitates the microscopic protrusion of the aculeus; this membrane is covered with a distinctive scale-like pattern. The tergite and sternite of this membrane are split into cuticular narrow bands with smooth surfaces, and they are called taeniae, which structurally strengthen the eversible sheath. Between each of the taeniae there exists an intertaenial membrane. There are also cuticular rods on the aculeus portion. The ones made of sternal rods are called valvae, while the tergal rods are found dorsally. The valvae are separated by a flexible membrane that allows for the movement of eggs suitable during oviposition. In some species, a suture can be seen at the apex of the aculeus. After the aculeus, come the cerci, where at the base a cloacal opening is seen (Kozanek and Belcari 2002).

A female first recognizes the host then oviposits her eggs because of the flexibility of the ovipositor; these activities are controlled by different types of sensillae situated all along the ovipositor. The morphology of the ovipositors and their associated sensillae is important to distinguish between different species of fruit flies.

The surface of the oviscape is covered by both microtrichia sensillae and mechanoreceptor sensillae; mechanoreceptors are also found on the base of the eversible membrane attached to the taenia and are called basitaenial sensillae. Chaetica sensillae are found on the cerci and the apex of aculeus, they are either situated inside specialized lateral grooves or distributed randomly. Cloaca may also contain microtrichia (Kozanek and Belcari 2002).

e. Wings

The pair of *Tephritidae* wings is located on the mesothoracic segment of the thorax; they are 1.8-3.0 times as long as wide. Usually, the apex of the wing is rounded or tapered. Different taxa have characterized patterns on their wings, making them very helpful in taxonomy. The pattern can involve streaks, spots, bands or have variety of colors. They can also be hyaline with the absence of any pattern. Another characteristic feature of wings is venation and the shapes of cells. The stigma, is the region situated at the apex of subcostal cell (Friedberg and Kugler 1989), it is also crucial in the classification of fruit flies.

D. Life History Strategies

There are 3 groups of tephritids according to their life history strategies:

Group I are the frugivorous ones that have a wide host range. These are generalist, multivoltine flies and relatively long lived as adults. In general, a frugivorous

tephritid female lays between 800 to 3000 eggs, and the larva developmental time inside the fruits ranges between 5-25 days (Zwölfer 1983).

Group II includes both specialist and generalist frugivorous species. However, the adults are short lived. Each female oviposits 50 to 400 eggs. Their larva developmental time inside the fruits ranges between 15 to 30 days.

Group III includes non-frugivorous species like *Trypetini*, *Myopitinae* and *Tephritinae* (White 1988). They are associated with the vegetative structures of their hosts. The females lay 50- 150 eggs. The larvae develop in 20- 40 days and sometimes undergo winter diapause, pupae might also overwinter.

E. Mating

In the majority of cases, mating occurs on the host plant. Chemical signals, or sex pheromones are emitted by the male from a specialized anal pouch (Friedberg and Kugler 1989).

In *Tephritinae* and *Myopitinae*, the male selects the flower head that will serve as the rendezvous site for mating, this selection is based on the flower head developmental stage, shape and smell. Afterwards, the male defends its territory. The mates recognize each other's by interesting courtship behaviors where body coloration and wing pattern play an important role. The mating rituals are continued by species-specific wing movements. All these create prezygotic reproductive isolation mechanisms, especially among sympatric species. In some species, the male delivers a "nuptial gift" to the female, also some males display post-mating trophallaxis where they touch the proboscis of females to present them a meal (Friedberg and Kugler 1989).

F. Movement and Oviposition

Fruit flies move in order to feed, mate and oviposit. There are two types of movement in fruit flies, dispersive and non-dispersive. The former group of flies tends to move frequently due to a decrease in host availability. Such flies travel long distances. The latter group of flies inhabits areas where the host plants are abundant and suitable for oviposition; such flies rarely disperse away to other areas.

The first step to oviposition is searching for a convenient site; it may be a vegetative structure or a fruit, depending on the specie. This decision is based on visual, olfactory and tactile cues. The female inserts the tip of the ovipositor and punctures the host tissue to finally extrude the eggs (Foote *et al*. 1993). The host site is then marked by deterring pheromones; this prevents other females from depositing their eggs on the same area (Averill and Prokopy 1989). The longevity of flies increases as oviposition sites decrease, since they use their energy for survival rather than oviposition.

G. Nutrition and Feeding

The *Tephritidae* adults need carbohydrate for energy and water for survival. A diet mixture consisting of carbohydrate, amino acids, vitamins and minerals are important for attaining sexual maturity, and to attain normal fertility, fecundity and egg production (Bateman 1972). While in some species, male gonadal maturation is independent of diet, and in females the absence of vitamins do not affect ovarian maturation (Knio, Goeden and Headrick 2007a).

In nature, adults feed on juices and tissues of damaged or decaying fruit, nectar from flowers, plant sap and bird feces. Honeydew secreted by *Homoptera* is also considered important in diet. Honeydew consists of hydrolyzed proteins, vitamin and minerals. Flies may also most likely feed in yeast cells and hyphal strands of fungus.

Increasing dietary essentials mentioned above has had a positive effect on the longevity of flies (Christenson and Foote 1960). Bacteria are ingested from fruit surfaces that offer an important protein source and result in increased fecundity (Knio *et al*. 2007a).

Feeding during larval stage and the number of new flies are correlated, it is known that a poor diet during the larval stage produces adults that have behavior and fecundity deficiencies (Bateman 1972).

The host plant is the main location where searching for food occurs. Visual and olfactory cues help the fly find its feeding area (Fletcher 1987). In some species females have been observed to survey the host surface then make a minute puncture in it with its ovipositor to return later on and feed on the exudates (Christenson and Foote 1960). Bacteria enter the alimentary canal of flies during their feeding that involves regurgitation and reingestion (Knio, Goeden and Headrick 2007b).

H. Abiotic Factors

1. Temperature

Temperature has a major effect on the rhythm of fruit flies; it affects their development, in which warm temperatures decrease their overwintering and cold temperature forces them to diapause.

Fecundity is also affected, where it is highest in warm temperatures and oviposition occurs at cooler ones. Temperature also plays an indirect role in fly abundance, since their hosts are affected by it.

2. Sunlight

Sunlight affects many aspects of the fruit flies including their fecundity, oviposition and eclosion. Mostly, a bright sunlight has a positive correlation of the

above mentioned parameters. Even mating habits are influenced by sunlight, where most tephritids mate at the gradually reducing light at dusk (Bateman 1972).

I. Host Races and Sympatric Speciation

There undoubtedly exists a large array of host plant-specific phytophagous insects and particularly the very well-studied cases of fruit flies (Bolnick and Fitzpatrick 2007). The relationship between plant and host can lead to reproductive isolation as a pleiotropic by-product. The plant in this case can be observed as exerting selection pressure on the insect and isolation can occur in the absence of geographical barriers (Feder, Berlocher and Opp. 1998). As such, host races have often been used as evidence for sympatric speciation (Drès and Mallet 2002).

Sympatric speciation is still a controversial topic between evolutionary biologists. The importance of the geographical context of speciation can't be ignored, but, it hinders the advancement in studies of divergence by debating on classifying taxonomies into certain types of speciation. It is more constructive to study modeling and measurable quantities, such as gene flow and selection (Fitzpatrick, Fordyce and Gavrilets 2008).

Host races as defined by Drès and Mallet, are "intermediates in the continuum between polymorphism and full species". To be identified as host race a previously panmictic population must acquire genetic polymorphisms. A panmictic population is one that individuals interbreed freely on any host (Fitzpatrick *et al*. 2008). The shift from host race to speciation requires the races to be reproductively isolated from each other with the cessation of gene flow. In the case of fruit fly *Rhagoletis* the degree of genetic variation is the key to adaptation to new hosts. The advancement of evolution and molecular phylogenetics can help identify different loci that may be responsible for

the adaptation to a host and host preference leading to lineage divergence (Bird *et al*. 2012).

J. Terellia Serratulae

1. Hosts and Phenology

An extensive study was conducted in Lebanon for the first time on flower-head infesting tephritids on thistles (*Asteraceae*). The Tephritids that were reared belonged to 18 species from 20 different species of thistles. Out of the 18 species 15 were recorded for the first time in Lebanon, of which the fly *Terellia serratulae* (Linnaeus) was shown to infest the flower-heads of *Cirsium phyllocephalum, Picnomon acarna* (formerly named *Cirsium acarna*) , *Cirsium lappaceum, Cirsium libanoticum* and *Carduus argentatus* (Knio *et al*. 2002). The different species in different host plants differ in their phenology and the host *P. acarna* seems to harbor a distinct host race of *Terellia serratulae* (Haddad, Smith, Al-Zein and Knio 2017).

2. Morphology

The adult is mostly small sized. The head region has a yellowish or brownish color and contains the proboscis and palps. It has setae of light brown color and dark brown ocellar spot, the antennal third segment is yellow. The mesotonum usually contains a black pattern, and the thoracic pleura have yellow and white stripes. Setae and yellow hears are present on the thorax. The abdomen can be of yellow or brown color and contains four spots that are black and triangular on all terga except the first one. The male has a distinct fifth abdominal tergum that is almost equal in length to the three proceeding terga combined and the oviscape is approximately of equal length to the three last terga of the abdomen. The wings are hyaline without any patterns

(Friedberg and Kugler 1989). The ovipositor morphology of *Terellia* populations might show a distinction between different host plants due to an adaptation undergone by females on their hosts (Haddad *et al*. 2017).

The larvae pupate within the flower-head of the host. They have an orange color with a brown caudal. The pupae can be of orange, reddish brown or dark brown color and range from 3.9 mm to 4.6 mm in length (White 1988).

K. Research on *Terellia serratulae* in Lebanon

In a previous study (Haddad *et al*. 2017), it was observed that *Terellia serratulae* larvae fed and destroyed seeds of their host plants, which can make them good candidates as biological agents for weed control. The fly was found abundantly on *Cirsium* species, but occurred at a low density on *Picnomon* species. Morphometric analysis using canonical discriminant analysis based on two head and three wing measurements could separate *T. serratulae* adults reared from different plant hosts into four distinct groups. The most important predictors in separating adults associated with different plant hosts were head length, wing width, in addition to the ovipositor (egg-laying tube) length in females (Haddad *et al*. 2017). Moreover, mitochondrial DNA sequencing demonstrated that the fly populations associated with *P. acarna* seem genetically distinct from the rest of the *T. serratulae* populations (Haddad *et al*. 2017). This genetic divergence may reflect a distinct host race. But, further molecular analysis and nuclear markers may provide more details. Also, the ultrastructure of the ovipositor needs to be investigated to further differentiate between females associated with the different host plants.

L. Aims of the Study

The major aim of this study is exploring the host race formation and host adapation of *T. serratulae* on *Picnomon acarna* in Lebanon and to evaluate the extent of genetic divergence between the populations associated with *P. acarna* and the rest of the plant host populations. The specific aims are:

- DNA sequencing of both mitochondrial and nuclear genes to determine the intraspecific variations in different hosts, through constructing and analyzing phylogenetic trees and haplotype networks of *T. serratulae* populations to determine the possibility of a distinct host race and host adaptation by studying genetic variation among different *T. serratulae* populations.
- Carrying out comparative studies of the ultrastructure of females' ovipositors reared from the different plant hosts using scanning electron microscope (SEM).

CHAPTER II

MATERIALS AND METHODS

A. Flower Head Collection

Flower heads from the *Picnomon*, *Cirsium* and *Carduus* genera were collected from different regions, at different elevations in Lebanon, from summer of 2014 until 2015. The flower heads were identified morphologically using Tohmé and Tohmé (2014), Post (1933) and Edgecombe (1970). The regions where the flower heads were collected from are the following: Ayoun El Siman, Faqra, Kartaba, Ehden, Falougha, Faraya, Hammana, Labweh, Laklouk and Halat.

