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Phylogenetic studies of tephritid flies (Diptera, Tephritidae) and their symbiotic bacteria

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Riassunto

I tefritidi costituiscono un'importante famiglia di ditteri fitofagi molti dei quali rivestono un notevole interesse economico in quanto dannosi alle colture agrarie. Le specie di maggior importanza sono quelle che si riproducono a carico del frutto tra cui *Ceratitis capitata* (Wiedemann) (mosca mediterranea della frutta), *Bactrocera oleae* (Rossi) (olivo), *Rhagoletis cerasi* (L.) (ciliegia), *Rhagoletis completa* (Cresson) (noce) e altre specie esotiche. Altre specie invece, come quelle appartenenti alla sottofamiglia Tephritinae vivono soprattutto a carico dei capolini fiorali delle Composite (Asteraceae).

Nella famiglia dei tefritidi sono presenti simbiosi batteriche note per alcune specie da quasi un secolo (Petri, 1909; Stammer, 1929). Recentemente, grazie alle tecniche biomolecolari, è stata identificata e sequenziata la specie batterica simbionte della mosca dell'olivo. Per tale simbionte, che è risultato appartenere alle Enterobacteriaceae è stato proposto il nome "*Candidatus* Erwinia dacicola".

Il presente lavoro ha come obiettivo l'approfondimento delle conoscenze sulle relazioni tra i ditteri tefritidi e i loro batteri simbionti e si articola in tre parti la prima delle quali è già stata oggetto di una pubblicazione.

1. In continuazione della tesi di dottorato della dottoressa Alessia Piscedda, l'identità dei batteri simbionti è stata studiata in 25 specie della sottofamiglia Tephritinae (Diptera: Tephritidae) provenienti da diverse zone del nordest d'Italia e da paesi limitrofi.

Per queste specie si è provveduto alla coltivazione del contenuto del mesointestino di mosche provenienti da pupe preventivamente sterilizzate, all'osservazione di preparati microscopici di questo stesso tratto dell'intestino con LIVE/DAD BacLight e all'utilizzo di tecniche biomolecolari. In accordo con quanto riportato da Stammer (1929) le indagini hanno consentito di accertare la presenza di batteri simbionti non coltivabili in numerose specie dei generi: *Tephritis*, *Campiglossa.*, *Trupanea*, *Acanthiophilus*, *Sphenella*, e *Oxyna.* Simbiosi batteriche sono state rinvenute anche in alcuni generi non considerati da Stammer (*Capitites*, *Dioxyna*, *Noeeta*). I batteri, di cui è stato sequenziato un frammento del 16S rDNA di oltre 1000 bp, risultano specifici

per ogni specie di insetto ospite e, come il simbionte della mosca dell'olivo (*Bactrocera oleae)*, appartengono tutti alla famiglia delle Enterobacteriaceae. I batteri simbionti riscontrati nelle specie del genere *Tephritis* per la loro affinità filogenetica sono stati designati come "*Candidatus* Stammerula tephritidis". L'estensione dell'indagine ad altre tribù paleartiche della sottofamiglia Tephritinae (Xyphosiini, Myopitini e Terellini) con le medesime tecniche, sia tradizionali che biomolecolari, non ha evidenziato la presenza di batteri simbionti come suggerito da Stammer (1929).

2. Nella seconda parte del lavoro sono state studiate le relazioni filogenetiche tra i tefritidi, appartenenti alla sottofamiglia Tephritinae, analizzando due regioni del DNA mitocondriale, 16S rDNA e COI-LeutRNA-COII. Gli alberi filogenetici risultati da una analisi bayesiana e di maximum-likelihood hanno evidenziato la presenza di 5 cluster monofilettici e di regola altamente supportati corrispondenti alle 5 tribù della sottofamiglia Tephritinae: Tephritini, Myopitini, Xyphosiini, Noeetini e Terellini. La ricostruzione filogenetica ottenuta dal COI-tRNALeu-COII data set è risultata più risolta e supportata nei nodi interni rispetto a quella del 16S rDNA, contribuendo maggiormente a definire i rapporti di parentela tra le tribù.

La disponibilità di una filogenesi dei batteri simbionti e dei loro insetti ospiti ha consentito inoltre lo studio della congruenza filogenetica. I diversi test di cofilogenesi addottati hanno evidenziato la presenza di una congruenza, seppur imperfetta, tra ospiti e simbionti. Dalle ricostruzioni si riconoscono due principali eventi di acquisizione il più importante e antico dei quali è quello avvenuto a carico dell'antenato comune della Tribù Tephritini. La causa di una non perfetta congruenza è da imputare all'esistenza di perdite, riacquisizioni e trasferimenti orizzontali. È importante ricordare che, essendo tali simbionti extracellulari, il ciclo biologico di questi insetti potenzialmente offre parecchie occasioni per trasferimenti orizzontali accidentali. Essendo nello stadio larvale i simbionti presenti nei cechi gastrici, parzialmente a contatto con il bolo alimentare, risulterebbero vulnerabili e sostituibili da altri batteri. Anche la frequentazione, da parte di specie diverse, delle stesse piante ospiti potrebbe essere occasione per trasferimenti orizzontali e sostituzioni. A fronte di queste molteplici possibilità la congruenza filogenetica riscontrata, seppure imperfetta, risulta a maggior ragione particolarmente interessante e va probabilmente spiegata con il coinvolgimento

di altri fattori quali l'esistenza di una compatibilità fisiologica tra l'insetto ospite ed il battere.

3. Nella terza parte del lavoro l'analisi filogenetica degli insetti è stata ampliata a specie paleartiche appartenenti ad altre sottofamiglie (Trypetinae e Dacinae) sempre basandosi su due regione del DNA mitocondriale (16S e COI-LeutRNA-COII). La disponibilità in GenBank di sequenze del 16S di altre specie appartenenti a diverse regioni zoogeografiche ha consentito di allargare almeno per questo gene il data set. L'elaborazione dei dati, ancora parzialmente in corso, conferma in generale la tradizionale classificazione condotta su base morfologica ma offre anche spunti di discussione per eventuali riarrangiamenti di alcuni taxa. É stato realizzato anche un tentativo di affiancare alla ricostruzione filogenetica, oltre agli aspetti legati al ciclo biologico della specie, anche le diverse caratteristiche morfologiche degli organi adibiti ad ospitare i batteri simbionti nell'adulto. Risulta interessante notare come, tutte le specie paleartiche analizzate che svernano come adulti, ospitano batteri simbionti. Al contrario (tranne in un caso), tutte le specie che non svernano come adulto, sono risultate prive di batteri simbionti. Tali acquisizioni lasciano supporre che la presenza dei simbionti a livello del mesointestino, più che una opportunità per integrare la dieta larvale probabilmente già relativamente ricca, possano rappresentare, per quelle specie che hanno scelto di svernare allo stadio di adulto, una componente indispensabile.

Summary

Tephritidae, commonly known as "fruit flies" is a large and complex family. Most, particularly the frugivorous species, are notorious pests. These include *Ceratitis capitata* (Wiedemann) (Mediterranean fruit fly), *Bactrocera oleae* (Rossi) (olive fly), *Rhagoletis cerasi* (L.) (cherry fly), *Rhagoletis completa* (Cresson) (walnut husk fly) and other exotic species. Other species, however, live on the flower heads of Asteraceae.

Since the beginning of the last century, some authors (Petri 1909, Stammer, 1929) report the presence of symbiotic bacteria in flies belonging to the subfamily Tephritinae. Recently the olive fly symbiont has been described and designated as 'Candidatus Erwinia dacicola' by (Capuzzo et al., 2005)

The present work aims to study the relationships between species of the family Tephritidae and their symbiotic bacteria. It is based upon three main studies, the first of which has already been published.

1.- The first study is the continuation of Alessia Piscedda PhD. thesis and deals with the identity of symbiotic bacteria, in 25 flies belonging to the subfamily Tephritinae (Diptera: Tephritidae), which were collected mainly in northern Italy. In order to detect and identify symbiotic bacteria, the first tract of the midgut of flies emerging from previously sterilized pupae, was plated on different microbiological media, LIVE/DAD BacLight staining was performed and biomolecular techniques were used. According to Stammer, (1929) the presence of non culturable symbiotic bacteria has been detected in species of genera *Tephritis*, *Campiglossa*, *Trupanea, Acanthiophilus*, *Sphenella*, and *Oxyna*. Symbiotic bacteria have also been found in other genera (*Capitites, Dioxyna, Noeeta*), which were not studied by Stammer. Sequencing 1000 bp of the small subunit rDNA gene from these symbiotic bacteria has indicated that they belong to the family Enterobacteriaceae and a novel candidate organism has been proposed for the symbiotic bacteria of the genus Tephritis, under the designation '*Candidatus* Stammerula tephritidis'.

These analyses have been extended to other tribes of the subfamily Tephritinae (Xyphosiini, Myopitini e Terellini), using the same techniques reported above, but non symbiotic bacteria have been detected in these tribes, as suggested by Stammer (1929).

2.- The second study of the present work analyzes the phylogenetic relationships between tephritid flies of the subfamily Tephritinae. Two regions of the mitochondrial

DNA, 16S rDNA e COI-tRNALeu-COII, were examined. The phylogenetic trees obtained from a Bayesian Inference and a Maximum-Likelihood analysis have suggested, as a rule, the presence of five monophyletic clusters corresponding to the fives tribes of this subfamily: Tephritini, Myopitini, Xyphosiini, Noeetini e Terellini. The phylogenetic tree obtained from the analysis of the COI-tRNALeu-COII showed more highly resolved trees and the internal nodes more highly supported than the phylogeny inferred from the 16S data set, and defined the relationships among the tribes better. Cophylogenetic analysis has been carried out, and the presence of congruence between hosts and symbionts, even if imperfect, has been suggested. The reconstructions obtained showed two principal events. The most important and probably earliest event corresponds with the acquisition of symbiotic bacteria by the common ancestor of the tribe. The presence of non-strict congruence is probably due to other events such as losses, duplications and host-switchings. Indeed, these bacteria are extracellular symbionts and some opportunities for host-switching occur during the biological cycle of the fly. In the larval stadium, for instance, bacteria are located in the intestinal caeca (Petri 1909; Stammer, 1929), without the protection of the peritrophic membrane and are thus, in contact with free living bacteria present in the intestinal lumen. The contemporaneous presence of different species in the same host plant could also be an opportunity for host-switching. Considering all of these aspects, the presence of congruence, even if not strict, results particularly interesting and a physiological compatibility between host and symbiont seems to appear.

3.- In the third part of my PhD. thesis, the phylogenetic analysis of insects has been extended to Paleartic species belonging to other subfamilies (Trypetinae e Dacinae). It has been based on the analysis of two regions of the mitochondrial DNA: 16S e COI-tRNALeu-COII. The availability of sequences of the 16S rDNA of several species in GenBank, has allowed extending this data set. These phylogenetic analysis still in progress, confirms the traditional classification based on a morphological approach but suggests also interesting relationships among the tribes. I have also attempted to associate the phylogeny obtained with morphological symbiotic arrangements and biological characteristics. Interestingly, it was pointed out that all the species of the subfamily Tephritinae that overwinter as adults, present symbiotic bacteria in the first tract of the midgut. The presence of these bacteria seems to be essential for the overwintering adults. Indeed, while

the diet of larval stages includes relatively rich substrates such as flower tissue and seeds, glyciphagous adults have access to less resources. Thus the presence of bacteria could be more critical for their survival than that in the earlier stages.

Chapter 1

Introduction

INTRODUCTION

TEPHRITIDAE

Tephritidae, commonly known as "fruit flies" is a large and complex family. More than 4,200 species and subspecies of fruit flies are recognized worldwide, grouped in 471 genera (Thompson, 1999).

Within the order Diptera, the family Tephritidae belongs to the suborder Brachycera, infraorder Muscomorpha (= Cyclorrhapha), section Schizophora, and superfamily Tephritoidea (J.F. McAlpine 1989 in Norrbom *et al.*, 1999).

The diversified behaviour, evolution and population genetics, insect-plant interactions and biosystematics makes them a fascinating family (H. Zwölfer, 1983)

Taxonomy

Fruit flies were recognized as a group for the first time in 1795 by Schrank (as "Bohrfliege") but the valid family name was proposed in 1834 by Newman (Norrbom *et al.*, 1999).

The basis of modern tephritid classification began with the work of Loew. Most of the generic concepts of Loew (1862, 1873) survive today as modern genera or higher taxa.

Further significant advances in fruit fly higher classification were made by Bezzi and Hendel during the first third of the last century. They named many of the larger, and/or most distinctive family groups, taxa and produced important monographs and revisions that permitted important advances (Norrbom *et al.*, 1999). Later Hering (with publications dating 1927-1961) followed in the footsteps of Bezzi and Hendel and was the most prolific author of fruit fly names. Although he produced few revisions, his classification of 1947 was the basis for tephritid classification for the next 30 years (Norrbom *et al.*, 1999).

Succeeding tephritid specialists were more regional in their approach. Among the most prominent were Munro (who concentrated on the Afro-tropical Region), Hardy (Oriental and Australasian Regions) Foote (Nearctic region) and Aczél (Neo-tropical).

The last decades have seen an increase in the study of fruit fly phylogenetic relationships and corresponding revision of the higher classification of the family by authors such as Drew, Freidberg, Norrbom, White, Han, and especially Korneyev and Hancock (Norrbom *et al.*, 1999).

Recently, biomolecular techniques represent a useful tool having improved tephritid higher classification. Han and McPheron have produced important phylogenetic studies that have enhanced the higher classification of tephritid flies (Norrbom et al., 1999).

Tephritid higher classifications have suffered several changes since the beginning. Different authors have proposed competing classifications, numerous new groups have recently been proposed, and many genera have been transferred.

Loew in 1850, subdivided the family in two subfamilies: Trypetinae and Dacinae (Tab. 1.1).

FAMILY TRYPETIDAE (=Tephritidae)		
TRYPETINA		
Gen. *Platyparea*	Gen. *Euphranta*	Gen. *Aciura*
Gen. *Hemilea*	Gen. *Anomoea*	Gen. *Acidia*
Gen. *Spilographa*	Gen. *Zonosema*	Gen. *Rhagoletis*
Gen. *Oedaspis*	Gen *Rhacochlaena*	Gen. *Trypeta*
Gen. *Ensina*	Gen. *Myopites*	Gen. *Urophora*
Gen. *Sphenella*	Gen. *Carphotricha*	Gen. *Oxyphora*
Gen. *Oxyna*	Gen. *Tephritis*	Gen. *Urellia*
DACINA		
Gen. *Ceratitis*	Gen. *Dacus*	

Tab. 1.1. – Systematic subdivision of Family Trypetidae (=Tephritidae) (Loew, 1850).

White (1988) subdivided Tephritidae in four subfamilies of Paleartic species: Subfamily Dacinae, Subfamily Myopitinae, Subfamily Trypetinae and Subfamily Tephritinae (Fig. 1.1).