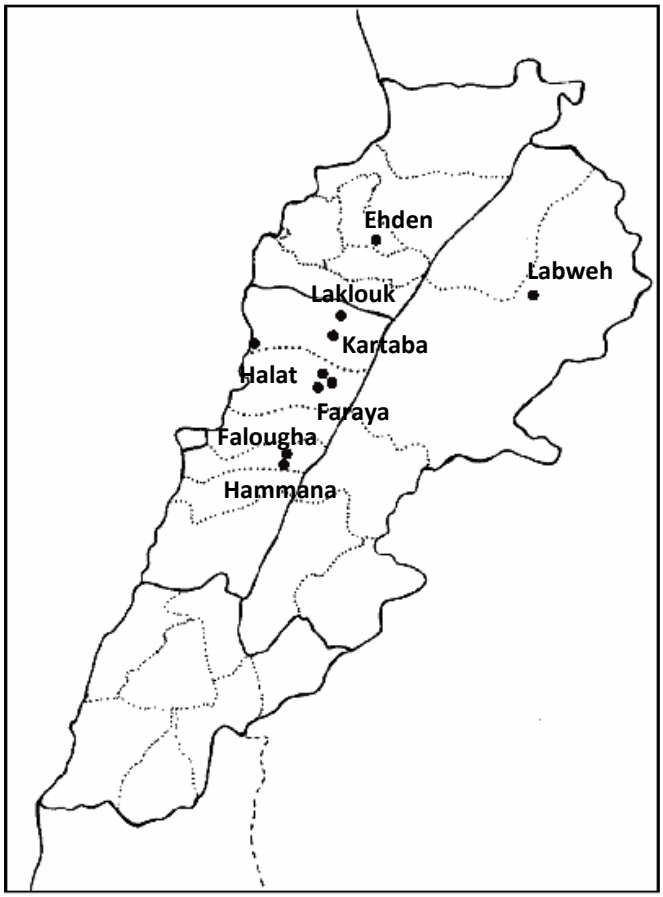


Fig. 2.1. Map of sampling locations in Lebanon

B. Fly Identification and Fly Rearing

The collected flower heads were placed in glass-topped, sleeved insectary cages where they were monitored daily for emergence of adult flies. Emerged flies were fed on honey streaked on the inner side of the cage glasses. Tephritid adults were identified using Freidberg and Kugler (1989) and White (1988), and stored at -70˚C in a Fisher Scientific Isotemp Freezer to be subsequent molecular analysis. It should be noted that many of the collected flower heads samples did not yield any tephritids; consequently, some regions were not represented in the consequent analyses.

C. Ultrastructure of the Female Ovipositor

The ovipositors of T. serratulae (N=12) were fixed in 2% gluteraldehyde for 12 hours, washed twice in distilled water, and post-fixed in 2% osmium tetroxide overnight. Then, they were washed twice in distilled water, dehydrated in an increasing concentration series of ethanol, and finally washed twice with absolute ethanol (with minimum 15 minutes at each concentration).

At the KAS Central Research Science Laboratory (American University of Beirut), the specimens were subjected to sonication using Elma D-78224 sonicator; then, they were critically point-dried using Quorum EMS K850-Critical Point Drier, mounted on stubs, and gold coated with 90% gold and 10% palladium (20nm gold thickness; 25 mA) using Carbon/Metal Coater Quorum Q150T ES before examination with a scanning electron microscope (SEM), at 15 KV accelerating voltage (Table 2.1).

Table 2.1. *Terellia serratulae* females reared from different host plants and used for studying the ultra-structure of the ovipositor using SEM

Fly Code	**Host Species**	**Location**
NM501	*Cirsium phyllocephalum*	Ehden
NM225	*Cirsium phyllocephalum*	Ehden
NM503	*Cirsium phyllocephalum*	Ehden
NM500	*Cirsium phyllocephalum*	Ehden
NM234	*Cirsium phyllocephalum*	Ehden
NM536	*Picnomon acarna*	Faraya
NM153	*Picnomon acarna*	Falougha
NM140	*Picnomon acarna*	Ayoun El Siman
NM141	*Picnomon acarna*	Ayoun El Siman
NM542	*Picnomon acarna*	Faraya
NM62	*Cirsium lappaceum*	Ayoun El Siman
NM49	*Cirsium lappaceum*	Ayoun El Siman

D. Molecular Studies on Adult Flies

1. DNA Extraction

The adult flies stored at -70˚C were used for DNA extraction. Each fly was removed from the freezer and directly placed in a 1.5 ml Eppendorf tube and homogenized for 1 min with 200µl of grinding buffer (10mM Tris-HCl PH 7.8, 10mM EDTA pH 8, 60 mM NaCl, 30 mM sucrose) using KONTES Pellet pestle. Then, 200 µl of lysis buffer (300 mM Tris-HCL pH 7.8, 1% SDS, 20 mM EDTA pH 8) were added to the sample, followed by mixing for about 30 seconds. The sample was kept on ice for 5 minutes. Next, it was centrifuged using Sigma 1-14 Microfuge at 15,000 rpm for 5 minutes. A volume of 200 µl of supernatant was transferred to a new tube, to which 260 µl of 4 M NaCl were added. The tube was then centrifuged again at 15,000 rpm for 5 minutes, and 400 µl of supernatant were transferred to a new tube over which 800 µl of absolute ethanol were added. The tube was then kept on ice for 5 minutes, and then centrifuged at 15,000 rpm for 5 minutes. The supernatant that forms was discarded and 500 µL of 75% ethanol were added to the tube which was then centrifuged for 5 minutes

at 15,000 rpm. The supernatant was discarded once again, and 150 µL of 75% ethanol were added to the tube. After a final centrifugation for 5 minutes at 15,000 rpm, the supernatant was pipetted out, and the pellet was left to dry at room temperature in open air. After drying, 200 µL of TE (10 mM Tris-HCL pH 7.8, 1 mM EDTA pH 8) were added to the tube and mixed by pipetting. The tube of DNA extract was then stored at -20˚C awaiting PCR amplification. It should be noted that samples of DNA stored from previous studies (Haddad *et al*. 2017) were also used in subsequent analyses.

2. Polymerase Chain Reaction Amplification and Sequencing of Nuclear DNA

A 480bp fragment of each of the ribosomal DNA nuclear gene ITS2 (Internal Transcribed Spacer Region 2) and D18S, a 438bp fragment of 18S ribosomal RNA nuclear gene was also amplified and sequenced using a different primer.

For the gene amplifications, the primers were: ITS2-F (5'-CCGAATTCTGTGAACTGCAGGACACAT-3') and ITS2-R (5'-CCCGGGATCCAAGCTTTATGCTTAAAATCAGGGGGT-3') modified with addition of EcoRI, HindIII, and BamHI sites for cloning from Porter and Collins (1991), and the primer pair D18SF1 (5'-GCAAAGCTGAAACTTAAAGG-3') and D18SR1 (5'-CTCGGTCTAGGAAATACACG-3') of Sayar, Smith, White, and Knio (2009), and also the primer pair 18H(5'-GCTGAAACTTAAAGGAATTGA-3') and 18J (5'-TCTAAGGGCATCACAGACCTGTTATTG-3') from Han and McPheron (1994).

Each of the PCR amplifications were conducted in a 50 µl total volume containing a 2 µl DNA template, 5 µl of PCR buffer (50 mM KCl, 10mM Tris-HCl pH 8.3 at 25˚C), 2.5 µL of 500 mM forward primer mix, 2.5 µL of 500 mM reverse primer mix, 5 µL of 200 µM each dNTP mix, 5 µL of 1.5 mM MgCl2, 26 µL of distilled water,

and 2 μL of 1 unit Taq polymerase diluted 1/25. The amplification reaction was conducted in a Bio-Rad DNA engine thermocycler with the following program: one cycle of 4 minutes at 94˚C; 35 cycles of 30 seconds at 93˚C, 30 seconds at 50˚C, 45 seconds at 72˚C; and one cycle of 10 minutes at 72˚C. To make sure if the PCR was successful, the products were electrophoresed on 1% agarose gel. The purifications of the PCR products were completed using a commercial kit (Illustra DNA and Gel Band Purification Kit, GE Healthcare and then re-checked for DNA by electrophoresis. Then, the purified products were all sent to sequencing to Macrogen Inc. (S. Korea).

3. Polymerase Chain Reaction Amplification and Sequencing of Mitochondrial DNA

A 690-base-pair (bp) of the mitochondrial gene NADH1 (Nicotinamide adenine dinucleotide dehydrogenase subunit 1) was amplified using the primers of Smith, Mcpheron and Kambhampati (2002): ND1-F: 5'-ATCATAACGAAAYCGAGGTAA-3' and ND1-R: 5'- CAA CCT TTT WGT GAT GC-3'. The 692-bp fragment of the COI (Cytochrome oxidase subunit I) gene was amplified and sequenced using the primers of Sayar *et al*. (2009): COX1F: 5'-TCAACWAATCATAAAGATATTGG-3' and COX1R: 5'-CCAAARAATCAAAATAAATGTTG-3'. The degeneracies (Y=C+T, W=A+T and R=A+G) were chosen because there is a single nucleotide polymorphism that would hinder the amplification process. The PCR amplifications were conducted using the same procedure in the sequencing of Nuclear DNA, the purified products were also sent for sequencing same as the nuclear ones.

4. Alignment of Sequences

The chromatograms of each of the obtained nuclear and mitochondrial

sequences were inspected for errors using ‘ChromasLite’ application. The chromatograms of those sequences which had irregular peaks were removed or excised manually. Then, the updated sequences were aligned using the ‘Clustal W Multiple Alignment’ method in the ‘BioEdit’ software.

5. Phylogenetic Trees

Phylogenetic trees were generated by Bayesian inference of phylogeny using the software MrBayes (Huelsenbeck and Ronquist 2001). The analysis was performed with the General Time Reversible model of nucleotide substitution. In the GTR model of nucleotide substitutions, all four DNA bases have unequal frequencies and each type of substitution has different rates of occurrence (Figure 2.2). Moreover, a gamma distribution model was assumed where all sites in a DNA sequence do not evolve at the same rate. The wide variation of DNA base frequencies among species that can be explained by the theory of directional mutation pressure should be taken into consideration during molecular analyses. Rates of mutation vary differently in different parts of the chromosome, this might be due to the structural differences of chromatin and this means that both DNA replication and repairing events may have different mechanisms in different domains of the chromosome (Cox 1972). In MrBayes the ‘invgamma’ command was chosen, where it allows for both rate variation among sites in the DNA and also accounts for a proportion of nucleotide sites that remain invariable. MrBayes uses Markov chain Monte Carlo to approximate the posterior probability depending on the sampled trees, the number of generations that the chain went through ranged from 20,000 to up to 1,000,000 trees, then a consensus tree was chosen, in other words, trees were scanned and either rejected or accepted based on the MCMC method.

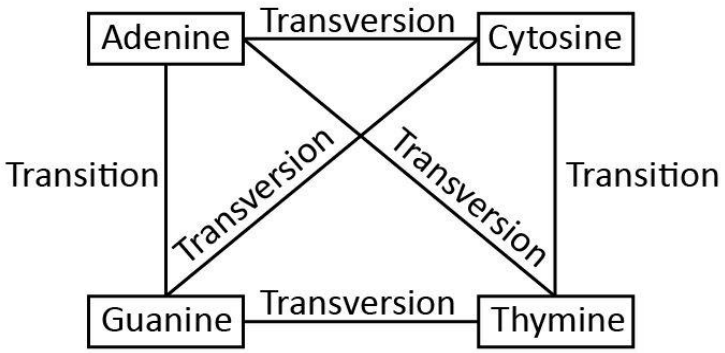


Fig. 2.2. The different types of single nucleotide substitutions

Graphs were generated using SPSS v. 20.0 from the output files to ensure that the analyzed sampled trees have reached stationarity, and that they have converged well. Also, the total tree length was examined for each sequence, short tree length being an indicator of a more parsimonious tree than a longer one. Finally, the output trees were drawn using FigTree v.1.4.2 (Bouckaert, Heled, Kühnert, Vaughan, Wu, Xie, Suchard, Rambaut and Drummond 2014).

All phylogenies contained their corresponding Drosophila melanogaster sequences as outgroups, Table 2.2 shows each of the outgroups. Three different trees were generated for the following nuclear primers: ITS2, D18S and 18H, one tree was the result of a concatenation of primers D18S and ITS2. Two mitochondrial gene trees were generated for the following primers: ND1 and COX1. For the concatenated D18S and ITS2 sequences, the outgroups RefSeq NR_133559.1 and Genbank: EU306667.1 were merged.

Table 2.2. Reference sequences for *Drosophila melanogaster*, the outgroup used in phylogenetic analyses

DNA Segment	**Outgroup, *Drosophila melanogaster***
D18S	RefSeq: NR_133559.1
ITS2	Genbank: EU306667.1
Concatenation of D18S with ITS2	Concatenation of RefSeq NR_133559.1 and Genbank: EU306667.1
COI	Genbank: AH005265.2
NADH1	RefSeq: NC_001709.1

6. Haplotype Analyses

Haplotype networks were generated using the PopART (Population Analysis with Reticulate Trees, Clement *et al*. 2002) software for both the nuclear and mitochondrial loci. The software collapses identical sequences into haplotypes. Gaps were considered as a fifth state. Two different methods were used for the networks; the first was the method of Templeton, Crandall and Sing (1992) (TCS method) and the second was Integer Neighbour-Joining (IntNJ) method.

7. Sequencing of Nuclear and Mitochondrial Gene Fragments

For the mitochondrial COX1 gene, a 607 bp fragment, corresponding to Genbank: AH005265.2 (1151-1757), could be read in all 15 fly sequences. For the mitochondrial ND1, a 532 bp region, aligned with RefSeq: NC_001709.1 (11880-12410), could be read in all 29 fly sequences.

For the nuclear genes, a 350 bp 18S fragment, corresponding to ReSeq: NR_133559.1 (1289- 1637), was read in all 30 fly sequences. For the ITS2 nuclear fragment, a 419 bp region, corresponding to Genbank: EU306667.1 (12- 430), could be read in all 20 fly sequences.

8. Bayesian Phylogenetic Analysis

To examine relatedness between the host populations and construct phylogenetic relationships between the different *T. serratulae* host populations, Bayesian phylogenetic analysis was conducted. This was based on the consensus trees generated for each of the gene fragments using MrBayes (Huelsenbeck and Ronquist 2001). Bayesian analysis allows for the determination of posterior probabilities for each node. These probabilities display clade support and consequently their reliability, while branch lengths of the trees are the average lengths of all the trees that were included in the consensus analysis. Mitochondrial sequences yielded more resolved trees than nuclear ones.

CHAPTER III

RESULTS

A. Molecular Studies on Adult *Terellia serratulae* Populations

Phylogenetic relationships, haplotype networks and extent of genetic divergence occurring in *T. serratulae* populations associated with *P. acarna* and other plant hosts were assessed using DNA sequences of nuclear and mitochondrial loci.

The numbers of flies associated with different plant hosts and used for nuclear and mitochondrial DNA analysis are summarized in Tables 3.1-3.3 and Tables 3.4-3.5, respectively.

Table 3.1. Number of flies whose ITS2 fragment was sequenced using the ITS2 primer per host plant

Host	**ITS2 fragment**
Cirsium phyllocephalum	5
Picnomon acarna	13
Cirsium lappaceum	2
Total	20

Table 3.2. Number of flies whose 18S fragment was sequenced using D18S1 primer per host plant

Host	**18S fragment**
Picnomon acarna	19
Cirsium lappaceum	3
Cirsium phyllocephalum	5
Carduus pycnocephalus	1
Carduus argentatus	2
Total	**30**

Table 3.3. Number of flies whose 18S fragment was sequenced using 18H primer per host per plant

Host	**18S fragment**
Cirsium phyllocephalum	5
Picnomon acarna	9
Cirsium lappaceum	1
Total	15

Table 3.4. Number of flies whose NADH1 fragment was sequenced using ND1 primer per host per plant

Host	**NADH1 fragment**
Cirsium phyllocephalum	6
Picnomon acarna	16
Carduus argentatus	3
Carduus pynocephalus	2
Cirsium lappaceum	2
Total	29

Table 3.5. Number of flies whose COI fragment was sequenced using COX1 primer per host per plant

Host	**COI fragment**
Cirsium phyllocephalum	4
Picnomon acarna	9
Cirsium lappaceum	2
Total	15

1. Sequencing of Nuclear and Mitochondrial Gene Fragments

For the mitochondrial COX1 gene, a 607 bp fragment, corresponding to Genbank: AH005265.2 (1151-1757), could be read in all 15 fly sequences. For the mitochondrial ND1, a 532 bp region, aligned with RefSeq: NC_001709.1 (11880-12410), could be read in all 29 fly sequences.

For the nuclear genes, a 350 bp 18S fragment, corresponding to ReSeq:

NR_133559.1 (1289- 1637), was read in all 30 fly sequences. For the ITS2 nuclear fragment, a 419 bp region, corresponding to Genbank: EU306667.1 (12- 430), could be read in all 20 fly sequences.

2. Bayesian Phylogenetic Analysis

To examine relatedness between the host populations and construct phylogenetic relationships between the different *T. serratulae* host populations, Bayesian phylogenetic analysis was conducted. This was based on the consensus trees generated for each of the gene fragments using MrBayes (Huelsenbeck and Ronquist 2001). Bayesian analysis allows for the determination of posterior probabilities for each node. These probabilities display clade support and consequently their reliability, while branch lengths of the trees are the average lengths of all the trees that were included in the consensus analysis. Mitochondrial sequences yielded more resolved trees than nuclear ones.

a. Trees Generated by Nuclear Loci

Two different phylogenetic trees for the two nuclear genes were constructed; a third tree was constructed by combining the two nuclear sequences, 18S (sequenced with D18S1) and ITS2.

The tree for the ITS2 nuclear gene (Figure 3.1) shows short branch lengths in most individuals, especially close to the root, indicating no rapid divergence among the host populations; in other words, relationships among them are not fully resolved. Although five flies reared from *P. acarna* formed a distinct group, ITS2 does not seem to be a good genetic marker to resolve the complex genetic relations within *T. serratulae* hosts.

Fig. 3.1. Phylogeney of *Terellia serratulae* host populations using ITS2 sequences

The phylogenetic tree constructed using 18S sequences obtained using the two primers (Figures 3.2 and 3.3) shows unresolved relationships between the vast majorities of the flies analyzed. The 18H primer yielded a very short gene fragment compared to the D18S1 primer; sequences, generated using this primer, were excluded from any further analyses.

It seems that although nuclear markers are useful for differentiating between closely related species of tephritids, they have inconsistent modes of molecular evolution and are of limited utility to resolve relationships within variable species (Muraji, Nakahara and Sugimoto 1998).

Fig. 3.2. Phylogeney of *Terellia serratulae* host populations using 18S sequences with D18S1

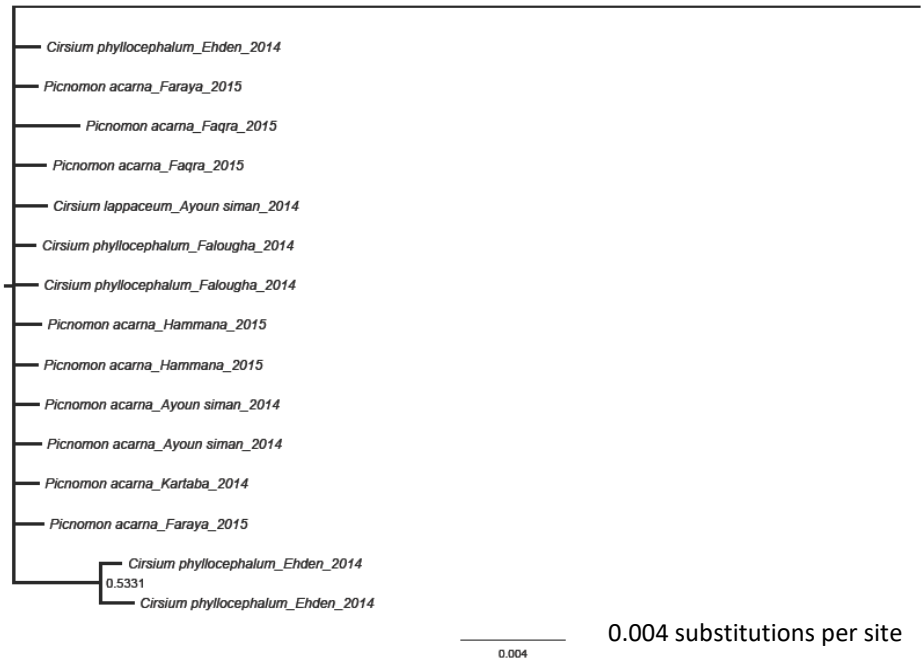


Fig. 3.3. Phylogeny of *Terellia serratulae* host populations using I8S sequences with 18H

Since nuclear loci alone did not resolve the relations among *T. serratulae* hosts, both 18S and ITS2 were concatenated and analyzed, yielding a consensus tree that separated *C. lappaceum* associated flies into a clade with 0.8 probability support (Figure 3.4). There was also one separate clade comprised of some *P. acarna* associated flies. However, the rest of *P. acarna* and *C. phyllocephalum* flies were not clearly resolved.

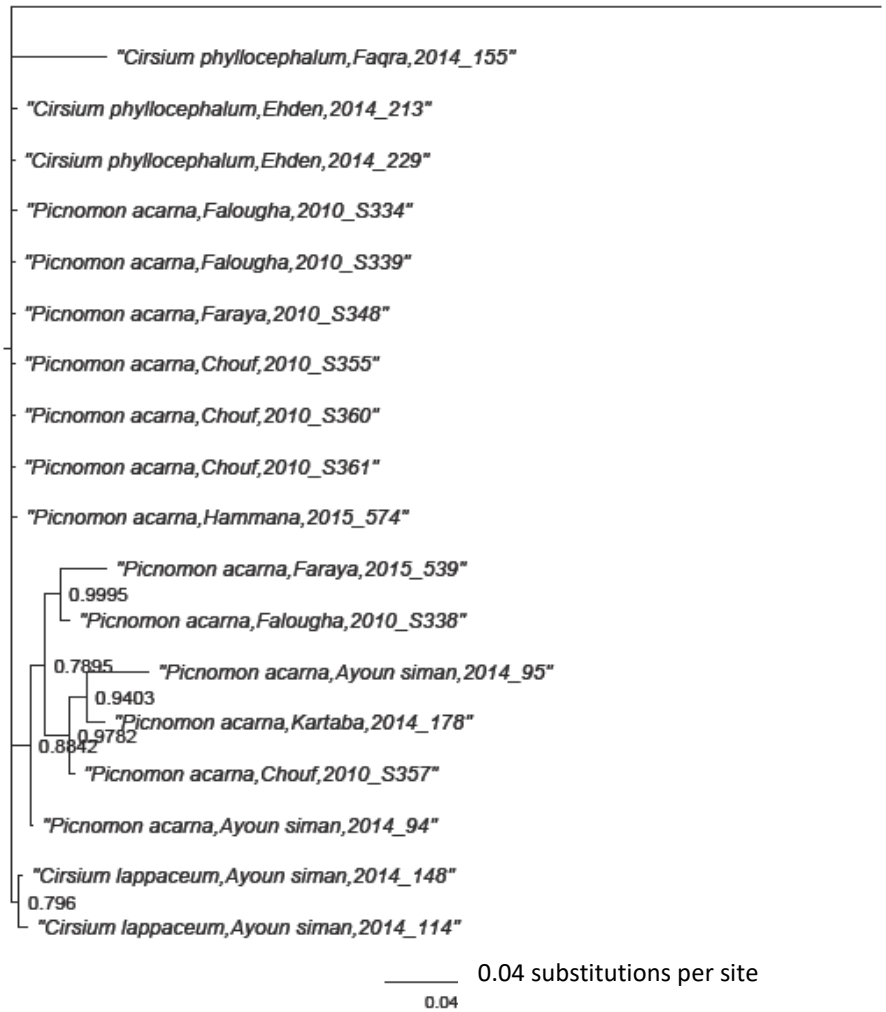


Fig. 3.4. Phylogeny of *Terellia serratulae* host populations using concatenated 18S and ITS2

b. Trees Generated by Mitochondrial Loci

Phylogenetic trees constructed using the two mtDNA sequences, ND1 and

COX1 (Figures 3.5 and 3.6), show better resolved relationships among host populations of *T. serratulae*.

Results for ND1 analysis showed two distinct clades, I and II (Figure 3.5). Within clade I, *C. lappaceum* associated flies formed a separate clade with 0.97 probability support. As for clade II, it was comprised predominantly of *P. acarna* associated flies; it also included two *C. phyllocephalum* associated flies.

Fig. 3.5. Phylogeny of *Terellia serratulae* host populations using ND1 sequences

Results for COXI analysis also showed two distinct clades, I and II (Figure 3.6). Within clade I, flies associated with *C. phyllocephalum* clustered with few *P. acarna* flies. Clade II was further divided into two clades with high posterior probabilities (greater than 0.99): one distinct clade comprised of *C. lappaceum* associated flies and another comprised of *P. acarna* associated flies but with one *C. phyllocephalum* associated fly.