More recently, the subfamily Myopitinae, among others, has been considered as a tribe of the Subfamily Tephritinae. Tribe Tephrerellini, Tribe Terellini, Tribe Oedaspidina previously considered a subfamily are now also considered a tribe of the Subfamily Tephritinae (Hering, 1947; Cogan & Munro, 1980).

Korneyev (1999) divided the family Tephritidae into Higher Tephritinae: "Subfamily Tephritinae + Subfamily Trypetinae + Subfamily Dacinae and Lower Tephritinae: "Subfamily Tachiniscinae + Subfamily Blepharoneurinae + Subfamily Phytalmiinae" (Tab. 1.2). He also produced an exhaustive analysis of Subfamily Tephritinae (Fig. 1.2). In the present work, we will follow this classification.

The Dacina, has been usually ranked as a subfamily, or even as a separate family (Munro, 1984), but some authors Norrbom et al. (1999) included it as a subtribe of Trypetinae and included this subtribe in the tribe Dacini with the Ceratitidina and Gastrozonina.

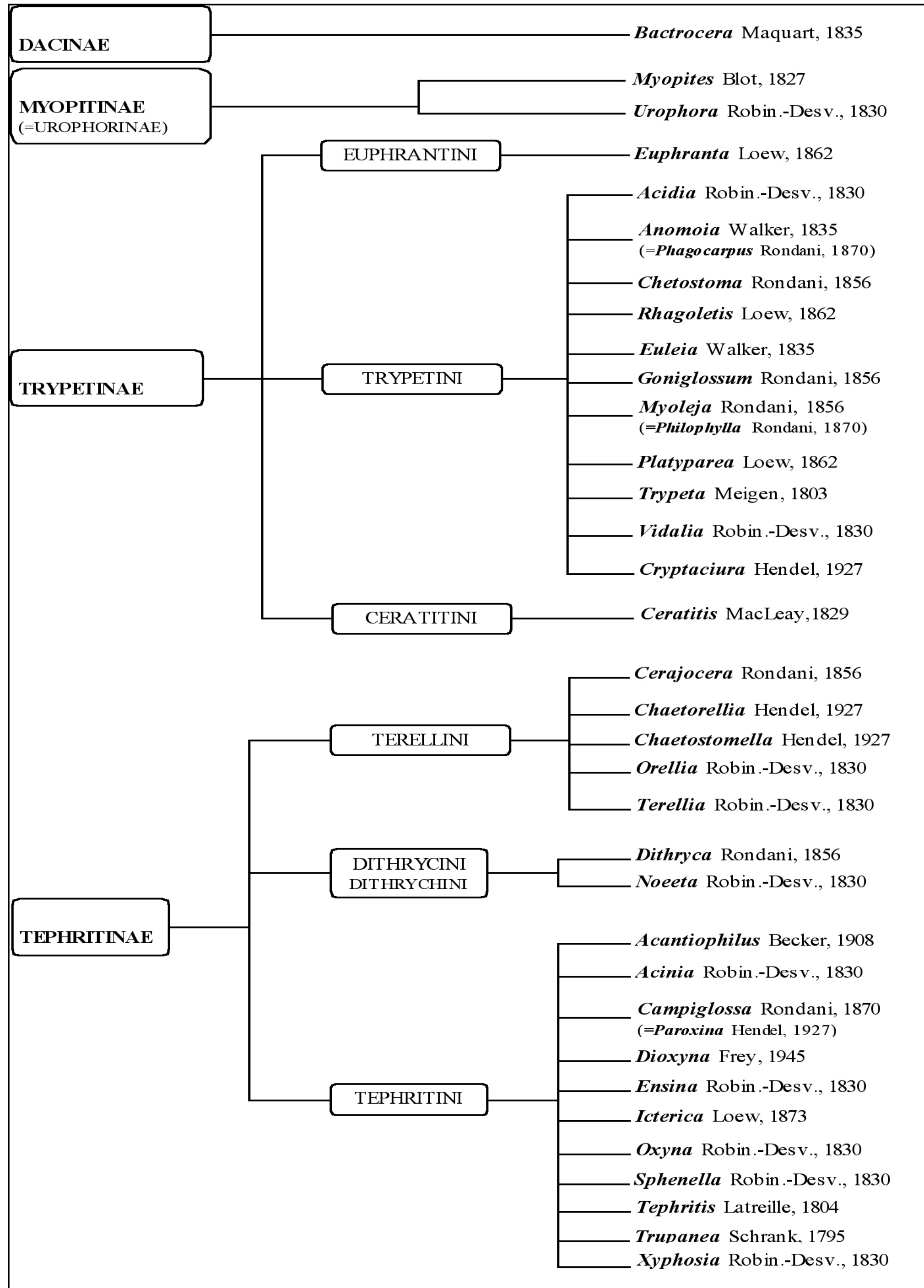

Fig. 1.1. – Systematic subdivision of Paleartic species of the Family Tephritidae (White, 1988).

Tephritidae	
Higher Tephritidae	**Lower Tephritidae**
Tephritinae	Tachiniscinae
Trypetinae	Blepharoneurinae
Dacinae	Phytalmiinae

Tab. 1.2. – Subdivision of Family Tephritidae according to Korneyev (1999)

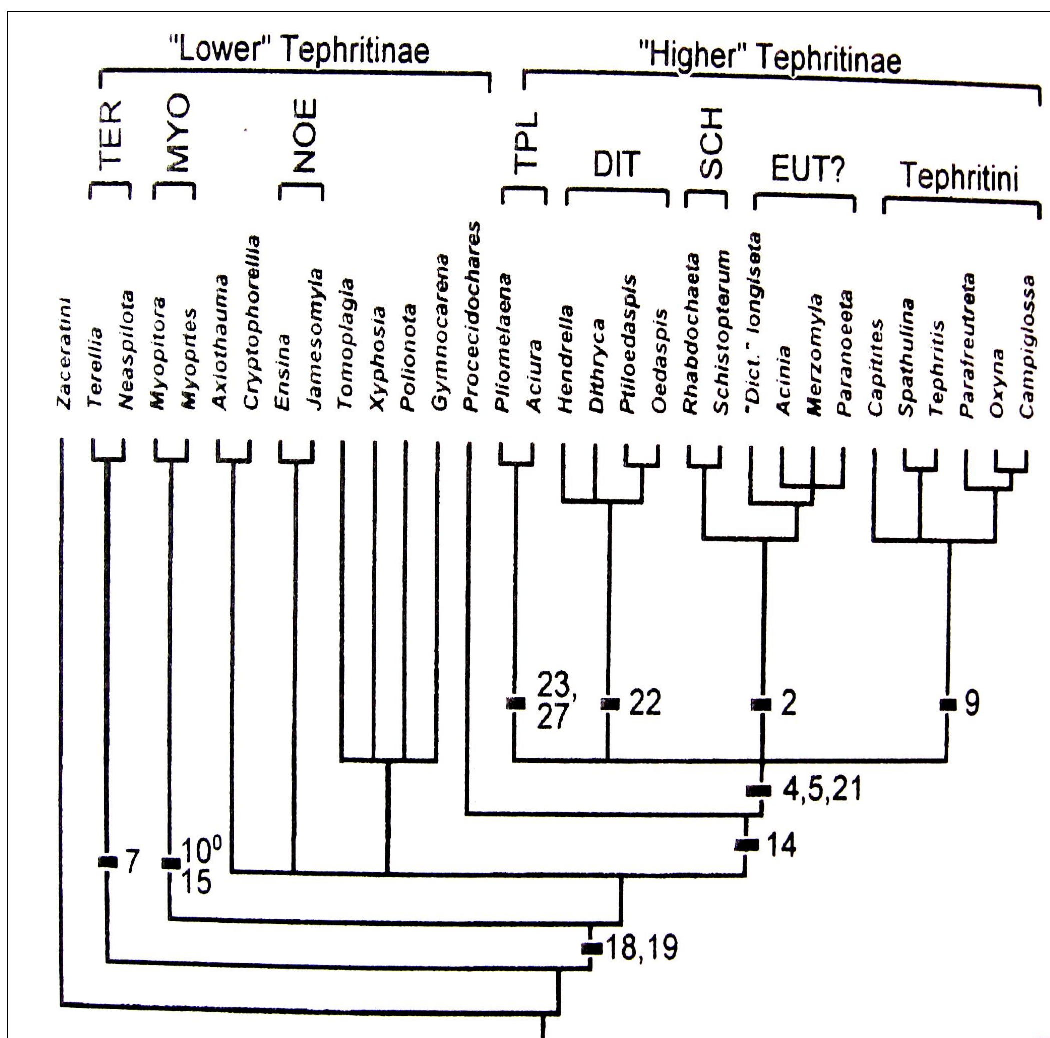

Fig. 1.2.- Phylogenetic relationships of Subfamily Tephritinae according to Korneyev (1999)

Biology

The tephritid life cycle includes the stages of: egg, three larval instars, pupa (formed inside the hardened third stage larval cuticle, or puparium), and adult. Species may be uni- bi- or multivoltine (Norrbom, 1989). In some species, adults live only for a few weeks but in others females can survive for long periods (e.g., up to 12 months in *Anastrepha ludens* (Shaw *et al.*, 1967).

Diapause is common, mainly in temperate species, but many species of subfamily Dacinae do not diapause.

Larva feed on different parts of the plant. Zwölfer (1983) divided the family into three groups based on resource exploitation strategies:

a) Generalist frugivorous species: larvae feed and develop in the pulp of fleshy fruits: *Dacus* sp. *Ceratitis* sp. *Anastrepha* spp.
b) Specialist frugivorous species: *Rhagoletis* spp.
c) Non frugivorous species: larvae feed on vegetative structures such as leaves, shoots or roots and the inflorescences of Asteraceae, Lamiaceae and Capparidaceae.

Adults feed on different substrates like fruit juices, honeydew, extrafloral glandular exudates, nectar from flowers, pollen, grains, bird feces, yeast and bacteria (Hagen, 1958; Christenson and Foote, 1960; Bateman, 1972; Prokopy, 1976; Nishida, 1980; Fitt and O'brien, 1985; Tsisipis, 1989; Hendrichs & Hendrichs, 1990; Hendrichs *et al.* 1991; 1993b; Hendrichs & Prokopy, 1994; Aluja 1994; Warburg & Yuval, 1997b).

Host range varies considerably, often among closely related species (Norrbom & Kim, 1988; Goeden, 1992, 1993, 1994). Probably the majority of Tephritidae are oligophagous, breeding in a few related or ecologically and chemically similar hosts (Norrbom *et al.*, 1999). However, some of them are strictly monophagous, as *Bactrocera oleae* (Rossi), which breeds only in olives, but some pest species are remarkably polyphagous, as *Ceratitis capitata* (Widemann), which has been reported from more than 300 hosts (Liquido *et al,.* 1991). Although phytophagy is the predominant mode of feeding in the Tephritidae some species of tribe Phytalmiini and Acanthonevrini are saprophagous (Norrbom et al., 1999).

Larvae usually spend most part of their lives in the same host plant. They usually pupate within the plant. Other tephritid larvae may move relatively long distances, first inside the plant tissues and then outside the plant, which they leave in order to pupate in the soil (Norrbom et al., 1999). Some species of *Blepharoneura* are dispersed by frugivourous bats that carry their host fruits (Condon & Norrbom 1994).

Adults of many species, especially those that are univoltine and/or narrowly host specific, may spend most of their life on one plant or adjacent plants of the same species. Other species, especially those that are polyphagous, may have dispersive phases and may fly distances as great as tens or hundreds of kilometers (Norrbom *et al.*, 1999).

Distribution

Fruit flies are distributed throughout the temperate and tropical areas of the world, being absent only from the high Arctic and Antarctic (Thompson, 1999). The Oriental Region appears to have the greatest fruit fly diversity in both genera and species, although the Afrotropical Region is a close second and may eventually prove to have more genera and species. The Nearctic fauna is by far the least diverse, in both genera and species, and the Neotropical Region is second least diverse, although it has almost as many species as the Australasian Region (Norrbom et al., 1999).

The Tephritidae probably originated in the Paleotropics in post-Gondwanan times, based on the diversity of higher-level taxa, in that part of the world (Norrbom *et al.*, 1999). Various authors, including Aczél, in Hardy (1957), Hancock (1986), and Norrbom (1994), suggested that the majority of tephritid higher groups originated there, or at least in the Old World, and only some dispersed to the Americas.

Dacini are concentrated in Afro-tropical region and Southeast Asia to northeastern Australia. Only smaller numbers of species are present away from these centres (Drew & Hancock, 2000).

Drew studied the distribution of some Dacini, correlated to the distribution of their hosts plants. Most species in Asia and the South Pacific occur in rain forest habitats where they oviposit in the fruits of many different plant families. Drew & Hancock (2000) suggested that Indo-Madagascan plate of Gondwanaland is a probable centre of origin of Dacini.

From this original centre these may have spread to other regions such as Australia or Africa.

Other authors, however, do not agree with this hypothesis. Munro (1984) considered *B. oleae* to be native to Africa. Recently White (2006) has discounted the hypothesis that the Dacina evolved on the Indo-Madagascan plate of Gondwanaland. He has suggested the hypothesis of an initial spread and subsequent evolution of *Dacus* in Africa, and a later, climatically filtered spread, of dry-tolerant *Dacus* subgenera into Asia. Even though, he considers his hypothesis still quite speculative, and proposed a future detailed study. In any case, the majority of *Bactrocera* spp. are Indo-Australasian and only 11 are native to Africa. Conversely, the genus *Dacus* is primarily African with 777 species recognized in a recent work (White, 2006), compared to only 71 in Indo-Australasia.

The subfamily Trypetini are most different in the Palearctic and Oriental Regions, but some genera occur in the other regions, including the Nearctic and Neotropical (Norrbom *et al*., 1999) *R. completa* and *R. pomonella*, for example, are native to North America.

Freidberg (1984) reports that subfamily Tephritinae, is largely restricted to the Holarctic Region or temperate, higher altitude areas of the Afro-tropical and Neotropical Region. The paucity of Tephritinae species in the Oriental and Australasian Regions suggests a northern origin for this subfamily (Norrbom *et al*., 1999).

On the other hand, Merz (1999) noticed that the presumed sister group of the *Tephritis* group, *Spathulina*, *Campiglossa* and *Sphenella* groups of genera, have a major centre of diversity in high altitudes of Eastern and Southern Africa (Munro, 1938; 1957a, b.; Freidberg 1987). Consequently, the *Tephritis* group could have originated in that region, and some genera, like *Tephritis*, *Trupanea* or *Actinoptera*, penetrated into other biogeographic realms at a later time. Thus, the origin of the *Tephritis* group is still in question. However, other Tephritinae as *Chaetorellia* spp. and *Terellia* spp., *Ensina sonchi* and *Urophora* spp. have a Paleartic origin (Norrbom *et al*., 1999).