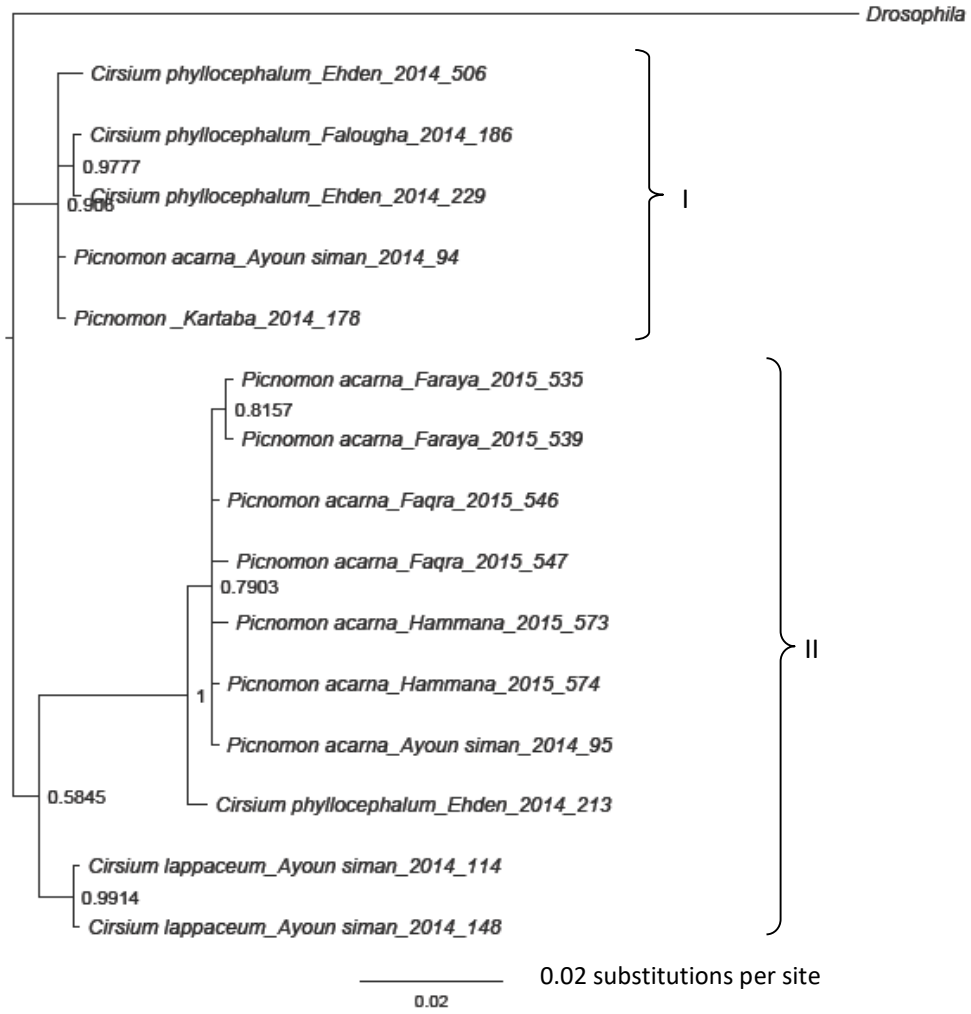


Fig. 3.6. Phylogeny of *Terellia serratulae* host populations using COX1 sequences

3. Haplotype Networks

At the population level, genetic relationships within and among populations are better represented by haplotype networks. This is because within species, a bifurcating tree phylogeny is not a suitable representation of the underlying history (Posada and Crandall 2001). Haplotype networks were generated using both the TCS and integer neighbor joining methods. Although both methods gave similar results, we adopted those of the TCS method because they were more parsimonious.

A total of 94 sequences were analyzed: 30 sequences belonging to 5

haplotypes for the 18S fragment (Table 3.6), 20 sequences belonging to 13 haplotypes for ITS2 (Table 3.7), 15 sequences belonging to 7 haplotypes for COX1 (Table 3.8) and 29 sequences belonging to 13 haplotypes for ND1 (Table 3.9).

Table 3.6. Haplotypes of *T. serratulae* populations based on 18S ribosomal DNA

Haplotype (D18S)	**Host**	**Location**	**Number of Sequences**
H1	*Cirsium phyllocephalum* (155)	Faqra	4
	Cirsium phyllocephalum (229)	Ehden	
	Picnomon acarna (S338)	Falougha	
	Picnomon acarna (S360)	Chouf	
H2	*Picnomon acarna* (539, S345, S348, 535)	Faraya	22
	Picnomon acarna (94, 95)	Ayoun siman	
	Picnomon acarna (178, 184)	Kartaba	
	Picnomon acarna (546, 547)	Faqra	
	Cirsium phyllocephalum (213)	Ehden	
	Cirsium lappaceum (S217)	Faraya	
	Carduus pycnocephalus (S255)	Koubba	
	Carduus argentatus (S316, S320)	Baysour	
	Picnomon acarna (S339)	Falougha	
	Picnomon acarna (S355, S357, S361)	Chouf	
	Picnomon acarna (573, 574)	Hammana	
	Cirsium phyllocephalum (186)	Falougha	
H3	*Cirsium phyllocephalum* (506)	Ehden	2
	Cirsium lappaceum (114)	Ayoun siman	
H4	*Picnomon acarna* (S334)	Falougha	1
H5	*Cirsium lappaceum* (148)	Ayoun siman	1

Table 3.7. Haplotypes of *T. serratulae* populations based on ITS2 ribosomal DNA

Haplotype (ITS2)	**Host**	**Location**	**Number of Sequences**
H1	*Picnomon acarna* (S334, S339)	Falougha	7
	Picnomon acarna (S348)	Faraya	
	Picnomon acarna (S355, S360, S361)	Chouf	
	Picnomon acarna (574)	Hammana	
H2	*Cirsium phyllocephalum* (213, 229)	Ehden	2
H3	*Picnomon acarna* (95)	Ayoun siman	1
H4	*Picnomon acarna* (S357)	Chouf	1
H5	*Picnomon acarna* (94)	Ayoun siman	1
H6	*Picnomon acarna* (S338)	Falougha	1
H7	*Picnomon acarna* (539)	Faraya	1
H8	*Picnomon acarna* (178)	Kartaba	1
H9	*Cirsium phyllocephalum* (185)	Falougha	1
H10	*Cirsium lappaceum* (148)	Ayoun siman	1
H11	*Cirsium lappaceum* (114)	Ayoun siman	1
H12	*Cirsium phyllocephalum* (233)	Ehden	1
H13	*Cirsium phyllocephalum* (155)	Faqra	1

Table 3.8. Haplotypes of *T. serratulae* populations based on COI mitochondrial DNA

Haplotype (COI)	**Host**	**Location**	**Number of Sequences**
H1	*Cirsium lappaceum* (114,148)	Ayoun siman	2
H2	*Cirsium phyllocephalum* (186)	Falougha	2
	Cirsium phyllocephalum (229)	Ehden	
H3	*Picnomon acarna* (94)	Ayoun siman	2
	Picnomon acarna (178)	Kartaba	
H4	*Picnomon acarna* (535, 539)	Faraya	6
	Picnomon acarna (546)	Faqra	
	Picnomon acarna (573, 574)	Hammana	
	Picnomon acarna (95)	Ayoun siman	
H5	*Picnomon acarna* (547)	Faqra	1
H6	*Cirsium phyllocephalum* (213)	Ehden	1
H7	*Cirsium phyllocephalum* (506)	Ehden	1

Table 3.9. Haplotypes of *T. serratulae* populations based on ND1 mitochondrial DNA

Haplotype (NAHD1)	**Host**	**Location**	**Number of Sequences**
H1	*Picnomon acarna* (539, S345)	Faraya	8
	Picnomon acarna (547)	Faqra	
	Picnomon acarna (574)	Hammana	
	Picnomon acarna (S361, S360)	Chouf	
	Picnomon acarna (S334, S338)	Falougha	
H2	*Picnomon acarna* (S348)	Faraya	2
	Picnomon acarna (S342)	Falougha	
H3	*Picnomon acarna* (573)	Hammana	2
	Cirsium phyllocephalum (229)	Ehden	
H4	*Cirsium lappaceum* (148)	Ayoun siman	2
	Cirsium lappaceum (S217)	Faraya	
H5	*Cirsium phyllocephalum* (506, 213)	Ehden	4
	Picnomon acarna (95)	Ayoun siman	
	Carduus argentatus (S316)	Baysour	
H6	*Carduus argentatus* (S257)	Baaklin	2
	Carduus pycnocephalus (S261)	Koubba	
H7	*Cirsium phyllocephalum* (185, 186)	Falougha	2
H8	*Picnomon acarna* (178)	Kartaba	1
H9	*Carduus pycnocephalus* (S255)	Koubba	1
H10	*Carduus argentatus* (S320)	Baysour	1
H11	*Picnomon acarna* (94)	Ayoun siman	1
H12	*Cirsium phyllocephalum* (155)	Faqra	1
H13	*Picnomon acarna* (S355)	Chouf	2
	Picnomon acarna (S339)	Falougha	

The ITS2 network revealed that all *Picnomon acarna* haplotypes (H1, H3, H4, H5, H6, H7, and H8) have a common hypothetical ancestor; they were divided into 3 clusters and all are nested within each other (Figure 3.7). Moreover, the two haplotypes associated with *C. lappaceum* branched separately, but were more closely related to haplotypes of *C. phyllocephalum* associated flies.

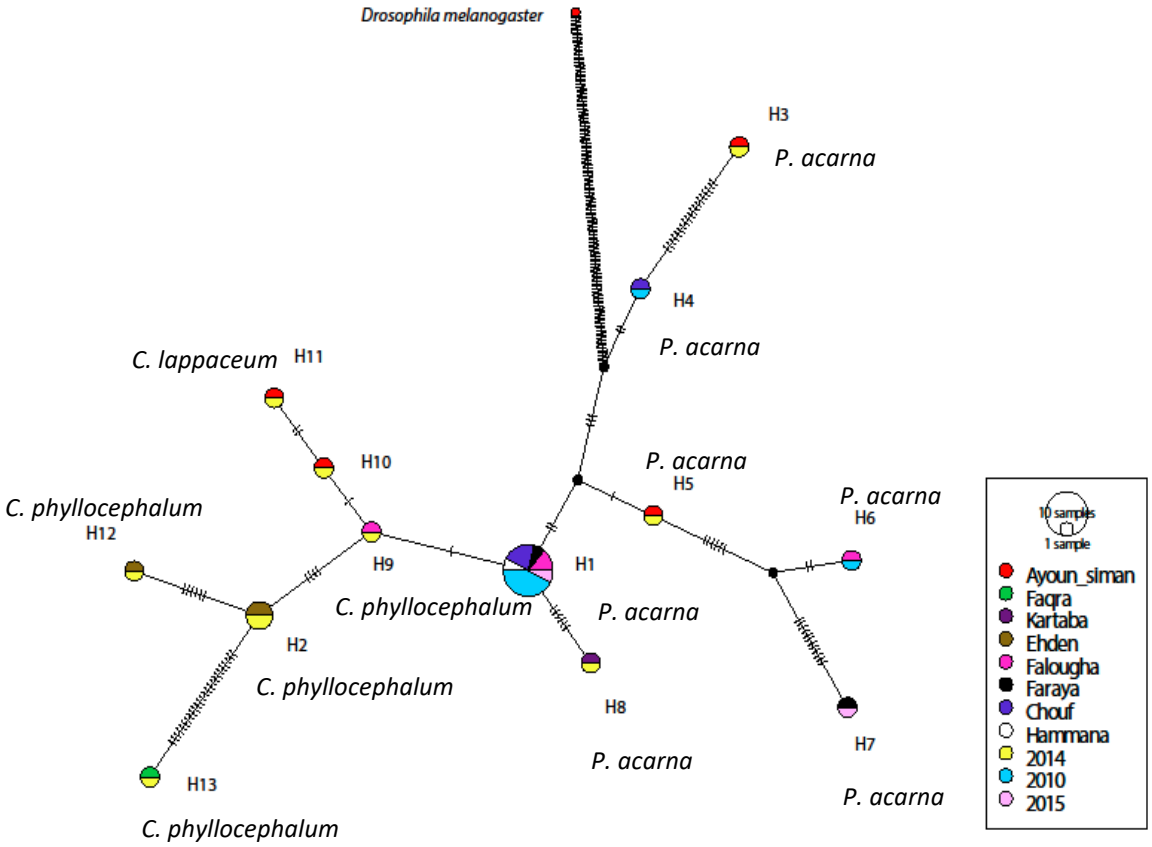


Fig. 3.7. Haplotype network generated using ITS2 sequences

The 18S network (Figure 3.8) was unresolved, showing that different flies associated with different hosts from different geographic locations had the same haplotype.

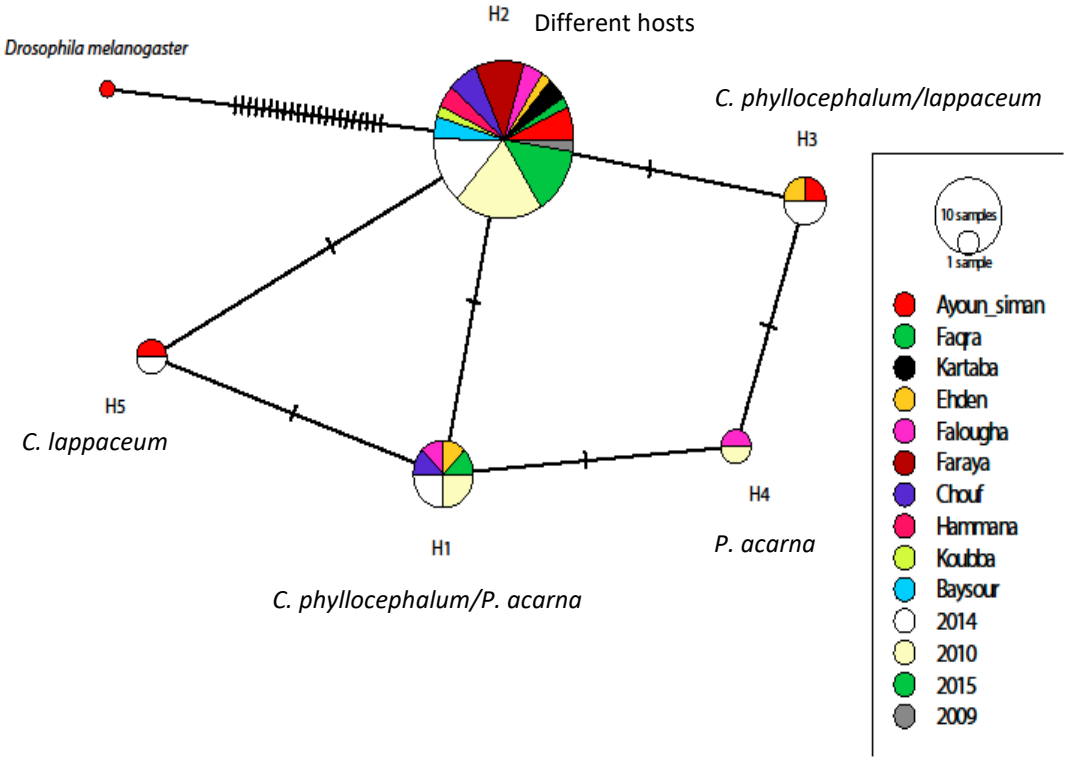


Fig. 3.8. Haplotype network generated using 18S sequences

The ND1 network (Figure 3.9) reveals that flies associated with *C. lappaceum* are somewhat genetically distant from the rest of the flies which clustered in two groups. The first one is primarily comprised of haplotypes from *P. acarna* associated flies while the second is comprised of haplotypes from *Carduus*, *C. phyllocephalum*, and few *P. acarna* associated flies. Similar results were obtained in the COX1 network (Figure 3.10).