Recently, however, mankind has played an important part in altering the distribution of some of the more polyphagous species where the hosts have been spread by man.

Economic importance

The carpophagous (frugivorous) species of Thephritid flies are of economic importance, and those such as *Anastrepha spp*, *C. capitata*, *B. oleae* and *Bactrocera tryoni* (Frogatt) are considered notorious pests; as indeed, are other non-frugivorous species such as *Platyparea poeciloptera* Schr. on asparagus and *Acanthiophilus helianthi* Rossi (H. Zwölfer, 1983).
Some other species, however, are considered beneficial, such as members of the genera *Procecidochares*, *Urophora* or *Tephritis* which are used in the biological control of noxious weeds (H. Zwölfer, 1983). *Urophora quadrifasciata* (Meigen) for example, has been introduced in Canada from Europe to control *Centaurea diffusa* and *C. biebersteineii*. This tephritid fly together with *U. affinis* Frfld. have contributed to the reduction of the total biomass of weeds (Harris and Myers, 1984; White, 1988).

Bacterial symbiosis in Tephritid flies

Several kinds of associations between microorganisms and insects are found in nature (Buchner, 1965; Baumann & Moran, 1997). Stable or obligate relationships and instances of coevolution with bacteria have been traced to aphids (Baumann *et al*., 1995; Clark 2000), whiteflies (Clark *et al*., 1992; Thao & Baumann, 2004), mealybugs (Munson *et al*., 1992; Downie & Gullan, 2005), plant hoppers (Noda *et al*., 1995), carpenter ants (Schröder *et al*., 1996; Degnan *et al*., 2004), weevils (Campbell *et al*., 1992; Lefevre *et al*., 2004), tsetse flies (Aksoy, 1995; Chen *et al*., 1999), cockroaches and termites (Bandi *et al*., 1994; Lo *et al,* 2003), psyllids (Thao *et al*., 2000), armoured scale insects (Gruwell *et al*., 2007) and leafhoppers and sharpshooters (Takiya *et al*., 2006). Among the benefits for the host, it is hypothesized that there may be a specific nutritional complementation, particularly for those insects living on a markedly imbalanced diet, as in the case of aphids (Rouhbakhsh *et al*., 1996).
These symbiotic bacteria are usually endocellular, in contrast to their closest free living bacteria, and display distinctive genetic properties including AT-biased base composition, accelerated molecular evolution, and, at least sometimes, small genome size; these features suggest increased genetic drift (Moran & Baumann, 2000). Additionally, some extracellular symbiotic bacteria have been found. Some of them are located in the gut and play an essential role in the life of these hosts (Dillon & Dillon, 2004). In some cases the presence

of a vertical transmission of these bacteria from the mother to the progeny has been noted. The stinkbugs of the family Platasipidae harbour a bacterial symbiotic in the posterior midgut and then transmit their symbiotic bacteria to the progeny by a mechanism called "symbiont capsule" (Hosokawa *et al*., 2006). When the female insects lay eggs on their host plant, small brownish particles containing symbiotic bacteria, are always deposited under the egg mass. In this way, the mother ensures that bacteria will be acquired by the nymphal (Fukatsu & Hosokawa, 2002).

In endocellular bacterial the host phylogeny generally mirrors the symbiont phylogeny, suggesting host-symbiont cospeciation over evolutionary time.

Extracellular associations are thought to be evolutionarily more casual than the endocellular associations, on the grounds that the symbionts are not isolated in the body cavity and vulnerable to invasion and replacement by foreign microbes (Buchner, 1965).

In the case of flies belonging to the family Tephritidae, the olive fly *B. oleae* (Subfamily Dacinae), was the first species for which a bacterial symbiosis was described.

Obligate endosymbionts multiply within a specialized organ in the insect head called the oesophageal bulb. From this organ, the bacteria are discharged into the oesophagus and thence to

the intestine where they are eventually digested (Petri, 1909). Recently, the olive fly symbiont has been described as '*Candidatus* Erwinia dacicola' (Capuzzo *et al.*, 2005) (Fig. 1.3).

a) b) c)

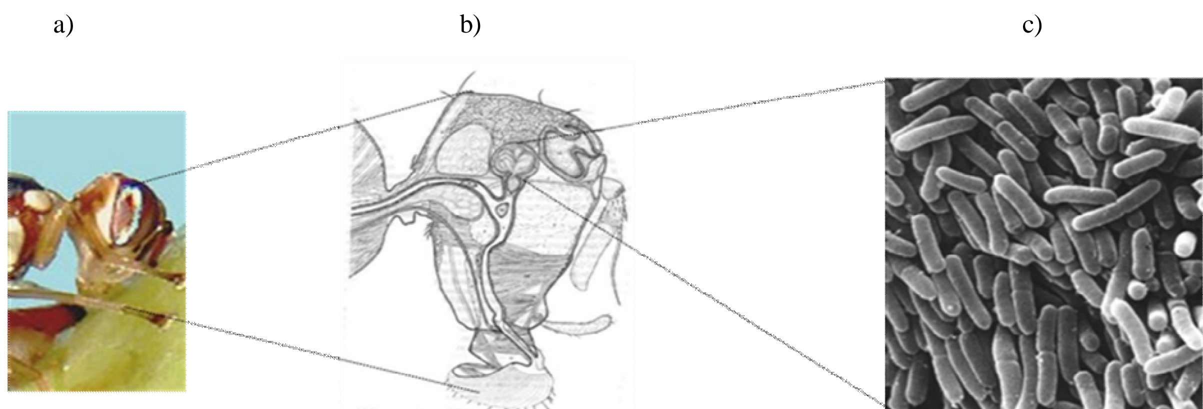

Fig. 1.3.- Bacteria location within adult *B. oleae*. a) Female fly of B. oleae; b) Anatomical depiction of the fly head in longitudinal section with indication of the oesophageal bulb (broken lines) leading to the visualization of its content (redrawn from Girolami, 1973); original realized by hand from a real fly specimen under a camera lucida-equipped microscope. c) Scanning electron micrograph of the bacterial content present in the oesophageal bulb.

The first studies relative to bacterial symbiosis in tephritid flies different from *B. oleae* were carried out by H. J. Stammer (1929). He studied 37 species belonging to the subfamily Dacinae, Trypetinae and Tephritinae. In particular, he described the presence of a symbiosis characteristic of some species of the Subfamily Tephritinae: *Tephritis, Oxyna, Paroxyna, Campiglossa, Trupanea, Acanthiophilus* and *Sphenella*. In adult stages of these species bacteria are located in the first tract of the midgut in contact with the epithelium but, as later reported by Girolami (1983), outside the peritrophic membrane (Fig. 1.4). The peritrophic membrane, which is present in different insect species, is a membranous film that forms a thin lining layer that surrounds the food bolus and separates it from the delicate midgut epithelium. *Acanthiophilus* and *Sphenella* present a specialized "evaginations" in this tract of the midgut were bacteria are located. In *A. helianthi* these evaginations are located only in one side of the midgut (Fig. 1.5) and in *S. marginata* the evaginations present a bilobate form, covered with villi, and in a slightly separated position with respect to the midgut (Fig. 1.6).
The oesophageal bulb, with some morphological differences, is also found in the subfamily Tephritinae, but in this case it is devoid of bacteria (Girolami, 1973). As described in *B. oleae* (Petri, 1909), females of several species of the subfamily Tephritinae smear the surface of their eggs with bacteria during oviposition in order to ensure the vertical transmission of the bacteria to the progeny.
The larval stages of the insects maintain the bacteria in their intestinal caeca (Petri, 1909; Stammer, 1929), presumably in contact with free living intestinal bacteria (Fig. 1.7).
In the rest of the Tephritinae (*Xiphosia* spp., *Ensina* spp., *Noeeta* spp., and Tribe Myopitini) Stammer found no symbiotic bacteria, nor special organs related to bacteria.
In species of subfamily Trypetinae and Dacinae presence of symbiotic bacteria are not known in adult stages. However, several works described the presence of symbiotic bacteria most of which belong to genus *Enterobacter*, *Klebsiella* and *Pantoea*. (Lloyd *et al.*, 1986; Drew & Lloyd, 1987; Daser & Brandl, 1992; Marchini *et al.*, 2002; Lauzon, 2003). For example, a predominance of the bacteria *K. oxytoca* has been detected in *R. completa* (Rossiter *et al.*, 1983). These bacteria are not vertically transmitted from the mother to the progeny, though the importance of these associations during the life of the insect can be considered facultative.

Oesophageal bulb and symbiosis

Oesophageal bulb

The first, well known description of symbiosis in fruit flies is by Petri, in which he shows that in *B. oleae,* obligate endosymbionts multiply within a specialized organ in the insect head, called the oesophageal gland or oesophageal bulb. Later, Dean (1935) showed a bulb in the oesophagus of *R. pomonella*, but without any indication of a relationship with bacteria. Only in the second part of the last century has the presence of an oesophageal bulb, in four morpho-histological types, been described in all the Tephritidae (Girolami, 1973) (Fig. 1.8).

- The first and the largest, typical of *B. oleae*, is spherical and is provided with a "neck" connecting it with the pharynx, without any evident muscular sheath. Its basal epithelial cells are elongated and symbiotic bacteria multiply next to them. Girolami (1973) describes this kind of oesophageal bulb as *Dacus* type, corresponding to the description by Petri (1909).
- A second type is also spherical but smaller than the first one. It is distinctive to the species of the subfamilies Trypetinae and Dacinae, (with the exception of *B. oleae)*, and is provided with elongated cells at the apex of oesophageal bulb and largely covered by a muscular sheath. Associated bacteria multiply in the lumen of this oesophageal bulb. Bacteria are easily visible in Trypetinae, however, one has to be careful not to put pressure on the oesophageal bulb during the dissection as this would allow the bacteria to escape. In Girolami (1973), this kind of oesophageal bulb is described as *Ceratitis* type.
- The third type, typical of the subfamily Tephritinae, (with the exception of the tribe Terellini), has an ovoid shape, with a strong, wide muscular sheath without any articulated cell elongations. No bacteria appear within this oesophageal bulb. Girolami (1973) described this kind of oesophageal bulb as *Ensina* type.
- The last type, characteristic of the tribe Terellini, shows a pharyngeal outward-deflection, whose apex closely resembles the oesophageal bulb of subfamily Trypetinae. In Girolami (1973), this is described as *Chaetorellia* type. Culturable bacteria have been detected here in the tribe Terellini, as well as in the oesophageal bulb of Trypetinae and Dacinae.

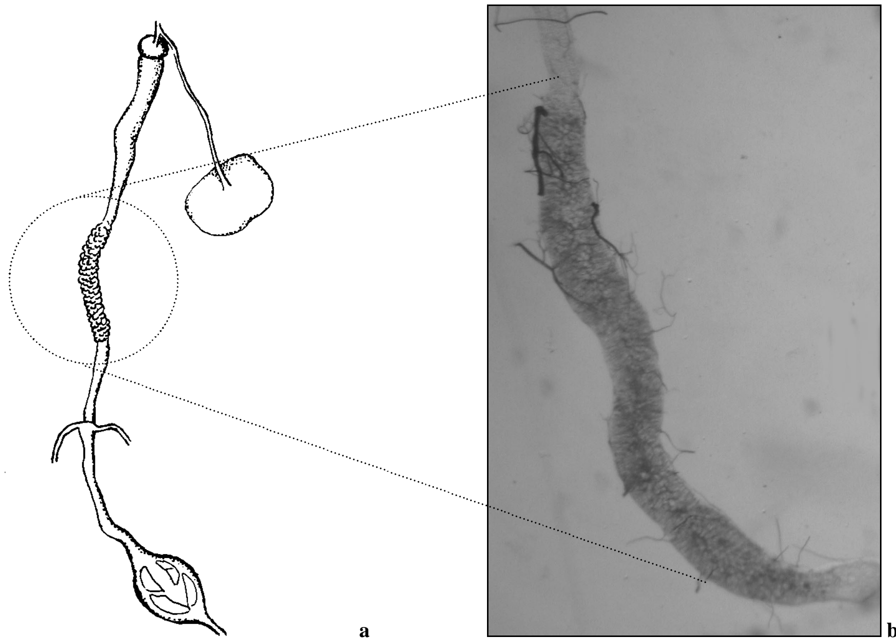

Fig. 1.4.- Midgut of an adult of *T. conura* (picture **a** by Buchner, 1965; **b** photo Capuzzo)

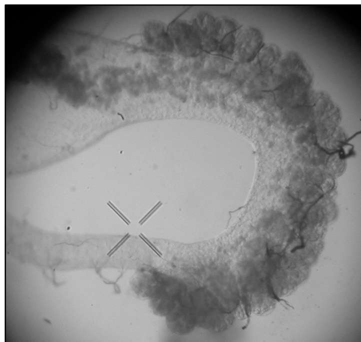

Fig. 1.5. – Midgut of an adult of *Acanthiophilus helianthi* showing the evagination where symbiotic bacteria are located (picture Capuzzo).

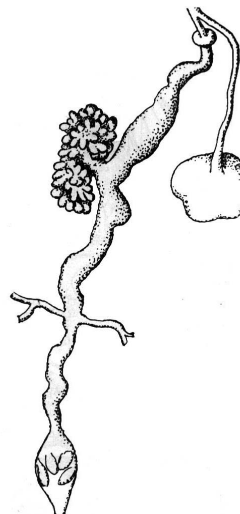

Fig. 1.6. – Midgut of an adult of *Sphenella marginata;* showing the evagination where symbiotic bacteria are located. (Buchner, 1965).

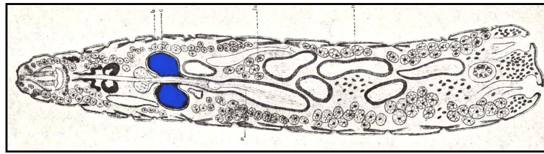

Fig. 1.7. – Larva of *B. oleae* with the symbiotic bacteria in their intestinal caeca (in blue) (Petri, 1909)

Membranous masses

In the species of the Subfamily Dacinae, (with the exception of *B. oleae*), Subfamily Trypetinae and the Tribe Terellini belonging to subfamily Tephritinae, "membranous masses" are produced inside the oesophageal bulbs (Girolami, 1973). This means that, Higher Tephritidae, except subfamily Tephritinae, but including Terellini, have an oesophageal bulb which continuously produces masses which are discharged into the midgut regardless of whether bacteria are present or not. Here they can be observed inside the peritrophic membrane of the intestinal part of the midgut (Girolami, 1973). In subfamily Trypetinae these "membranous masses" adopt the shape of the oesophageal bulb (Fig. 1.9). They are probably produced from a continuous delamination of the stomodeal intima of the bulb. In *B. oleae* they are produced more than ten times per day (Piscedda & Girolami, 2005). The production of membranous masses free of bacteria can be obtained in the laboratory (Fig 1.10.a &1.10.b) using disinfectant products on pupae and adults as reported in Capuzzo *et al.,* (2005).