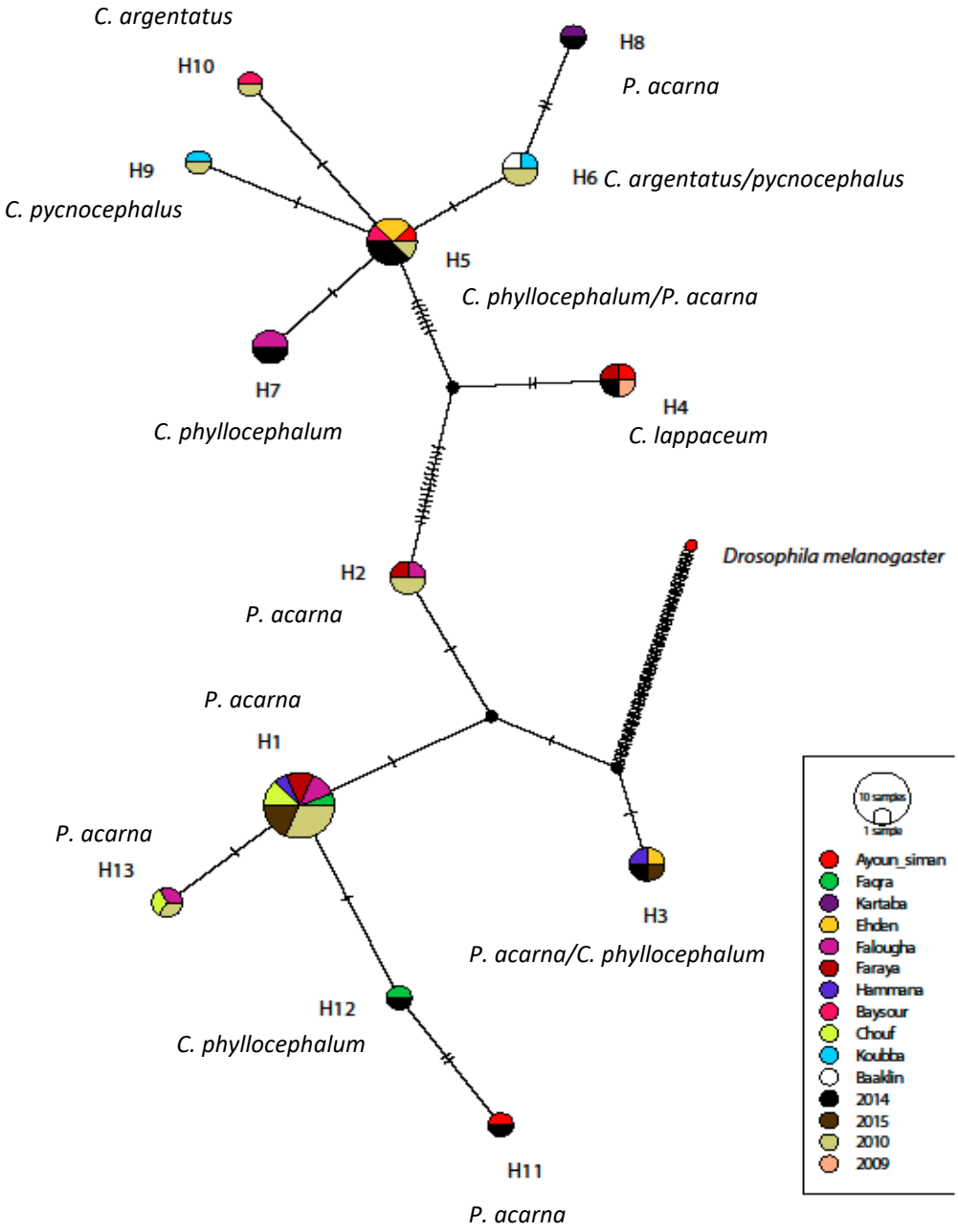


Fig. 3.9. Haplotype network generated using ND1 sequences

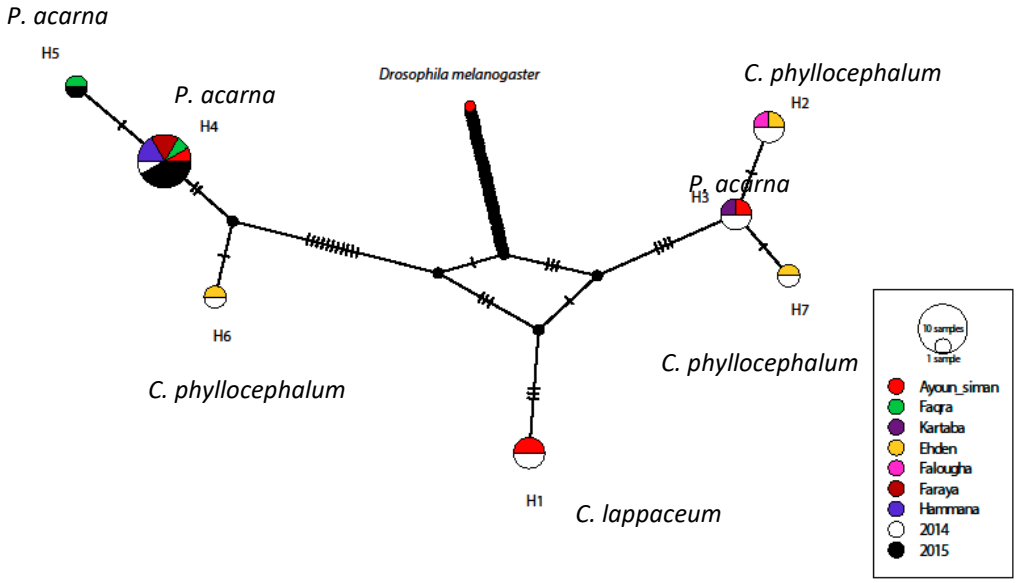


Fig. 3.10. Haplotype network generated using COX1 sequences

B. Ultrastucture of Ovipositors

The ultrustructure of the ovipositor *Terellia serratulae* was studied using electron microscopy. In addition, ovipositors from *T. serratulae* females reared from different plant hosts were compared.

1. Ovipositor of Terellia serratulae Females Reared from Picnomon acarna

The external anatomy of *T. serratulae* ovipositor is composed of the seventh abdominal segment (or oviscape), the eversible membrane, and the aculeus (ovipositor) (Figure 3.11). When not in use, the ovipositor is retracted and not visible; it rests in the modified seventh abdominal segment. During oviposition, mating, and defecation, the ovipositor is extended and becomes visible.

The seventh abdominal segment is sclerotized and conical in shape. It is

covered with tactile hairs (Zacharuk, Lee and Berube 1986) (Figure 3.11A).

The eversible membrane connects the ovipositor to the seventh abdominal segment (oviscape). It is composed of a basal and distal region. When the ovipositor is retracted, part of basal sheath of the eversible membrane is visible, but the distal sheath is not visible unless the ovipositor is fully extended. The eversible membrane is covered with heavily sclerotized and scale-like acanthae (Figure 3.11B; Figure 3.12B). The acanthae of *T. serratulae* point anteriad (toward the head); they are acuminate, and not sharply pointed as those of *Trupanea nigricornis* (*Coquillett*) and *Trupanea bisetosa* (*Coquillett*) (Knio *et al*. 2007).

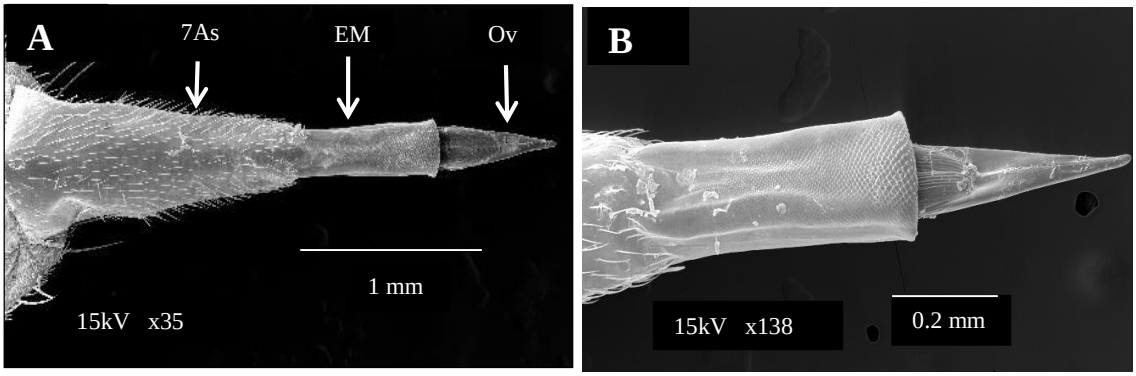


Fig. 3.11. **A.** Scanning electron micrographs of the ovipositor of *Terellia serratulae* reared from *Picnomon acarna* showing: A. the seventh abdominal segment (7As), eversible membrane (EM) and ovipositor (Ov). **B.** Scanning electron micrographs of the ovipositor of *Terellia serratulae* reared from *Picnomon acarna* showing: the basal region (EM) of the eversible membrane and the ventral region of the aculeus (Ov).

The ovipositor (aculeus) consists of three parallel tube-like processes, two short ventral processes and one longer dorsal process (Figure 3.12A, C). The two ventral processes are parallel and separated by a 'deepening fossa' or ventral groove and terminates in the cloaca, which is a common opening to the digestive and reproductive tracts (Stoffolano and Yin 1987). The surfaces of the ventral processes show a number

of sensilla (Figure 3.12 D), which are hair-like and found in a sunken, shallow socket. These were identified as mechanoreceptors, innervated by one neuron and not associated with chemoreception (Stoffolano and Yin 1987).

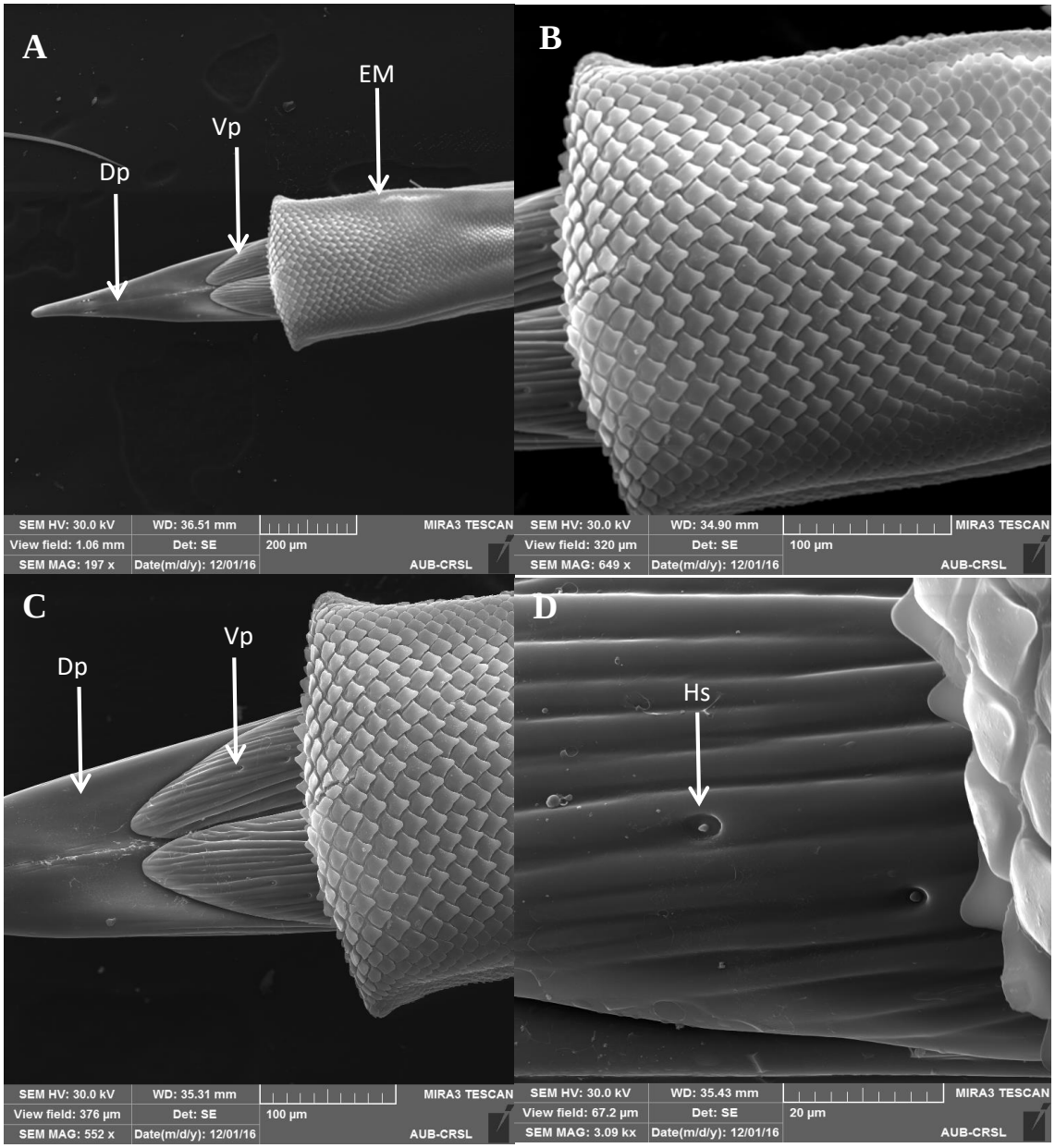


Fig. 3.12. **A.** Scanning electron micrographs of the ovipositor of *P. acarna* females showing the basal region of the eversible membrane (EM) and the ovipositor (Ov). **B.** Scanning electron micrographs of the ovipositor of *P. acarna* females showing the scale-like acanthae of the eversible membrane. **C.** Scanning electron micrographs of the ovipositor of *P. acarna* females showing the dorsal process and two ventral processes. **D.** Scanning electron micrographs of the ovipositor of *P. acarna* females showing the hair-like sensilla (Hs) found on the surface of the ventral processes

The tip of the dorsal process (ovipositor tip) has a blunt apex (tip) (Figure 3.13A). It bears two ventrolateral grooves, each located distally from the ventral processes. Each ventrolateral groove bears three elongated sensilla (Figure 3.13B). These function as chemosensilla and they have open tips. Each sensillum appears to contain 3-4 chemosensitive neurons and one mechanosensillum (Stoffolano and Yin 1987).

At the very tip of the aculeus, there are campaniform sensilla that we refer to as central campaniform sensilla (Figure 3.13D,E, F); these appear knob-like, enclosed in an ellipsoidal sunken socket. They are about 10, arranged in two semi-circular rows, and very close to the very tip of the ovipositor. These sensilla have a somewhat elevated knob, and a rather obtuse shape in *T. serratulae* females reared from *P. acarna*.

Basally on the dorsal process and beyond the ventral grooves (between the ventral processes and the ventral grooves), there are numerous campaniform sensilla at either sides of the midline of the ovipositor (at both edges), referred to as shallow-campaniform sensilla, as their knob-like process is less elevated and shallower; they are also enclosed in a sunken depression or socket (Figure 3.13 B, C).

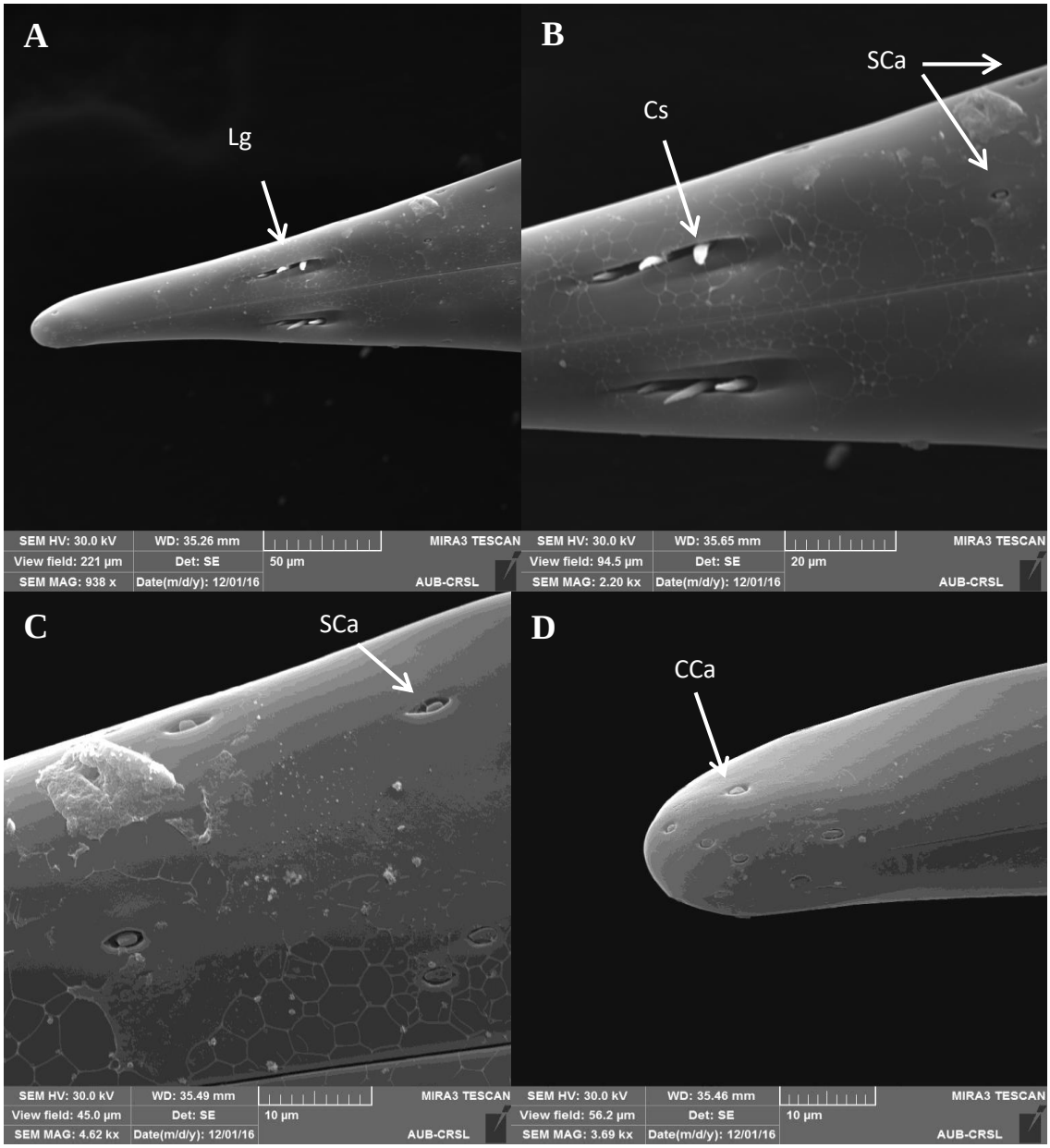
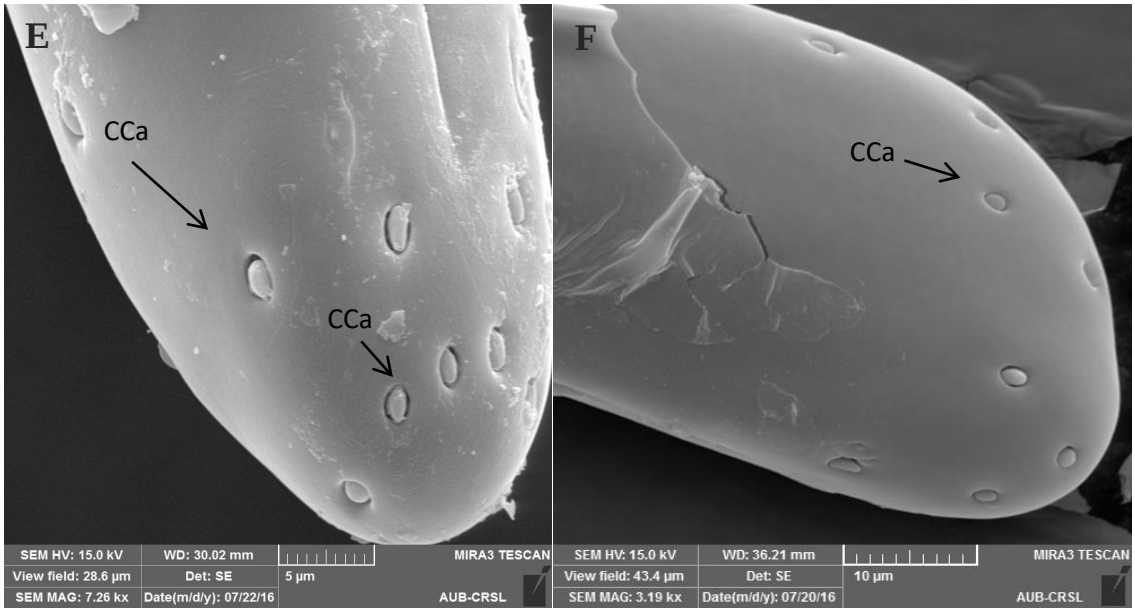


Fig. 3.13. **A.** Scanning electron micrographs of the ovipositor (aculeus) of *T. serratulae* showing the dorsal process with the two ventrolateral grooves (Lg). **B.** Scanning electron micrographs of the ovipositor (aculeus) of *T. serratulae* showing ventrolateral grooves containing each three chemosensilla (Cs). **C.** Scanning electron micrographs of the ovipositor (aculeus) of *T. serratulae* showing the campaniform sensilla (SCa) on the surface of the basal region of the dorsal process (between the lateral grooves and the ventral processes). **D.** Scanning electron micrographs of the ovipositor (aculeus) of *T. serratulae* showing the aculeus tip with its associated campaniform sensilla.



"Fig. 3.13 – *Cont'd*". **E.** Scanning electron micrographs of the ovipositor (aculeus) of *T. serratulae* showing the aculeus tip and the disposition of the elevated, central campaniform sensilla (CCa). **F.** Scanning electron micrographs of the ovipositor (aculeus) of *T. serratulae* showing the aculeus tip and the disposition of the elevated, central campaniform sensilla (CCa)

2. Ovipositor of Terellia serratulae Females Reared from Cirsium phyllocephalum

The ovipositor of *T. serratulae* reared from *C. phyllocephalum* is also composed of the seventh abdominal segment, the eversible membrane, and the aculeus (ovipositor) (Figure 3.14A).

The basal sheath of the eversible membrane bears heavily sclerotized and scale-like acanthae (Figure 3.14C) similar to those of *T. serratulae* females reared from *P. acarna*.

The ovipositor with its two short ventral processes and one longer dorsal process are shown in Figures 3.14B and 3.15A. The two ventral processes are also covered with hair-like sensilla enclosed in a shallow socket (Figure 3.14D).