Membranous masses and culturable bacteria

In nature all flies belonging to Trypetinae and Dacinae and Tribe Terellini have bacteria inside the oesophageal bulb (Girolami, *pers. com.*). Culturable bacteria can be observed both by dissecting the oesophageal bulb and by plating the contents of the oesophageal bulb in different microbiological media. Dissection must be done without putting pressure on the oesophageal bulb, in which case the contents could be lost (Girolami, *pers. com.*). These free living bacteria are embebed within these "membranous masses" where they multiply. The production of these membranous masses implies an energy cost. It is probable

therefore that the efficient multiplication of free living bacteria within these "membranous masses" represent an advantage for these Tephritid flies.

In Tephritinae, except Tribe Terellini, no bacteria and membranous masses have been found in the oesophageal bulb, and true symbiotic bacteria are located in the first tract of the midgut (Stammer, 1929; Mazzon *et al*., 2008), in contact with the epithelium but, outside the peritrophic membrane Girolami (1983).

As reported above, is common to find species of bacteria such as *Enterobacter*, *Klebsiella* and *Pantoea* in the midgut of Trypetinae . These free living bacteria are commonly present in fruits that have been attacked. Specific analyses of bacterial masses of the oesophageal bulb and midgut have been done recently using *R*. *completa*, where species belonging mainly to the genus *Klebsiella* have been found (90% of isolates) (Girolami *et al*., in preparation). *Klebsiella* spp. is also found in other *Rhagoletis* spp. such as, *R*. *pomonella* (Rossiter *et al*., 1983; Howard, 1989).

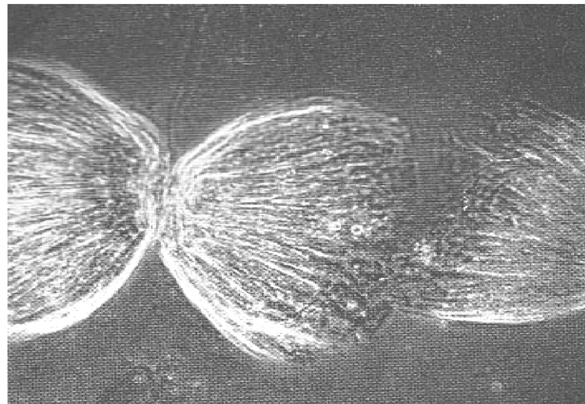

Fig. 1.9.- "Membranous masses" in *Ceratitis capitata* which adopt the shape of the internal intima of this oesophageal bulb (Girolami, 1973).

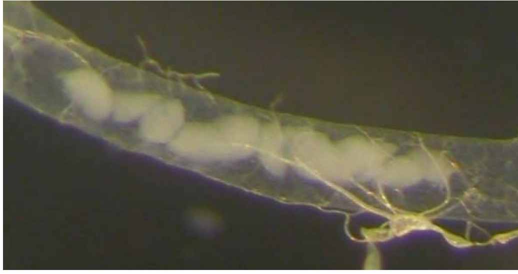

Fig. 1.10.a. – Bacterial masses (Membranous masses) in the midgut of *Bactrocera oleae* (picture Piscedda).

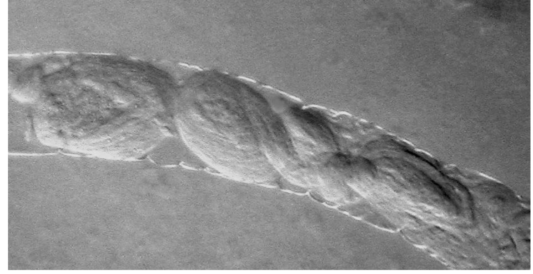

Fig. 1.10.b.- Membranous masses inside the peritrophic membrane Midgut of the *Bactrocera oleae* reared in laboratory in artificial media, are bacteria free and transparent (picture Girolami)

Transmission apparatus of bacteria

Another interesting structure related to symbiosis, is the presence of glands, containing bacteria, in the ovipositor of tephritid flies (Fig. 1.11). Petri in 1909, when studying *B. oleae* and Stammer when studying some Tephritinae noticed that during the oviposition the egg comes into contact with glands, which release symbiotic bacteria, which permeate the egg micropile. Thereafter, bacteria penetrate the newly formed larva, ensuring the vertical transmission of symbionts to the progeny.

These ovipositor glands are not present in the Tephritidae, which do not host symbiotic bacteria such as *Ensina sonchi*, *Urophora* spp., and *Xiphosia* spp. Furthermore no glands have been found in the ovipositors examined in Trypetinae and Dacinae; here again, *B.oleae*, is the exception.

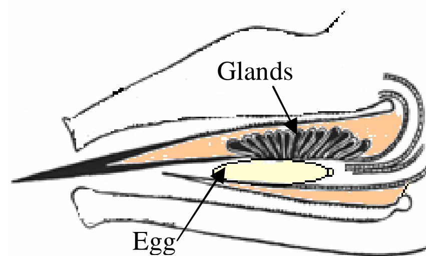

Fig. 1.11. – Glands in the ovipositor of *Bactrocera oleae* (Petri, 1909).

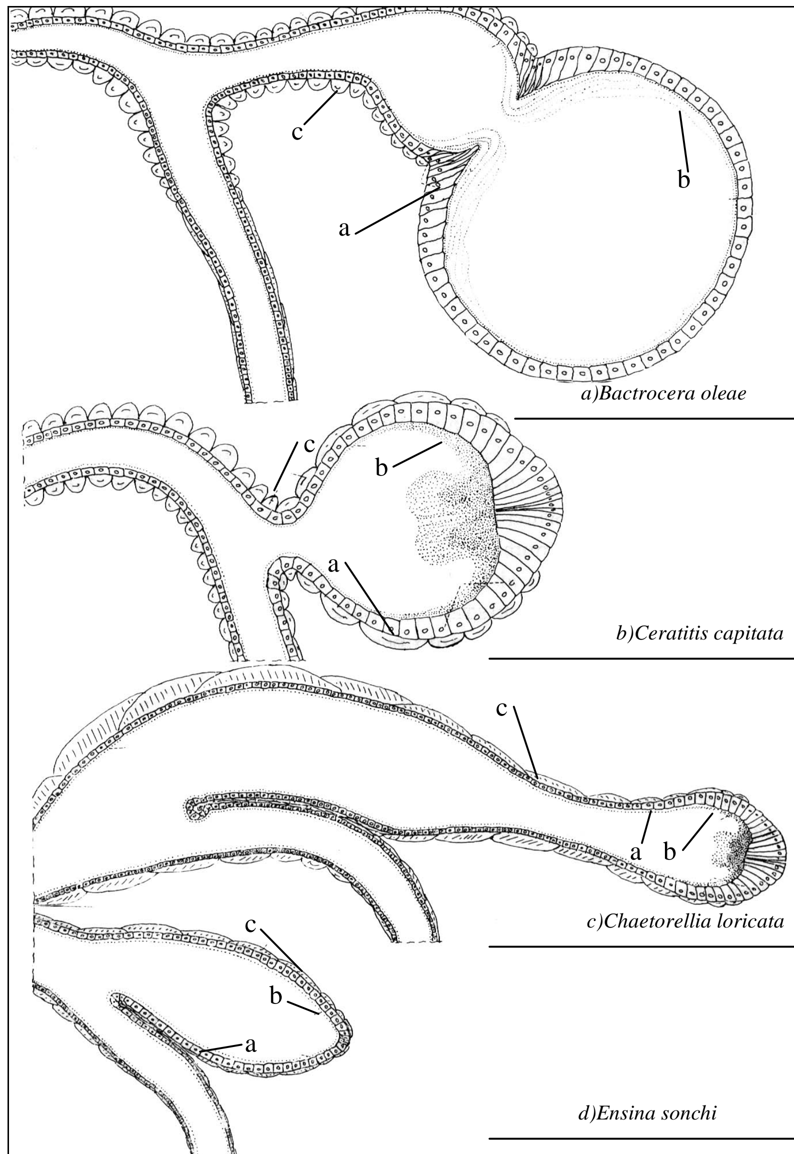

Fig. 1.8. – Four different types of oesophageal bulbs of tephritid flies;
a epithelium; b intima; c muscular tunic (Girolami, 1973).

Historical aspects of the knowledge of symbiosis

Petri described the oesophageal bulb in *B. oleae*, which contains bacteria that are successively discharged to the midgut. He specified that these bacteria multiply in a mucilaginous secretion of the oesophageal bulb; but considered that the bacterial masses were discharged into the midgut due to the multiplication of symbionts. The oesophageal bulb was also sought by Petri, in some species of Trypetinae, such as *Rhagoletis cerasi* L., and *C. capitata* but was not found. Later Stammer (1929) also sought the oesophageal bulb in the same species and genera *Carpomya* and *Trypeta* but he did not find it. Therefore, the presence of an oesophageal bulb in Tephritidae different from that of *B. oleae*, was denied by both Stammer and Petri. The oesophageal bulb was described for the first time in many Tephritidae by Girolami (1973). Nevertheless, in literature, it is often attribute that the oesophageal bulb of Trypetinae and Dacinae, (different from *B. oleae*) has been described by Buchner (1965). Apropos in this context, Buchner , basing his comments on the work of Petri and Stammer writes “Stammer had found not cephalic organ in the *Tephritis* species”. Dean described an oesophageal bulb in *R. pomonella*, but did not test its content. Later he writes “in all members of the subfamily of the Trypetinae, insofar as they have been investigated….larvae and images both lack specific symbiotic organs”. So, in different parts of his work, Buchner clearly writes that the oesophageal bulb is not present in fruit flies, with the exception of *B. oleae* and possibly in *R. pomonella.*

At the end of the chapter considering Dean’s drafts, Buchner (1953) writes “The female *Rhagoletis* has an oesopharyngeal bulb like that of *Dacus*”. Probably the last sentence, analyzed without the context of the Buchner work has lead many authors since the 70’s to refer to Buchner (1965) as the author of oesophageal bulb .

Aims of the research

The aim of this thesis is to improve the knowledge of tephritid flies and their symbiotic bacteria.

The first contribution (Chapter 2) aims at broadening the knowledge of the presence of symbiotic bacteria in flies of the Subfamily Tephritinae, previously presented in a PhD. thesis (Piscedda, 2006).

The second contribution (Chapter 3) analyzes the phylogenetic relationships of flies of Subfamily Tephritinae and studies the coevolution with their symbiotic bacteria. For this purpose two regions of the mitochondrial DNA of the different species have been amplified and sequenced in order to verify the phylogenetic congruence between symbiotic bacteria and their hosts. This concordance implies that the current distributions of symbionts among the tephritids result from their vertical inheritance from an ancestor host initially infected and excludes the presence of horizontal transfer of symbionts. The study of the congruence will allow us to answer some interesting questions like: i. Is the presence of symbiotic bacteria derived from a single event? ii. Do they depend on different events? iii. Why are symbiotic bacteria not present in some species belonging to subfamily Tephritinae?

The third study (Chapter 4) is based on the phylogenetic analysis of some Paleartic species belonging to the Higher Tephritidae : Subfamily Tephritinae + Subfamily Trypetinae + Subfamily Dacinae (Korneyev, 1999). This work is mainly focused on species of the Subfamilies Tephritinae and Trypetinae. Two regions of the mitochondrial DNA of the different species have been amplified, sequenced and analyzed. The phylogeny inferred has been compared to the taxonomical classification based on morphological characters and also to the phylogenetic study based on the 16S rDNA sequences presented by Han (Han *et al.*, 2006). I have also attempted to combine phylogenetic results with biological and symbiotic information, to try to give a general picture. Phylogenetic relationships of tephritid flies have been analyzed and successively compared to the symbiosis information and the morphological characteristics of some anatomical structures related to the symbiosis, in order to confirm some relationships between them. In the same way, biological characteristics of these flies have been compared with the phylogenetic studies.

Chapter 2

Presence of specific symbiotic bacteria in flies of the Subfamily Tephritinae (Diptera Tephritidae) and their phylogenetic relationships: Proposal of '*Candidatus* Stammerula tephritidis'

Published as:

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In the present wotk, I contributed to experimental parts.

Abstract

The presence of symbiotic bacteria in flies belonging to the subfamily Tephritinae, which predominantly infest flower heads of composits (Asteraceae) was investigated. Twenty-five species of flies, collected mainly in the North of Italy, were examined. The bacteria adhere to the midgut epithelium in a space external to the peritrophic membrane and therefore not in direct contact with food bolus. Specific, unique, live but unculturable bacteria were consistently found in the majority of the fly species, and their presence was shown to be persistent also in flies reared under microbiologically controlled conditions and devoid of any residual culturable intestinal bacteria. Sequencing of the small subunit ribosomal DNA from the bacteria indicate that they belong to the family Enterobacteriaceae. Three main strongly supported clades are delineated by phylogenetic trees, the first of which features a coherent set of sequences displaying an homology lower than 96% compared to known taxa. The second and third clades instead feature cases with higher sequence homology to culturable bacteria, including *Erwinia persicina* and *Ewingella americana,* respectively. Relative rate tests are supportive of a fast genetic evolution for the majority of the bacterial symbionts of Tephritinae. In agreement with the interpretation suggested in 1929 after pioneering observations made by H.J. Stammer, a symbiotic relationship between bacteria and tephritid flies is postulated, its apparently polyphyletic origin is discussed, and a novel genus is proposed for the first clade under the designation '*Candidatus* Stammerula tephritidis'.

Introduction

Several kinds of associations between microorganisms and insects are found in nature (Buchner, 1965; Baumann & Moran, 1997). Stable or obligate relationships and instances of co-evolution with bacteria have been traced for aphids (Baumann *et al.,* 1995), whiteflies (Clark *et al.,* 1992), mealybugs (Munson *et al.*, 1992), planthoppers (Noda *et al.*, 1995), carpenter ants (Schröeder *et al.*, 1996), weevils (Campbell *et al.*, 1992), tsetse flies (Aksoy, 1994), cockroaches and termites (Bandi *et al.*, 1994). Among the benefits for the host, a specific nutritional complementation is hypothesized, in particular for those insects living on a markedly imbalanced diet, as in the case of aphids (Roubaksh *et al.*, 1996). In the past, such studies mostly relied upon microscopy to define the morpho-histological features of

symbiotic organs and describe the bacteria hosted within. Indeed, many insect-microbe associations were already covered in Buchner's renowned treatise (Buchner, 1965). However, as most of the prokaryotic microsymbionts are not culturable *ex situ*, their characterization and taxonomical placement had to wait until the advent of biomolecular techniques, which enabled 16S rDNA-based taxonomy via amplification and sequencing.