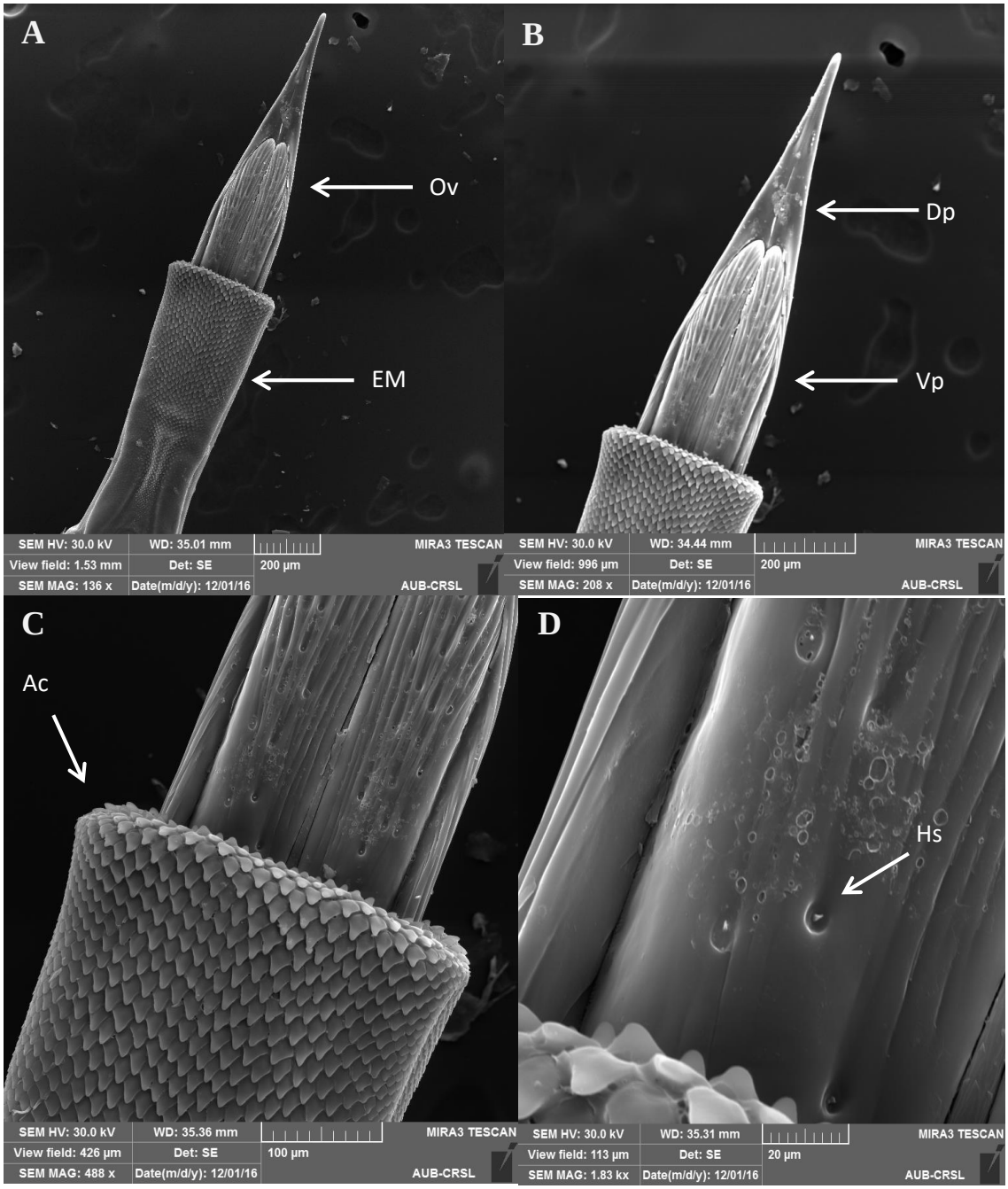


Fig. 3.14. **A.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing the eversible membrane (EM) and ovipositor (Ov). **B.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing the basal sheath of the eversible, the ovipositor with its ventral processes (Vp) and dorsal process (Dp). **C.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing the scale-like acanthae (Ac) on the basal sheath. **D.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing the hair-like sensilla (Hs) found on the surface of the ventral processes.

The tip of the dorsal process (ovipositor tip) has a narrow and blunt apex (tip), compared to the relatively wider ovipositor tip in females reared from *P. acarna* (Figures 3.15C and D). The ventrolateral grooves also bear three elongated sensilla (Figures 3.15B and C). The location of these ventrolateral grooves relative to the ovipositor tip were previously demonstrated to be further away from the tip in *T. serratulae* females associated with *C. phyllocephalum*, and much closer to the ovipositor tip in females associated with *P. acarna* (Haddad *et al.* 2017).

At the very tip of the aculeus, there are about 10 central campaniform sensilla, which are also knob-like and enclosed in an ellipsoidal sunken socket. However, these sensilla appear slightly more elevated than in *T. serratulae* females associated with *P. acarna*. Moreover, their disposition is different as they are dispersed and arranged in two longitudinal lines (Figures 3.15 D, E and F).

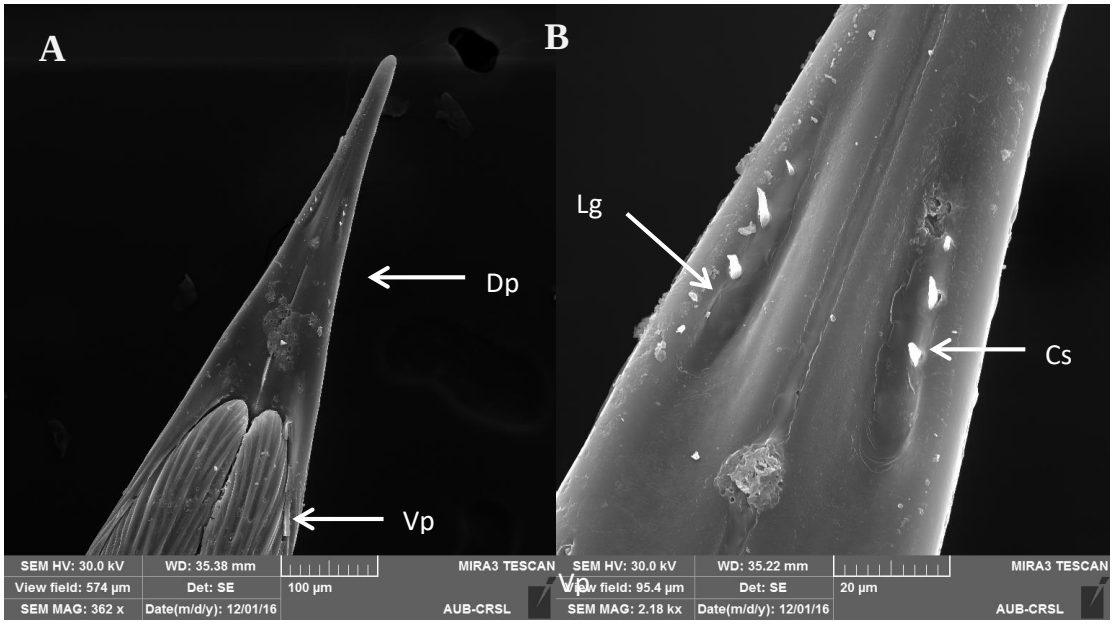
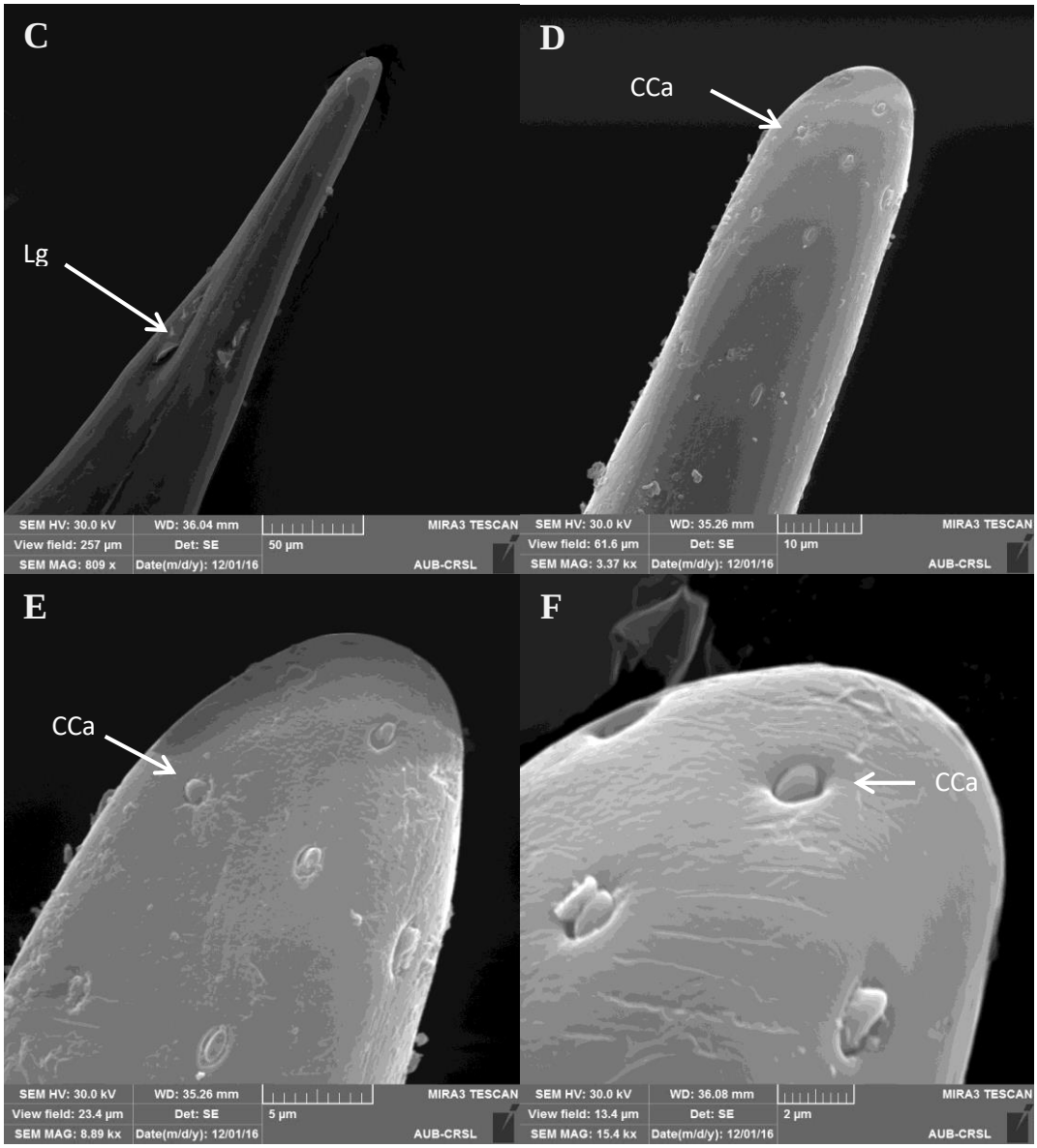


Fig. 3.15. **A.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing the ovipositor with its long dorsal process (Dp) and two ventral processes (Vp). **B.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing the lateral grooves, each bearing three long chemosensilla (Cs).



"Fig. 3.15 – *Cont'd*". **C.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing the position of the lateral grooves (Lg) at the lateral sides of the ovipositor tip. **D.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing the tip of the ovipositor with its associated central campaniform sensilla (CCa) (SCa). **E.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing central campaniform sensilla dispersed and position in two longitudinal rows. **F.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium phyllocephalum* showing central campaniform sensillae with its elevated knob-like shape, sunken in a socket.

3. Ovipositor of Terellia serratulae Females Reared from Cirsium lappaceum

The external anatomy of the ovipositor of *T. serratulae* females associated with *C. lappaceum* also shows an eversible membrane with sclerotized acanthae (Figure 3.16A). Similarly, the ovipositor bears two ventro-lateral grooves with three elongated chemosensilla per groove (Cs) (Figure 3.16B and D) and numerous shallow campaniform sensilla on the surface of the basal region of the dorsal process (Figure 3.16B and C).

On the other hand, unlike females reared from *P. acarna*, the tip of the ovipositor bears about 10 central central campaniform sensilla in two longitudinal rows. This disposition of the central campaniform sensilla is similar to females associated with *C. phyllocephalum*; however, the knob-like structure of the sensilla appear more pronounced and elevated in *C. lappaceum* female and the sensilla are not as dispersed or spaced (Figure 3.16E and H).