In the case of flies belonging to the Tephritidae, the object of the present investigation, the first species for which a bacterial symbiosis was described is the olive fly *Bactrocera oleae* (Rossi) (Subfamily Dacinae). In the adult insect's head obligate endosymbionts multiply within a specialized organ, the oesophageal bulb, and are thence discharged into the oesophagus and to the intestine where they are eventually digested (Petri, 1909). Recently the olive fly symbiont has been taxonomically described and designated has '*Candidatus* Erwinia dacicola' (Capuzzo *et al*., 2005). Other bacterial associations in tephritid flies have been described by Stammer (1929) and occur in several genera of the Tephritinae. In these cases, in adult stages, bacteria are located in the first tract of the mid gut, in contact with the epithelium but outside the peritrophic membrane (Girolami, 1983). The peritrophic membrane (Fig. 2.2), occurring in different insect species, is a membranous film, forming a thin lining layer that surrounds the food bolus and separates it from the delicate midgut epithelium. The oesophageal bulb, with some morphological differences, is also found in the subfamily Tephritinae, but in this case it is devoid of bacteria (Girolami, 1983). During oviposition, females of several Tephritinae, similarly to *B. oleae*, smear the egg's surface with bacteria in order to ensure their vertical transmission to the progeny. Larval stages maintain bacteria in their intestinal caeca (Petri, 1909; Stammer, 1929). In this case they are in direct contact with free-living intestinal bacteria.

The Tephritinae subfamily includes about 200 genera worldwide with over 1800 species (Foote *et al*., 1993). Tephritinae use vegetative and reproductive parts of host plants; larvae feed on flower heads and many species induce galls in these plant structures (Headrick & Goeden, 1998). With few exceptions, they feed solely on plants of the family Asteraceae, (Compositae), the daisy family (Zwölfer, 1983; White, 1988; Foote *et al*., 1993). As larvae do not develop on fruits, these insects are not regarded as pests of economical importance. Nevertheless some species may occasionally attack agricultural crops, as in the case of *Acanthiophilus helianthi* (Rossi) that can be found on sunflower (Belcari, 1985) and on

safflower (Ricci & Ciriciofolo, 1983). Some fly species have been tested as biological control agents of weeds (Zwölfer, 1983). A comprehensive body of literature on Tephritinae biology is reviewed by Headrick & Goeden (1998).

In the present work we investigated, using biomolecular methods, the bacterial symbioses first discovered by Stammer in the Tephritinae (1929) and reported later by Buchner (1965). The purpose of this work was to investigate the gut symbiotic bacteria in different species of the subfamily Tephritinae, exploring the instances described by Stammer (1929) and extending the analysis to uncover the symbionts' identities via 16S rDNA sequencing. This approach enables to verify the existence of a strict host-symbiont specificity and to trace the possible phylogenetic relationships occurring among hosted bacteria.

Materials and Methods

Origin and identification of biological material

Live specimens encompassing 25 different species of Tephritinae were collected from 20 field locations, ranging from central and northern Italy, to Slovenia and Croatia (Fig. 2.1). When possible, the same insect species was collected in different locations and in different years (Table 2.1).

Routinely, the floral heads (capitula) of plants, all belonging to the Asteraceae and containing their respective host insects, were collected. As for those insects that were found in more than one Asteraceae species, two or three alternative hosts were sampled. An in-field pre-screening of positive samples involved blade sectioning of the inflorescences and inspection of larval or pupal presence.

The harvested floral heads were kept within transparent plastic bags at room temperature in order to allow larvae to complete their development and pupate. When possible, capitula were subsequently sectioned and pupae were extracted, routed to surface sterilization and reared in aseptic conditions as described below. Adult flies that were occasionally captured in nature, or those that emerged during flower incubation in the plastic bags, were directly transferred into net cages.

Host plant determination was carried out using botanical keys (Pignatti, 1982) and supervised by European flora specialists. The taxonomical identification of the tephritid species was performed on morphological basis on adult stages using appropriate keys

(Hendel, 1927; Séguy, 1934; White, 1988; Merz, 1994). When necessary, identification was confirmed from the tephritid taxonomy specialist Dr. Bernhard Merz, (Museum of Natural History, Geneva, Switzerland).

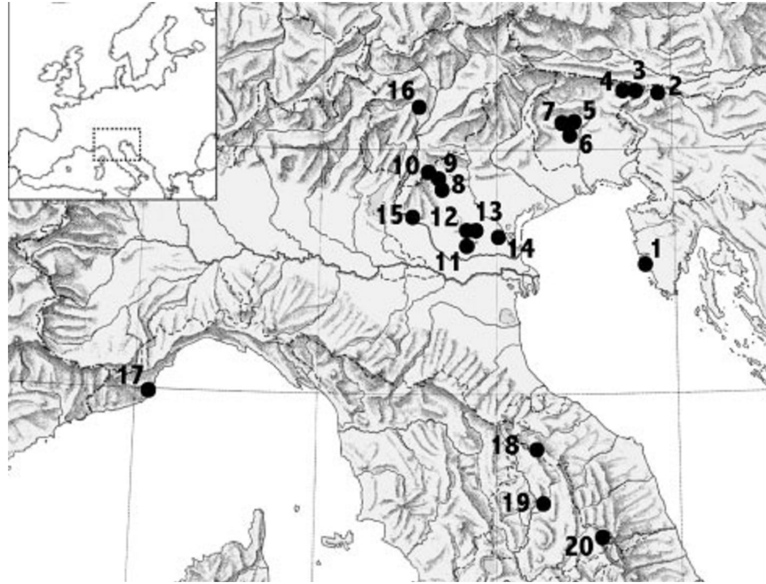

Fig. 2.1. Insect collection sites. Geographical map of central to northern Italy and surroundings. Numbers refer to the following localities (States or Provinces are indicated in brackets):
1: Rovigno (Croatia); 2: Kraniska Gora (Slovenia); 3: Tarvisio (Udine, including the sub-localities of 3a Tarvisio, 3b Mount Lussari and 3c Lake Predil); 4: Malborghetto, Val Saisera (Udine); 5: Fanna (Pordenone); 6: Vivaro (Pordenone); 7: Maniago (Pordenone); 8: Cogollo del Cengio, Mount Cengio (Vicenza); 9:Roana (Vicenza); 10: Asiago, Cima Larici (Vicenza); 11: Cinto Euganeo (Padova); 12: Teolo (Padova); 13: Torreglia (Padova); 14: Legnaro (Padova); 15: Verona (Verona); 16: Tres-Passo Predaia (Trento); 17: Diano Marina (Imperia); 18: Borgo Pace, Bocca Trabaria (Pesaro-Urbino); 19: Assisi (Perugia); 20: Norcia, Sibillini Mountain Range (Perugia).

Insect treatment and rearing

Pupae extracted from flower capitula were surface-sterilized by a 5 min immersion in 1% sodium hypochlorite, rinsed in sterile water at least two times, air-dried in sterile conditions and kept in sterile vials until adult emergence. The resulting flies were kept under microbiologically controlled conditions to avoid contamination with microorganisms other than the internal symbiotic bacteria. The flies were then aseptically transferred, under a laminar flow hood, into larger vials, containing a layer of Plate Count Agar on the bottom as a sterility check, and sealed with a sterilized transparent gas-permeable cellulose

membrane for dialysis (Sigma-Aldrich chemical co. S. Louis MO, USA). A drop of sterile glucose solution was placed on the internal side of the membrane to allow insect feeding. The drop of solution was re-wetted, whenever necessary, by spraying sterile water onto the vials.

All adult flies captured in nature, and those which emerged in the laboratory, were maintained in tulle-lined cages, and fed with a 50% sucrose solution containing 0.2% benzoic acid and 0.05% sorbic acid as antimicrobials.

Insect dissection

After a week of rearing under microbiologically controlled conditions, the flies were dissected for isolation of bacteria hosted in their midguts. Only specimens from which no microbial colonies had developed on the PCA agar on the bottom of the vials were selected. Flies were handled under a stereomicroscope in a laminar flow hood using sterile equipment and sterile water. Before dissection, insects were stunned by keeping vials for 20 minutes at 4 °C. After opening the insect's abdomen, the whole intestine was extracted, from which the midgut tract was selected by sectioning between cardial valve and malpighian tubes (Fig. 2.2). The resulting segment was transferred in a sterile Eppendorf tube and used for both bacterial culturability tests and for bacterial DNA amplification, in which case tubes were stored at -20°C until extraction.

Dissection was also done on adults who had not been reared under microbiologically controlled conditions (captured in nature or emerged from the flower capitula in the laboratory). In these cases the peritrophic membrane was pulled off from the midgut and discarded and the epithelium was gently rinsed in sterile water. These operations were intended to minimize the presence of bacteria from the alimentary bolus in these specimens which had not been fed with sterile solutions. The cleaned midgut, in these cases, was used for bacterial DNA extraction but not for microbial culturability tests.

Attempts at cultivation of the extraperitrophic bacteria

The possible culturability of the bacteria hosted in the midgut of insects, across the peritrophic sheat, was verified by plating the extracted midgut tissue on different standard microbiological media. Plate Count Agar, Potato Dextrose Agar, Malt Agar, Brain Hearth

Infusion, McConkey Agar, Trypticase soy agar, DeMan Rogosa Sharpe Agar and Luria Bertani agar (all media from Oxoid) were used. Only insects that had been surface-sterilized as pupae and reared in microbiologically controlled conditions from that stage onwards, were used.

In order to verify the actual presence and viability of bacterial cells in the specimens, a section of each midgut used for cultivation attempts, was observed under microscopy and analyzed with a physiological staining using the LIVE/DEAD® BacLight Bacterial Viability Kit (Molecular Probes Eugene CA, USA). Slides were also prepared for Gram staining and morphological characterization which was performed under an Olympus BX60 microscope.

DNA extraction and 16S rRNA gene amplification

The starting material for the molecular taxonomical analysis of the insect-associated prokaryotes was the midgut tract in which bacteria reside between the epithelium and the peritrophic membrane sheet as described (Fig. 2.2). The operation was performed either on insects emerged from surface-sterilized pupae and reared aseptically until the adult stage, on those that had reached the imaginal stage from stored freshly-harvested flower heads, or on those adults captured directly in the field. DNA was extracted from the content of midguts as described by Palmano et al. (2000). PCR amplification targeting the 16S rDNA was carried out in 25 µl containing 1 µl from the nucleic acid extract, 200 µM dNTPs, 0.8 µM of each primer, 0.625 U Taq DNA polymerase (Amersham Biosciences) and 2.5 µl 10X PCR buffer (500 mM KCL, 100 mM Tris/Hcl, pH 9, 15 mM $MgCl_2$). The universal bacterial 16S rRNA primers used were fD1 (5' – AGAGTTTGATCCTGGCTCAG – 3') and rP1 (5' – ACGGTTACCTTGTTACGACTT – 3') (Weisburg *et al.*, 1991), to yield an expected amplicon of approximately 1500 bp. The cycling program, carried out in an INC PTC-100 thermal controller (MJ Research Inc., San Francisco CA), consisted of a 95°C 2 min step followed by 35 cycles at 96°C for 30 s, 56°C for 30 s, 72°C for 90 s and a final extension at 72°C for 10 min.

The amplified products were separated on a 1% agarose gel and visualized under UV following staining with ethidium bromide.

DNA sequencing and bioinformatics analyses

The amplification products were analysed by dideoxynucleotide sequencing using primer FL4 (5' – CGGGTGAGTAATGTCTG – 3') or FL2 (5' – GGAACTGCATTCGAAACTG – 3').

Further primers were devised from the sequences obtained in order to walk through the entire 16S rRNA gene sequence. PCR products were purified with a QIAquick PCR purification kit (Qiagen spa. Milan, Italy) and used as template for sequencing in an ABI PRISM automatic DNA sequencer Applied Biosystems, Foster City CA). For those species in which PCR amplification of 16S tract would consistently yield more than a single product, individual bands were extracted from the gels with the (QIAquick Gel Extraction Kit – Qiagen spa.).

A BLASTN GenBank analysis of the sequences obtained was run through the NCBI website (http://www.ncbi.nlm.nih.gov/). Sequences were inspected and aligned using MEGA 3.1 (Kumar, 2004).

Phylogenetic relationships among sequences were estimated by three methods: (1) Maximum Parsimony (MP) using PAUP*4.0b10 (Swofford, 2002), (2) Approximate Maximum Likelihood (ML) using the software PHYML (Guindon & Gascuel, 2003) and (3) Bayesian Inference (BI) using MrBayes v3.1 (Huelsenbeck & Ronquist, 2001).

For MP analysis, best trees were obtained by full heuristic search using starting trees generated by stepwise addition of sequences (20 replicates with a randomized order), and the Tree Bisection Reconnection algorithm. Gaps were considered as a 5th state. Node support was obtained by 100 bootstrap replicates.

For ML analysis, the best-fit model of sequence evolution (TrN+I+G) was selected by Modeltest v3.06 (Posada & Crandall, 1998) using Akaike Information Criterion tests (Posada & Buckley, 2004). The best model found was used for approximate ML, using PHYML, with NJ starting trees and 100 replicates of bootstrap.

For the Bayesian analysis the best-fit model (GTR+I+G) was selected by the Akaike criterion using MrModeltest v2.1 (Nylander, 2004); gaps were also considered using the binary model implemented in MrBayes v3.1. Analysis started from random trees that were sampled for 3000000 generations with a sampling frequency of 10. The 50% majority rule

consensus tree and Bayesian posterior probability of support were obtained from 225000 sampled trees.

To verify faster evolution in putative endosymbionts Relative Rate Tests were carried out by using the Kimura's 2-parameter distance implemented in Phyltest software (Kumar, 1995) version 2.0.

Sequences with less than 99% similarity to GenBank database entries were screened for chimeras using the CHIMERA CHECK program of the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/).

Results and Discussion

Bacterial presence and location in Tephritinae flies

Twenty-five different fly species, belonging to 8 genera were studied. Ten of these had been dealt with in Stammer's original survey (Stammer, 1929). A list of the insect taxa analyzed, of the 34 plant species from which each was isolated, along with the place and time of the collection, is shown in Table 2.1. As some insect species are found on more than one host, and as we isolated the same fly species from more than one site, a total of 46 combinations of insect-plant-site were examined.

The bacteria associated with Tephritinae occupy an extracellular location. They are visible in the first part of the abdominal midgut (Fig. 2.2A) in all the analyzed Tephritinae. Bacteria appear to be adherent to the intestinal epithelium as already described by Stammer (1929), but external to the peritrophic membrane and therefore not inside the intestinal lumen in direct contact with the food bolus (Fig. 2.2B). They are consistently found in extracellular placement even when they are hosted inside a specialized evagination of the midgut epithelium, such as in *Sphenella marginata* (Stammer, 1929). On the contrary in the olive fly, a major agricultural pest not dwelling in Asteraceae flowers, and which hosts bacteria in its unique cephalic bulb (Capuzzo *et al.*, 2005), no extra-peritrophic gut symbionts are observed, and bacterial masses flow in the intestinal lumen, internal to the peritrophic membrane.

As visible by microscopy, samples from the midgut epithelium yielded bacteria that stain Gram-negative, the majority of them averaging 2,5 μm in length and often forming short

chains of several cells. Only from the insect *Campiglossa guttella* and *Noeeta* genus the bacteria appear small (1-2 µm), of coccoid shapes, and not forming chains.

The results observed confirm the regular presence of bacteria in many species of Tephritinae, although the function of this association is not yet fully understood. However a trophic advantage extending throughout the adult stage can be postulated. In fact, symbionts are found in both male and female adults. Therefore bacterial presence is not exclusively linked to a maternal transmission for the sole benefit of offspring stages. On the contrary they might play an important nutritional role in adult survival, as supported by the observation that flies emerged in the laboratory can live for over a year.

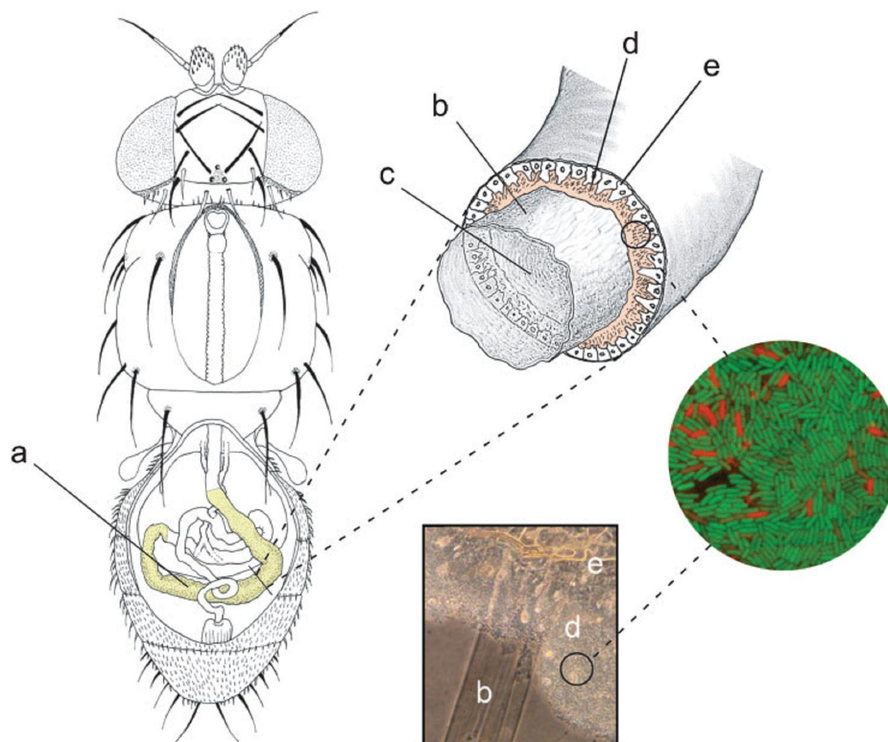

Fig. 2.2. Anatomical location of the bacteria examined. The drawing, portraying an insect observed under a dissecting microscope, shows the position of the midgut tract in which symbiotic bacteria reside (yellow portion) (a). The close-up detail (right) shows the co-axial presence of an inner thin vessel: the peritrophic membrane (b), in whose lumen (c) the regular alimentary bolus transit occurs, and many non-specific easily culturable bacteria can be found. Instead, in the interstitial gap space that runs all along (d), between peritrophic tube and the outer midgut epithelium (e), resident bacteria are observed, which constitute the target of the present analysis.

Insect species [1]	Host Plant	Collection site (Fig.2.1)	Collection year	n. of cases sequenced	Origin of PCR template[2]	GenBank accession n.
*Acanthiophilus helianthi**	*Centaurea jacea*	5	2004	1	A	EF469625
"	*Cirsium arvense*	14	2005	1	A	-
"	*Centaurea jacea*	16	2006	1	B	-
Campiglossa doronici	*Doronicum austriacum*	8	2005	3	A(1);B(2)	EF469636
Campiglossa guttella	*Hieracium murorum*	10	2005	1	B	EF469637
"	*Hieracium murorum*	3b	2005	1	B	-
"	*Hieracium glaucum*	4	2005	1	B	-
"	*Hieracium murorum*	16	2006	1	B	-
Capitites ramulosa (a)	*Phagnalon saxatile*	17	2006	6	A(3); B(3)	EF469628
*Dioxyna bidentis**	*Bidens tripartita*	5	2005	1	B	EF469631
"	*Bidens tripartita*	17	2006	3	B	
Noeeta bisetosa	*Hieracium piloselloides*	5	2006	1	A	EF469632
Noeeta pupillata	*Hieracium porrifolium*	3d	2005	1	B	EF469634
"	*Hieracium murorum*	8	2005	2	B	EF469635
"	*Hieracium umbellatum*	5	2005,'06	5	A(2); B(3)	EF469633
"	*Hieracium umbellatum*	13	2005, ,06	2	B	-
Oxyna flavipennis	*Achillea millefolium*	9	2005,'06	4	C	EF469630
*Sphenella marginata**	*Senecio inaequidens*	6	2004	1	B	-
"	*Senecio vulgaris*	14	2004,'05	2	A	-
"	*Senecio alpinus*	9	2006	2	B	EF469629
Tephritis arnicae	*Arnica montana*	10	2005	2	A	EF469616
*Tephritis bardanae**	*Arctium minus*	11	2004	1	B	EF469617
*Tephritis cometa**	*Cirsium arvense*	1	2006	1	B	EF469615
*Tephritis conura**	*Cirsium oleraceum*	5	2004	1	A	-
"	*Cirsium erisithales*	16	2004,'05	3	A	EF469618
Tephritis divisa	*Picris echioides*	17	2005,'06	2	B	EF469619
*Tephritis fallax** (c)	*Leontodon hispidus*	18	2005	2	B	EF469622
Tephritis formosa	*Sonchus oleraceus*	5	2004,'05,'06	3	A	EF469620
"	*Sonchus oleraceus*	13	2005	2	B	-
"	*Sonchus* sp.	3c	2005	2	B	-
"	*Sonchus* sp.	18	2005	1	B	-
Tephritis hendeliana (b)	*Carduus chrysacanthus*	20	2005	2	C	-
"	*Carduus nutans*	9	2005	1	B	EF469612
Tephritis hyoscyami (b)	*Carduus personata*	3b	2005	3	B	EF469613
*Tephritis leontodontis** (c)	*Leontodon autumnalis*	2	2005	1	B	EF469621
"	*Leontodon hispidus*	16	2006	1	B	-
Tephritis matricariae	*Crepis vesicaria*	14	2004,'05,'06	3	A(2);D(1)	EF469623
"	*Crepis vesicaria*	18	2005	2	B	-
Tephritis prope matricariae	*Crepis chondrilloides*	7	2005,'06	4	A(2);B(2)	EF469624
Tephritis postica (b)	*Onopordum acanthium*	20	2005	1	B	EF469611
Tephritis separata	*Picris hieracioides*	1	2006	1	B	EF469614
*Trupanea amoena**	*Lactuca serriola*	13	2004,'05	2	B(1);C(1)	EF469626
"	*Reichardia picroides*	17	2006	2	B	-
*Trupanea stellata** (a)	*Erigeron annuus*	15	2004	1	B	EF469627
"	*Erigeron annuus*	14	2005	1	B	-
"	*Crepis foetida*	14	2004	1	B	-

Table 2.1. Tephritinae species in which symbiotic bacteria have been observed and sequenced (*in these cases the presence of extraperitrophic bacteria had been previously reported by Stammer, 1929).
1Species in which the same letter is indicated in brackets contain symbionts sharing an identical 16rDNA sequence.
2A: adult obtained from surface sterilized pupa incubated in microbiologically-controlled conditions; B: adult emerged from flower heads in laboratory; C: adult captured in the field; D: surface-disinfected larva.

Viability and non-culturability of the extra-peritrophic bacteria

When performing the LIVE/DEAD® BacLight bacterial viability test on the bacteria adhering to the midgut epithelium, including the specimens reared in microbiologically controlled conditions, the majority of cells stained green, indicating a substantially viable population. However, attempts to culture the bacteria by plating, testing eight different microbiological solid media, never yielded colonies from any of the host species tested. In the same way, in the majority of the cases microbial colonies did not develop on the PCA culture medium on the bottom of the vials in which the adults from surface-sterilized pupae were introduced and survived for a week. Besides being a control for the aseptic conditions of the rearing technique, this also indicates the absence of culturable released bacteria in the faeces or in other excreta. Similar results were observed when we used the same methods for '*Candidatus* Erwinia dacicola' dwelling in the head organ of the olive fly (Capuzzo *et al.*, 2005), suggesting that in these, as in many other bacterial-insect associations, the prokaryotic partner is not culturable *ex situ*. In fact, several studies concur that a loss of the capability of multiplication outside the host correlates with a situation of symbiotic coevolution between insects and bacteria.

Bacterial 16S sequence analysis

Adult or larvae were used as outlined in Table 2.1. A total of 86 samples were processed, which accounts on average for three repetitions from each of the 25 insect species.

After cell lysis, amplification of the small subunit ribosomal gene using eubacterial universal primers, and nucleotide sequencing, the results could be aligned and compared.

It is remarkable to report that: (1) Seventeen out of the twenty-five insect species were found to possess a specific and unique single bacterial symbiont; (2) Three symbiont sequences were found to be shared by two or three insect species. Those insect species that share an identical symbiont sequence (*Tephritis postica-T. hendeliana-T. hyoscyami*;

Tephritis leontodontis-*T. fallax*; and *Trupanea stellata*-*Capitites ramulosa*) are known from insect taxonomy as very closely related; (3) there was a nearly complete reproducibility of the results for sequences of bacteria isolated from the same insect species, i.e., the full length 16S sequences of bacteria inhabiting a given insect species always turned out identical, irrespective of geographical site of isolation (from the Alps to the central Apennine range), year of collection, and of plant species. The latter distinction applies to oligophagous flies such as *Acanthiophilus* or *Sphenella,* in which the same insect species can lay eggs in different Asteraceae species;

(4) For one insect species, *Noeeta pupillata,* slightly different sequences were observed in flies isolated from three different plant species of the genus *Hieracium*. This result could, however, also suggest the existence of a group of sibling insect ecospecies, not yet resolved by entomology systematics due to their morphologically indistinguishable features.

The comparison of bacterial sequences in pairwise alignments indicates a range of identity spanning from a maximum of 99% (within the *Tephritis* genus group) to a minimum of 92% (across the symbiont of the *Acanthiophilus helianthi* and the symbiont group of the genus *Noeeta*).

A methodological consideration can be done in retrospect. Identical bacterial sequences were obtained from all the Tephritinae species analysed, irrespective of the individuals rearing history. In fact, either adults from surface-sterilized pupae hatched in sterile vials, or those emerged in non-sterile conditions or even the nature-borne ones that we occasionally captured, would yield the same PCR product. This indicates that the simple additional procedures of removing the peritrophic membrane and rinsing the midgut could ensure a clean PCR outcome even in a non-gnotobiotic situation, supposedly due to a prevailing amount of the specific putative endosymbiont DNA. As a consequence, rearing in a microbiologically controlled situation could be omitted and the simplified procedure recommended for studies of this kind. A critical issue, however, is to avoid newly-hatched adults and work with those which have emerged for at least a week; in the former, bacteria are usually too few and PCR may result template-limited. Stammer (1929) noticed that after about a week from fly emergence, substantial multiplication of endosymbionts takes place in the extra-peritrophic reservoir.

Phylogenetic placement of the symbiotic bacteria

A BLAST analysis of the sequences revealed the degree of 16S homology with known taxa. Results indicate that all of the samples belong to the family Enterobacteriaceae in the Gammaproteobacteria class. Sequences of the bacterial symbionts from genera *Tephritis*, *Acanthiophilus*, *Sphenella*, *Trupanea* and *Capitites* share no more than 96% identity with database taxa. Instead there are cases for which an identity level of about 99% with known culturable species indicate different relationships; this is the case of the symbiont from *Campiglossa guttella* with *Erwinia persicina* (AM184098), of that from *Dioxyna bidentis* with *Erwinia persicina* (Z96086), and of those from the *Noeeta* group with *Ewingella americana* (DQ383802).

Bacterial 16S rRNA sequences were subjected to crossed molecular phylogenetic analyses testing with maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). Results are shown in Fig. 2.3. We have included representative members of the closest known relatives indicated by the BLAST analysis. It is noteworthy that the tree topology of the bacterial symbionts mainly correspond to the phylogeny of Tephritinae based on morphological features.

Three main clades were clearly distinguished and supported by each of the clustering methods used.

Clade A includes symbionts from all the analyzed species within the genera: *Tephritis*, *Acanthiophilus*, *Sphenella*, *Trupanea*, *Capitites* and *Oxyna*, as well as the symbiont of *Campiglossa doronici*. None of the other sequences present in GenBank groups in this clade, suggesting that the symbionts in this group are likely monophyletic. The inference is well supported by the bootstrap values of all three methods used (77% for ML, 73% for MP, 56% for BI). Inside this clade the taxon assignment of host insects for the genus *Tephritis* is strongly aligned with the grouping of their symbiotic bacteria (ML 89%, MP 88%, BI 96%). The same agreement (100% ML, 100% MP, 100% BI) is seen for the two *Trupanea* species, although there is symbiont sequence identity between *Trupanea stellata* and *Capitites ramulosa.* For this clade the most similar free-living bacteria belong to the genus *Erwinia*, sharing 95-96% sequence identity.

Clade B, that groups together with free living *Erwinia* cases, stems out as an apparent sister group of Clade A; however the bootstrap value (66%) given by only one method (ML)

suggests a weak affinity. *Dioxyna bidentis* and *Campiglossa guttella* symbionts belong to this clade as well as our previously described Candidatus *Erwinia dacicola*, the olive fly symbiont (Capuzzo *et al*., 2005), with similarity values ranging from 97 to 99% with other *Erwinia.*

It is noteworthy that symbionts of the two analysed species of *Campiglossa* belong to different clades.

Clade C is statistically well supported (100% for all three methods) and includes symbionts found in all *Noeeta* species analyzed, whose most similar (99%) free-living culturable species turns out to be the human pathogen *Ewingella americana*. The symbionts of the *Noeeta* genus appear to have been acquired independently from those of the other Tephritinae considered. The bacterial symbiosis of the Tephritinae may therefore be considered not monophyletic. In this respect it is important to recall that the *Noeeta* genus belongs to the tribe Noeetini, which is phylogenetically well separated (Han *et al*., 2006) from the tribe Tephritini comprehensive of all the other species of the clade A e B (with the exception of *Candidatus* Erwinia dacicola belonging to the Dacinae subfamily).