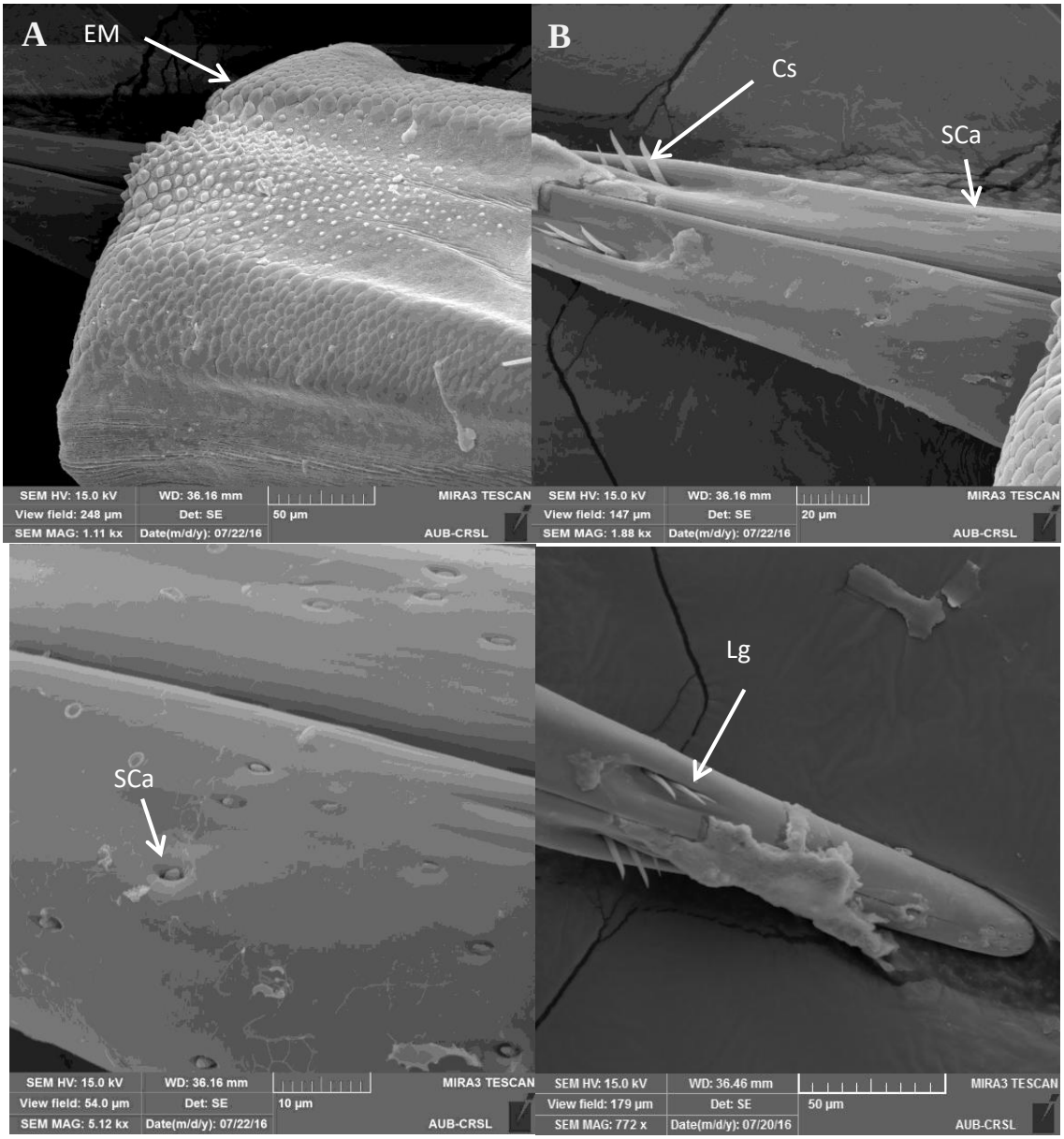
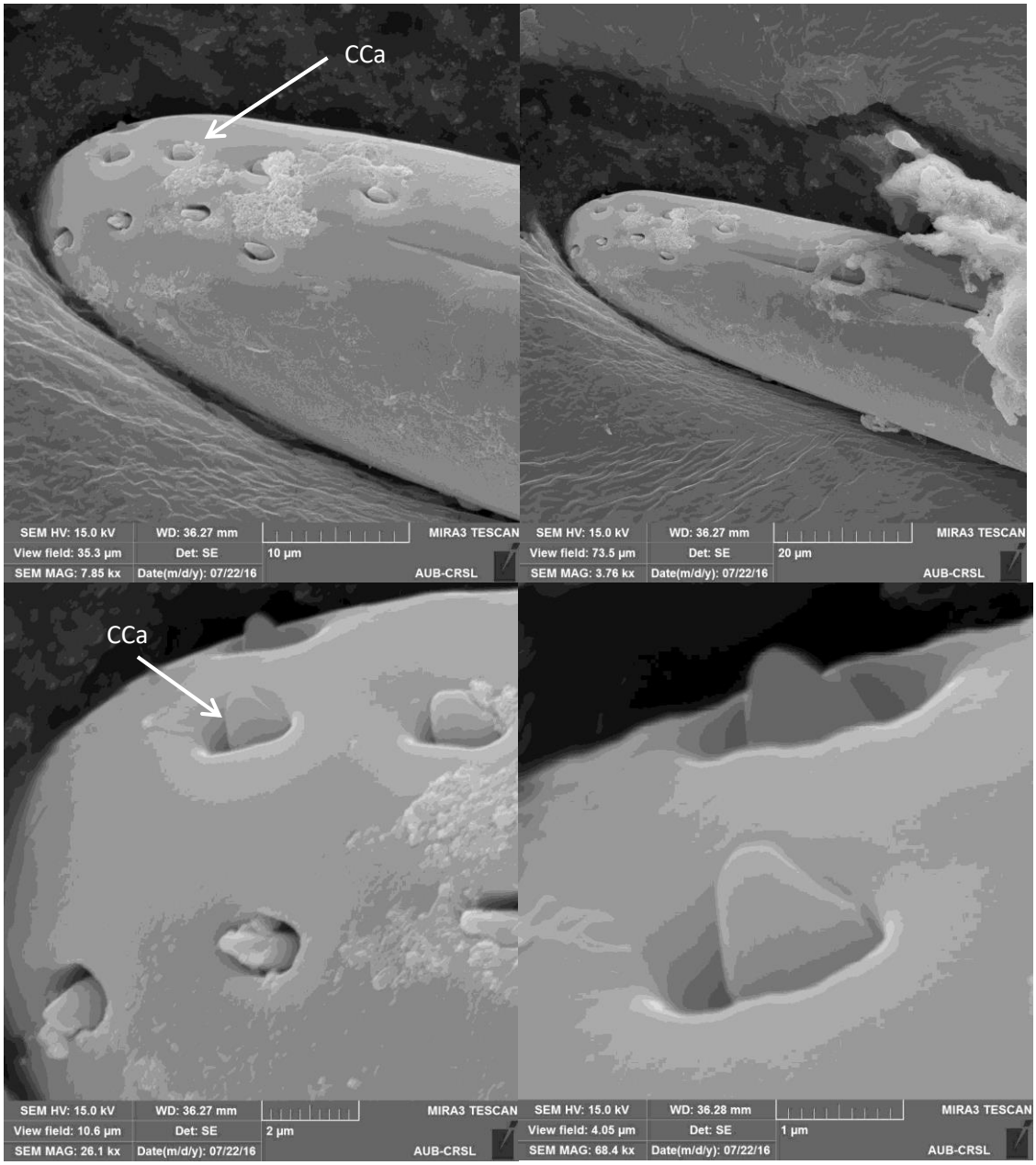


Fig. 3.16. **A.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium lappaceum* showing the eversible membrane covered with acanthae (Vp).
B. Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium lappaceum* showing the ventrolateral grooves containing each three elongated chemosensilla (Cs). **C.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium lappaceum* showing shallow campaniform sensilla (SCa) located on basal region of the dorsal process (between the lateral grooves and the ventral processes). **D.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium lappaceum* showing the position of the lateral grooves (Lg) at the lateral sides of the ovipositor tip



"Fig. 3.16 – *Cont'd*". **E.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium lappaceum* showing the tip of the ovipositor with its associated central campaniform sensilla (CCa). **F.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium lappaceum* showing the tip of the ovipositor with its associated central campaniform sensilla (CCa). **G.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium lappaceum* showing central campaniform sensilla with their elevated knob-like shape and ellipsoidal socket. **H.** Scanning electron micrographs of *Terellia serratulae* reared from *Cirsium lappaceum* showing central campaniform sensilla with their elevated knob-like shape and ellipsoidal socket.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Our results demonstrated that sequences of mitochondrial genes were more phylogenetically useful than nuclear genes. This is in accordance with the literature, where mitochondrial genes are favored in studies involving recently diverged populations and to study genetic variations within a species or a species complex (Moore 1995; Muraji *et al*. 1998). Phylogenetic inferences should be approached with caution for many reasons. One being the issue of inferring gene trees which are not in congruence with the species tree in both mitochondrial genes and nuclear genes; this is because of the incomplete lineage sorting that occurs in the gene branches. Another problem to consider when inferring species trees is the presence of gene flow at the population level. Mitochondrial genes have shown to perform better in cases where incomplete lineage sorting is evident, where they outperformed nuclear genes in correctly inferring species trees. Nuclear genes were shown to be more useful than mitochondrial genes when gene flow is involved in a population since the latter are inherited as a single linkage group while the former are found on different chromosomes such that each gene tree yields a separate estimate of the species tree (Moore 1995). Moreover, nuclear ribosomes have the advantage of containing both coding regions such as 18S and also having the rapidly evolving internal spacer regions such as ITS2.

Previous work on *Terellia serratulae* used mitochondrial DNA sequences for the phylogenetic analysis and employed parsimony methods (Haddad *et al*. 2017). This work showed that *P. acarna* flies are a distinct host race compared to all the other host

flies. In this study, it was demonstrated that populations associated with *C. lappaceum* were somewhat genetically distant from all other host populations while the majority of *P. acarna* populations were distinct but had some gene flow with flies associated with other hosts.

Traditional phylogenetic methods such as phylogenetic trees assume the relationship between the individuals under question to be strictly bifurcating, which is not the case in studies done at the population level. Some of the assumptions that are violated by traditional bifurcating trees are dealing with population-level phenomena like recombination events or recent divergences. Haplotype networking can be the answer to low resolved trees as seen in the ITS2 phylogenetic tree in our case (Templeton, Clark, Weiss, Nickerson, Boerwinkle and Sing 2000). Furthermore, a number of inferences can be made from these networks; for example, when a haplotype is found in the interior with many mutational connections, we can infer that this haplotype is ancestral (Posada and Crandall 2001). Such is the case with the H1 haplotype in the ITS2 network, where one can infer that the previously unresolved phylogenetic tree was a result of persisting ancestral alleles of *P. acarna* found in high frequencies in the population. The mitochondrial haplotypes cannot provide additional information to the already resolved mitochondrial phylogenies without any further investigations like recombination testing.

The monophyly of *C. lappaceum* was observed with a high clade support in both the mitochondrial and nuclear loci. To further confirm this observation, the number of flies associated with *C. lappaceum* should be increased and other geographic locations should be investigated for the presence of this plant which only occurs at very high elevation.

It would also be also interesting to study the coevolution of the flies and their

various thistle hosts to inspect if lineage splitting in hosts has derived any analogous lineage splitting in *T. serratulae*. Sequencing more genes may enhance the molecular analyses. For example, ND6 mitochondrial DNA and nuclear period DNA may be used (Barr, Copeland, De Meyer, Masiga, Kibogo, Billah, Osir, Wharton and McPheron 2006).

Because patches of *Cirsium* plants, namely *C. phyllocephalum*, are commonly heavily infested with *T. serratulae* flies while patches of *P. acarna* are usually less infested, it could be that few of the early emerging females (from overwintering pupae) exploit *P. acarna* flower heads at the beginning of the blooming season of *C. phyllocephalum* which overlaps with part of the blooming season of *P. acarna*. However, the phenology of the different host plants, at different elevations, should be further elucidated.

The ultrastructure of the ovipositor of *T. serratulae* was described using scanning electron microscopy. It showed variations in the position of the sensilla (central campaniform sensilla) at the tip of the ovipositor. Females reared from P. acarna, which have longer ovipositors with blunt tips (Haddad *et al*. 2017), had the sensilla arranged in semicircular rows near the very tip of the aculeus, suggesting that the eggs are laid in a shallow position among the florets. On the other hand, females reared from *Cirsium* spp., which had relatively shorter ovipositor with narrower tips (Haddad *et al*. 2017), had their central campaniform sensilla distributed in two vertical rows and ending at the tip of the ovipositor. These sensilla were less elevated and more dispersed in females associated with *C. phyllocephalum*.

The morphological variations in the position of the central campaniform sensilla together with the shape of the ovipositor tip in *T. serratulae* females associated with different plant hosts reveal adaptations to the morphology of the flower heads

exploited. The sensilla at the tip of the ovipositor are mechano-chemosensillae and they are used by female tephritids to locate and evaluated the quality of potential host plants for oviposition (Stoffolano 1989). Moreover, the position of the ventro-lateral grooves that bear chemosensilla differed in *T. serratulae* females associated with different host plants. These grooves were closer to the ovipositor tip in females associated with *P. acarna* (Haddad *et al*. 2017). Hence, variation in the ovipositor morphology apparently reflects adaptation of tephritid females to their oviposition substrate. In *P. acarna* associated females, the long ovipositor with a blunt tip and sensilla located very close to the tip may facilitate egg-laying in the narrow, tubular and long, flower heads which are surrounded by tough spines. Moreover, *P. acarna* flower heads bear few seeds that are loosely packed in comparison with the *Cirsium* flower heads. Therefore, the relatively wider and blunter ovipositor tip would be suitable to deposit the eggs in between the achene florets. The longer ovipositor might also help with the female curving its body abdomen and ovipositor to reach the achenes as the upper surface of *P. acarna* flower heads offers a narrow platform, which makes it difficult for females to position themselves for oviposition. On the other hand, the relatively larger, deeper and crowded flower heads of *Cirsiu*m spp. necessitate a slender and narrower aculeus tip for females to deposit their eggs deep within the flower head, which is densely packed with achenes.

In conclusion, the populations of *Terellia serratulae* associated with different host plants show genetic and morphological variations, which appear to reflect host adaptations.

It was previously established that the population associated with *Picnomon acarna* is genetically and morphometrically distinct. In this study, more evidence supports this claim and reveals reduced gene flow between *P. acarna* and *C.*

phyllocephalum in certain sites. Moreover, the population associated with *Cirsium lappaceum* was shown to form a separate clade from the rest of the populations.

Future studies are needed to resolve the extent of gene flow between the *C. phyllocephalum*, *P. acarna* and *Carduus* spp. and to determine whether elevation also influence the gene flow between different populations of *T. serratulae* in Lebanon.

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