The 16S rDNA sequences of *Klebsiella oxytoca* and *Pantoea agglomerans*, two of the most common intestinal fruit fly associated bacterial species for the Trypetinae and Dacinae (Rossiter *et al*., 1983; Loyd *et al*., 1986), do not show particular similarity with any of these three clades.

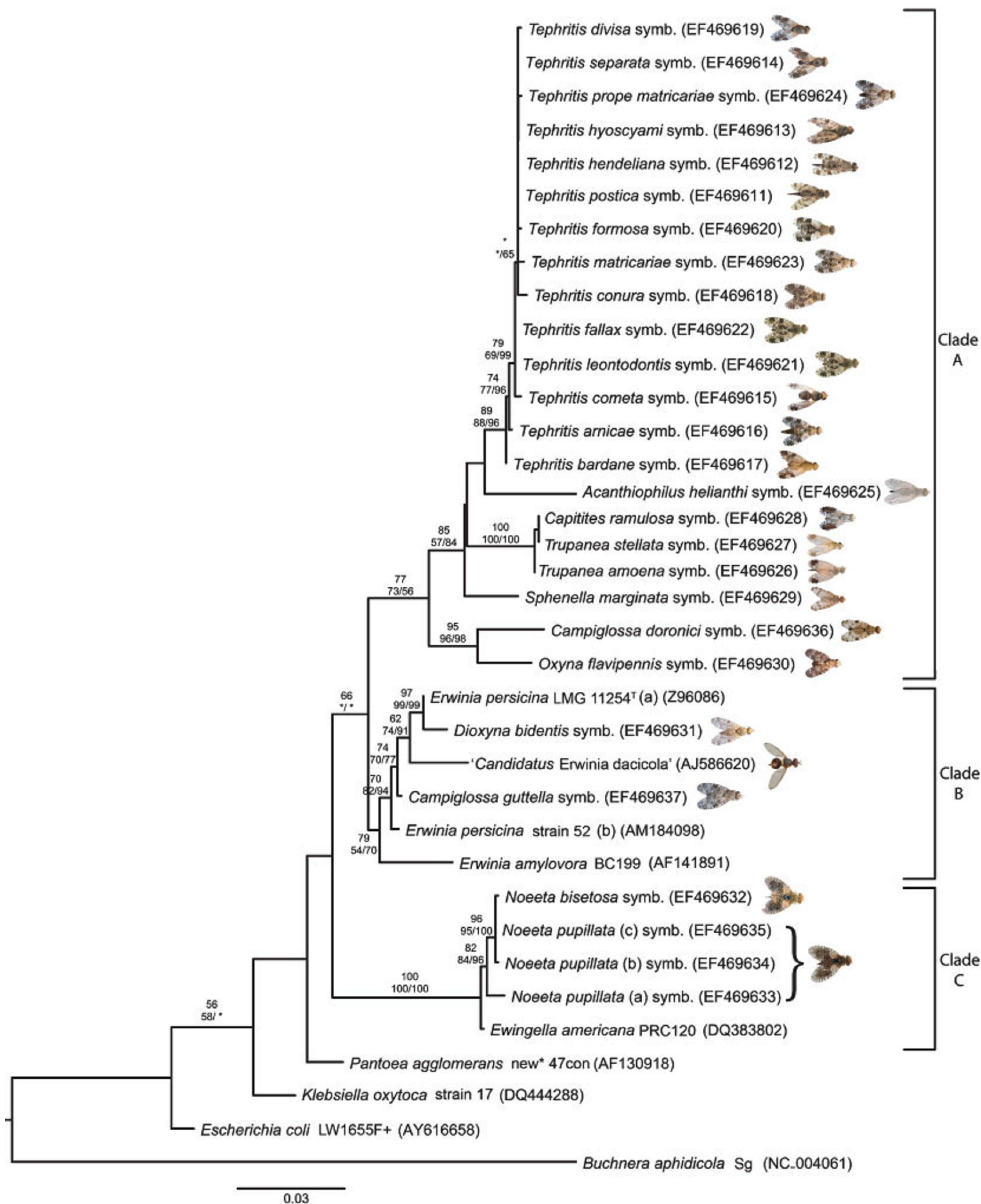

Fig. 2.3. Phylogenetic tree of Tephritinae bacterial symbionts and close relatives within the γ-Proteobacteria, based on the 16S rDNA gene sequence, constructed upon the alignment of a minimum common portion of 1324 nucleotides. The Maximum Likelihood tree is shown, with the bootstrap probabilities (maximum likelihood ML, maximum parsimony MP) and posterior probabilities (Bayesian Inference, BI) reported on the nodes scoring a support higher than 50% in at least one of the three methods (asterisks indicate bootstrap values lower than 50%). Position of the values at nodes: ML: top, MP/BI: bottom. For the three cases in which identical symbiont sequences were found (*Tephritis postica* =*T. hendeliana* =*T. hyoscyami*; *T. leontodontis* =*T. fallax*; and *Trupanea stellata* =*Capitites ramulosa*), only one member is listed in the branch.

Rates of evolution and biases of sequence composition

When bacteria live confined within isolated contexts, such a particularly fast rate of DNA sequence evolution is shown to take place. This is presumably due to the absence of recombination with external populations and because of a more pronounced effect of genetic drift (Moran, 1996, Brynnel *et al.*, 1998). Tests to verify whether a differential rate of evolution exists between a putative endosymbiont and its closest culturable free-living relative have been described (Wu & Li, 1985; Muse & Weir, 1992). The calculation (Relative Rate Test) uses the distance of a third outgroup species and tests the null hypothesis that species A and B have evolved at the same rate since divergence. We applied the test to different examples of our putative symbiotic bacteria in comparison to their near free-living neighbours. Results are summarized in Table 2.2. All 16S sequences of clade A exhibit a significantly high rate of substitution, ranging from 3.2- to 4.7-fold, when compared with their free-living sister lineage. Higher rates are observed in clade B, with *Candidatus* Erwinia dacicola and *Dioxyna bidentis* evolving 6.6 and 8.5-fold more rapidly than the non-symbiont counterpart *Erwinia persicina*. Relative substitution rates from *Campiglossa guttella* (clade B) and all *Noeeta* symbionts (clade C) were instead not significantly different from their nearest culturable bacteria.

The test further supports the instance that the extra-peritrophic bacteria of Tephritinae, sharing low homologies with free-living species, are bona fide insect symbionts.

In many insect symbioses, including the *Buchnera* species in aphids, the location of the bacterial symbiont is intracellular, while in our case bacteria occupy an extracellular cavity and are separated from the intestinal lumen only by the thin peritrophic membrane. Thus the chance of contacts with the outer environment is higher than in endocellular symbionts.

A strong A+T mutational bias is often observed in endosymbionts' genomes, supposedly due to a loss of repair genes (Wernergreen, 2002). *Buchnera aphidicola* has an A+T content of 50.6%, while the A+T content of Tephritinae symbionts in the 16S gene ranges from 44.6% to 47.2%, values which are not significantly different from 44.2% displayed by their free-living relative *Erwinia persicina*. Other exceptions in this A+T richness are observed in symbiont bacteria presumed to have recently established association with their hosts (Heddi *et al.*, 1998; Baumann *et al.*, 2002; Lefèvre *et al.*, 2004). On the other hand,

maintaining traits common to those of culturable free-living species could be envisaged as not unlikely in some symbioses; in the present case it has to be stressed that symbionts (a) are extracellular, (b) undergo cycles of rapid multiplication in newly-emerged adults, and (c) are exposed to direct contact, and possibly competition, with other bacteria from the food bolus when hosted in gastric caeca at larval stages.

Taxon 1[a]	Taxon 2[b]	L1[c]	L2[c]	L1-L2[d]±SE	z^e	Rate Ratio[f]
Tephritis group	*E. amylovora*	0.034	0.008	0.026±0.0055	4.76*	4.3
Acanthiophilus helianthi	*E. amylovora*	0.044	0.009	0.034±0.0065	5.31*	4.7
Trupanea group; *Capitites ramulosa*	*E. amylovora*	0.039	0.012	0.027±0.0062	4.33*	3.2
Sphenella marginata	*E. amylovora*	0.037	0.012	0.026±0.0060	4.26*	3.2
Oxyna flavipennis; Campiglossa doronici	*E. amylovora*	0.040	0.012	0.028±0.0059	4.80*	3.4
Campiglossa guttella	*E. persicina a*	0.003	0.001	0.002±0.0020	0.82	2.1
Erwinia dacicola	*E. persicina b*	0.016	0.002	0.013±0.0038	3.43*	6.6
Dioxyna bidentis	*E. persicina b*	0.005	0.001	0.005±0.0020	2.44*	8.5
Noeeta group	*E. americana*	0.004	0.002	0.002±0.0012	1.70	2.1

Table 2.2.- Relative rate tests for the 16S rDNA gene between taxa of extracellular symbionts of Tephritinae (taxon 1) and related free-living bacteria (taxon 2), with *Escherichia coli* (AY616658) as third (outgroup) member.
[a]Tephritinae symbionts.
[b]Related free-living bacteria: *Erwinia amylovora* (AF141891); *Erwinia persicina* a (AM184098); *E. persicina* b (Z96086); *Ewingella americana* (DQ383802).
[c]Average of observed number of substitutions per site in comparison to the common ancestor of Taxon 1 and Taxon 2.
[d]Distance Difference.
[e]Z-statistics: Z-values higher than 1.96 (marked with*) are requested to reject the rate constancy at 5% level (Kumar, 1996).
[f]L1/L2.

Considerations on the origin of the symbiosis

A thorough evaluation of the evolutionary relationships between hosts and symbionts will require the expansion of the present analysis in two main directions: (a) investigating the symbionts of Tephritinae species inhabiting other continents, and (b) carrying out a parallel phylogenetic analysis on the insect hosts by sequencing their ribosomal DNA or other genes to verify the congruence of the resulting tree with the one based on bacterial sequences. Regarding hypotheses on the origin of this symbiosis it appears that at least three distinct events could have taken place. The earliest concerns clade A and possibly

involves the putative ancestor of the present representatives of the genus *Erwinia*. More recent independent events of lateral genetic transfer would instead be supported by the situation observed in the two other clades, in both of which Tephritinae symbionts appear intermingled with free living Enterobacteriaceae (*Erwinia persicina* in clade B and *Ewingella americana* in clade C) or with symbionts of more distantly related insects (e.g., *Bactrocera oleae*, belonging to the Dacinae subfamily and hosting '*Candidatus* Erwinia dacicola'). For the latter case we envisage the possibility of an acquisition in relatively recent time, involving the descent of free-living species commonly occurring on the vegetation. Other cases of insect-bacteria symbioses of apparently polyphyletic origin, invoking an interpretation based on lateral gene transfer events are reported in the literature, as for example in the case of the closely related bacteria found in *Sitophilus* weevils and *Glossinia* tsetse flies (Lefèvre *et al*., 2004).

Proposal and description of "*Candidatus* Stammerula tephritidis" gen nov. sp.nov.

The phylogenetic analyses delineated above describe three main phenons. For one of these, namely clade A, a deep and robust separation from the other branches exists, and the levels of homology with known taxa are below 96%. The degree of 16S sequence divergence that currently separates different genera within the family Enterobacteriaceae ranges between 2% and 8% (Moran *et al*., 2005). On the basis of these premises we propose the designation of a novel genus, *Stammerula*, to include symbionts of Tephritinae flies occurring in clade A. Among these, as evidenced from Fig. 2.3, an extremely coherent subset is represented by the sequences originating from all insects belonging to the *Tephritis* genus, sharing among themselves 99% of identity. We propose that this group be representative of a novel species within the perspective genus, under the designation '*Candidatus* Stammerula tephritidis'.

Regarding the more variable remaining sequences that also cluster in clade A, encompassing the symbionts from *Acanthiophilus, Trupanea, Sphenella, Campiglossa* and *Oxyna*, we propose that these be gathered under the same genus and we envisage a possible future ascription either to the species described above or to new ones once a higher number of insect hosts are investigated in order to refine the rank attribution of their symbionts.

Stammerula (Stam.me.ru.la. N.L. fem.n., in honor of the German biologist Hans-Jurgen Stammer who first described bacteria associated with Tephritinae flies; L. fem. dim. suff -*ula*;) *Stammerula tephritidis* (teph.ri.ti.dis. N.L. gen. n. *tephritidis*, of the tephritis, the insect genus to which the bacterium is associated).

[(Enterobacteriaceae) NC; G2; R; NAS (Genbank 16S sequences EF469611 - EF469624); oligonucleotide sequences of unique regions of the 16S rRNA gene specific for the genus are nn 1109-1123 GGACCTyAT**y**AAAGT; The y at position 1118 is an insertion peculiar of this genus; oligonucleotide sequences of unique regions of the 16S rRNA gene specific for the species are: nn 5-18 GATGTCGTAAGACC; nn 86-104 GAGGTAATGGCTTACCTAA; nn 288-301 GAGGTTAATAACCC. S (*Tephritis* spp., extracellular space between gut epithelium and peritrophic membrane); M]. Straight rod-shaped cells, 2–3 µm. Negative to Gram staining. Unculturable on microbiological media. Symbiont of *Tephritis arnicae, T. bardanae, T. cometa, T. conura, T. divisa, T. fallax, T. formosa, T. hendeliana, T. hyoscyami , T. leontodontis, T. matricariae, T. postica, T. separata* (Diptera, Tephritidae). Located exclusively in association with its host species within the following structures: extraperitrophic space of the midgut. Basis of assignment: 16S rRNA gene sequences. G+C content of the 16S rRNA gene sequence is 54.5 mol%.

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Chapter 3

Phylogenetic relationships of the Tephritinae subfamily (Diptera, Tephritidae) and concordant evolution with their symbiotic bacteria

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I contributed to experimental parts data analysis and paper writing.

Introduction

Several kind of intimate mutual associations, between bacteria and insects are known. In these insects, symbiotic bacteria are endocellular, and housed in the cytoplasm of specialized cells called bacteriocytes (or mycetocytes). Thus, they are obliged to live in a close environment with limitated possibilities of gene exchange (Buchner, 1965; Moran & Baumann, 2000; Gil *et al.*, 2004; Wernegreen, 2004). These symbionts are vertically transmitted to the next generation and they are not able to live outside of their host. Technological progress in the field of molecular phylogeny has permitted us to study and explore the world of these non culturable bacteria. Several works report that the host phylogeny mirrors the symbiont phylogeny (Chen *et al.*, 1999; Clark *et al.*, 2000; Moran, 2001; Moran *et al.*, 2003; Gruwell *et al.*, 2007). This congruence between host and symbiont implies a single bacterial infection of the host ancestor which was followed by co-evolution of both partners (Gil *et al.*, 2004). Endocellular bacteria, in contrast to their closest free-living bacteria, display distinctive genetic properties including AT-biased base composition, accelerated molecular evolution, and, at least sometimes, small genome size; these features suggest increased genetic drift (Moran & Baumann, 2000).

For insects belonging to different systematic groups, the presence of extracellular symbiotic bacteria has been described. These symbiotic bacteria are harboured in their gut cavity and some of them are known to play substantial biological roles for their hosts (Dillon & Dillon, 2004). For some of them the presence of a vertical transmission has been noted. The stinkbugs of the family Platasipidae harbour a bacterial symbiont in the midgut which is transmitted to the new generation orally, by a capsule containing the bacteria placed under the egg mass. (Fukatsu & Hosokawa, 2002).

Such extracellular associations are thought to be evolutionarily more ocasional than the endocellular associations, on the grounds that the symbionts are not isolated in the body cavity and vulnerable to invasion and replacement by foreign microbes. Thus, in these cases, a phylogenetic congruence between extracellular bacteria and host is not common (Buchner, 1965; Donovan, *et al.*, 2004).

A strict cocladogenesis between two species belonging to the family Plataspidae and their gut symbionts has been described for the first time recently, demonstrating strict host-symbiont cospeciation (Hosokawa *et al*., 2006).

The family Tephritidae, the subject of the present work, is commonly known as fruit flies and in the world includes more than 4000 species, divided into about 500 genera (White, 2006). Tephritid larvae develop mainly in fruit, leaves, or within the flower heads of Asteraceae (White, 1988). Some species, mostly the carpophagous species, are considered a notorious group of agricultural pests.

There is extensive literature describing, the presence of "associated bacteria" belonging to genera *Enterobacter* and *Klebsiella* in some Tephritid flies (Lloyd *et al.*, 1986; Drew & Lloyd, 1987; Daser & Brandl, 1992; Marchini *et al*., 2002; Lauzon, 2003). These associations, despite their importance during the life of the insect, can be considered facultative (Drew & Lloyd, 1991).

The first hereditary symbiosis in the Tephritidae family has been described in the olive fly *Bactrocera oleae* (Rossi) by Petri (1909). The mother transmits symbiotic bacteria to the new generation, smearing the surface of its eggs with bacteria. The symbiont are extracellular but multiply inside the intestinal caeca at the larval stage (Petri, 1909; Stammer, 1929), presumably in contact with free living intestinal bacteria. In the adult insect's head, obligate symbionts multiply within a foregut diverticulum that is present in all the adult tephritids flies and is called oesophageal bulb (Petri, 1909; Capuzzo *et al*., 2005).

Recently, the presence of a hereditary symbiotic bacteria, designated as '*Candidatus* Erwinia Dacicola', has been confirmed in adults emerging from previously surface-sterilized pupae (Capuzzo *et al.*, 2005). As well as, in *B. oleae*, the presence of symbiotic bacteria has been described in some species (genus *Tephritis*, *Campiglossa* (=Paroxina), *Trupanea* (=Trypanea), *Acanthiophilus*, *Sphenella* and *Oxyna)* belonging to the subfamily Tephritinae (Stammer, 1929). In this case the oesophageal bulb is smaller than *B. oleae'*s and devoid of bacteria. The symbiotic bacteria are located in the first tract of the midgut, in contact with the epithelium but, as later reported by Girolami (1983), outside the peritrophic membrane. Thus it is not in contact with the food bolus. According to Stammer, Mazzon *et al.* (2008) described the presence of symbiotic bacteria in flies belonging to

these and other new genera. For all these symbiotic bacteria, the bimolecular analyses carried out in 25 insect species have suggested a correspondence between the species of host fly and the sequences of bacterial 16S rDNA. The phylogenetic analyses delineated 3 main clades; the symbionts of one of them are monophyletic and homogeneous and are designated as '*Candidatus* Stammerula tephritidis' (Mazzon *et al.*, 2008).

The phylogenesy of this important family, primarily based on morphological data, has been recently improved, by taxonomic research, using molecular techniques based on nucleotide sequence data. These recent studies have suggested several previously unknown relationships and have offered new possibilities for Tephritid classification (Han & McPheron, 1997; 1999). Subsequent important studies have improved and confirmed some relationships between several subfamilies ad tribes, as the monophyly of the tribe Trypetini (Han, 2000) and the subfamily Tephritinae (Han *et al.*, 2006).

In the present work, we have sequenced a region of the 16S rDNA and COI e COII of all the species, in which the presence of symbiotic bacteria has been described (Mazzon *et al.*, 2008) and expanded the analysis to other tribes of Tephritinae coming from the same geographic area.

These allow us to: i. Study the molecular relationship among the tribes of the Tephritinae harbouring symbiotic bacteria; ii. Verify the phylogenetic congruence between tephritid flies and their symbiotic bacteria, despite the fact that they are extracellular symbionts. This concordance implies that the current distributions of symbionts among the tephritids results from their vertical inheritance from an ancestor host initially infected and excludes the presence of horizontal transfer of symbionts; iii. Answer some interesting questions such as, is the presence of symbiotic bacteria derived from a single event or do they depend on different events? Do all tribes belonging to subfamily Tephritinae present symbiotic bacteria?

Materials and Methods

Insect host

Origin of material

In the present work, 46 species belonging to three tephritid subfamilies have been analyzed, emphasizing the Paleartic Tephritinae subfamily (Tab. 3.2). For this subfamily, besides the 25 species in which symbiotic bacteria have been detected previously (Mazzon *et al*., 2008) 17 other species, have been sequenced. A total of 42 species belonging to 17 different genera currently placed in five (Norrbom *et al.*., 1999) of the largest Paleartic Tribe (a total of nine) have been studied. Family Platystomatidae have been considered as an outgroup. All the specimens added have been collected mainly in the North of Italy, in the same locations as the preceding research (Mazzon *et al*., 2008).

These specimens (pupae or adults) have been obtained in the laboratory from infested flower heads and previously collected in the field following the methodology described in Mazzon *et al.* (2008). Samples were treated as described by Mazzon *et al*. (2008) and routinely preserved in 95% ethanol and stored at -80°C until processed.

With some exceptions, the specimens sequenced were the same as those used in the previous work in which bacterial DNA has been extracted (Mazzon *et al.* 2008).

Tephritid higher-level classification and specific names follow Norrbom *et al*. (1999), Korneyev (1999) and Merz (1994, 1999).

A set of samples from each of the studied species were dried, pinned and deposited in the Department of Environmental Agronomy and Crop Sciences – Entomology (DAAPV), University of Padova, Italy.

DNA extraction

DNA of the whole insect body (or part of it), was extracted with the salting-out protocol (Patwary, 1994). At least two samples for each species were processed. In order to assess the quality of the DNA, extract products were separated in a 1% agarose gel and viewed under UV after staining with ethidium bromide.

Amplification and sequencing of host genes

Two regions of the mitochondrial DNA, a fragment of 16S rDNA and a fragment including the 3’ region of cytochrome oxidase sub-unit I, tRNA-Leu and the 5’ region of cytochrome oxydase sub-unit II, were amplified.

PCR amplification was carried out in a 20µl volume containing 2µl from the nucleic acid extract, PCR Buffer 10X, 25 mM MgCl, 2mM dNTPs, 10 µM of each primer and Taq (5U/µl).

A combination of universal and specific primers were used for PCR amplification and sequencing (Tab. 3.1). Specific primers were designed comparing some of our sequences with some mitochondrial 16S rDNA sequences of Tephritidae available in GenBank: *Ceratitis capitata* AJ242872, *Tephritis signatipennis* AF177124, *Urophora misakiana* DQ471388, *Ensina sonchi* DQ471390, *Noeeta pupilata* DQ471392, *Campiglosa californica* DQ471404.

The cycling program was carried out in an Eppendorf Mastercycler Gradient and consisted of a first step at 96°C for 5 min followed by 35 cycles with a denaturation step of 96 °C for 1 min., an annealing step ranging between 52°C and 62°C for 1min. and an extension step of 72°C from 1-2 min. followed by a final extension at 72°C for 5 min. The amplified products were separated in a 1% agarose gel and viewed under UV with an ethidium bromide or SYBR safe (Invitrogen) staining**.** PCR products were purified with the ExoSAP-IT kit (Amersham Biosciences) and directly sequenced. Sequencing was performed at the BMR Genomics service (Padova, Italy).

Host sequences alignment

Sequences of 16S rDNA and COI-tRNALeu-COII were inspected and corrected using MEGA 4.0 (Tamura *et al.*, 2007). The 16S sequences presented some ambiguous portions which were difficult to align. In order to resolve this problem we decided to align our sequences considering the rDNA secondary structure, thus avoiding the deletion of these ambiguous portions.

Considering the secondary structure of rRNA in the alignment, we took into account the correlation between nucleotide sites in the stem regions; this information, when ignored, could lead to a bias in the supporting confidence of the clades. Moreover, a phylogenetic

study based on the secondary structure seems also to have advantage with closely related species (Buckley *et al.*, 2000).

Target gene	Primer gene	Sequence	Source
16S	LR-J -12883	(5’- CTCCGGTTTGAACTCAGATC – 3’)	(Xiong & Kocher, 1991)
	TV-N-14202	(5’ – AGCATTTCATTTACATTGAA – 3’)	(Han & McPheron, 1996)
	DFI	(5’ – CATTGGGCAGGTYARACT – 3’)	(this study)
	DFI2	(5’ – GATTTATAGGGTCTTCTCGTC – 3’)	(this study)
	DR	(5’ – GATGTACCGGAAGGTGTATCT – 3’)*	(this study)
	DRI	(5’ – GTTATTCGTTTATAAAGRTATC – 3’)*	(this study)
	LRN13398	(5’ - CGCCTGTTTAACAAAAACAT – 3’)	(Simon *et al.*, 1994,2006)
	SR – N14220	(5’ – ATATG(CT)ACA(CT)ATTGCCCGTC – 3’)*	(Simon *et al.*, 1994,2006)
	N1 – J12261m	(5’ – TACTTCGTAAGAAATTGTTTGAGC – 3’)	(Simon *et al.*, 1994,2006)
	SR-J-13342	(5’ – CCTTTGTAC (AG)GT CAA AAT AC(CT) GC – 3’)	(Simon *et al.*, 1994,2006)
	SR-N14745	(5’ – GTGCCAGCAG(CT)(CT)GCGGTTA(AGCT)AC – 3’)*	(Simon *et a..*, 1994,2006)
	SR-N14588	(5' AAACTAGGATTAGATACCCTATTAT – 3’)*	(Simon *et al.*, 1994,2006)
COI-LeutRNA-COII	C1-J-2195	(5’- TTGATTTTTTGGTCATCCAGAAGT– 3’)	(Simon *et al.*, 1994,2006)
	TKN3796	(5’ – ACTATAAAATGGTTTAAGAG – 3’)	(Simon *et al.*, 1994,2006)
	LCO1490	(5’ – GGTCAACAAATCATAAAGATATTGG – 3’)	(Simon *et al.*, 1994,2006)
	C1-J-2183	(5’ – CAACATTTATTTTGATTTTTTGG – 3’)	(Simon *et al.*, 1994,2006)
	C1 -J -2792	(5’ – ATACCTCGACGTTATTCAGA – 3’)	(Simon *et al..*, 1994,2006)
	TL2-N-3014	(5’ – TCCATTGCACTAATCTGCCATATTA – 3’)	(Simon *et al.*, 1994,2006)
	C1-J-2441	(5’ – CCTACAGGAATTAAAATTTTTAGATGATTA – 3’)	(Simon *et al.*, 1994,2006)

Tab. 3.1. – Oligonucleotide primer sequences used in the polymerase chain and sequencing reactions for 16S rDNA and COI-LeutRNA-COII. Primers indicated with an asterisk (*) were used only in polymerase chain reaction.

Secondary structures of LSU 16S rDNA were aligned using the rDNA secondary structure of *Drosophila melanogaster* as a guide (Cannone *et al.,* 2002, http://www.rna.icmb.utexas.edu/).

For the stems we considered both the Watson-Crick and the GU-UG pairs. Stems regions were carefully checked to allow at least 75% of the sequences to match the secondary structure guide.

When the 75% of sequences did not follow the secondary structure guide a consensus secondary structure was created for that stem using the "secondary struct consensus " option available in the software *PHASE* Version 2.0 (Jow *et al*., 2005)

Phylogenetic analysis

For the 16S fragment and for the COI-COII coding fragments, substitution saturation was checked with the index of Xia *et al*. (2003), calculated in DAMBE version 4.2.13 (Xia & Xie 2001).

Phylogenetic relationships among sequences of insects, for the 16S data set, the COI-tRNALeu-COII data set and the combined 16S and COI-tRNALeu-COII data sets, were estimated using two methods: approximate maximum-likelihood (ML) and Bayesian inference (BI) analysis.

For ML analysis, the best-fit model of sequence evolution, was selected by MODEL TEST v3.06 (Posada & Crandall, 1998) using Akaike Information Criterion tests (Posada & Buckley, 2004) for both 16S dataset and COI-tRNALeu-COII dataset. The best model found was used for approximate ML, using PHYML_v2.4.4 software (Guindon & Gascuel, 2003), with neighbour-joining starting trees and 100 bootstrap replications. The software GARLIv0.951 (Zwickl, 2006) with 100 replicates of bootstrap was also used to obtain trees for the following analysis.

For the BI analysis of the host data set we used MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003).

For the 16S rRNA dataset we applied a doublet model to the stem regions and a standard 4by4 nucleotide model for the loop regions**;** for the COI-tRNALeu-COII dataset we used a codon site partioning scheme, with a 4by4 nucleotide model for each codon position. Two independent iterations were run for 5,000000 generations and sampled every 100 generations.

The 50% majority rule consensus tree and Bayesian posterior probability of support were obtained discarding the first 25% of sampled generations (burn in of 12500).

As shown in several studies, topologies recovered from combined data are generally better resolved than those based on individual partitions (Han, 2004, Baker and Dessalle 1997). Treefinder software (Jobb, 2004) was used in order to confirm the possibility of combining

molecular data sets for the host analysis. We used GTR+I+G as substitution model for the sequences and both the 16S and COI-tRNALeu-COII tree topologies obtained from GARLIv0.951 to achieve a p-value. If the p-value is not lower than 5% the two dataset can be considered suitable to be combined.

Symbiont

In order to enhance the knowledge of the presence of symbiosis in the subfamily Tephritinae the analysis has been extended, using the techniques described in Mazzon *et al.*, (2008), to other tribes which, symbiotic bacteria, Stammer (1929) did not detect, using the traditional microscopic techniques.

At least two samples per species were analyzed (Tab. 1). For each sample pupa of every sample was surface-sterilized by a 5 min immersion in 1% sodium hypochlorite, rinsed in sterile water at least two times, air-dried in sterile conditions and kept in sterile vials until adult emergence. Resulting flies were kept under microbiologically controlled conditions to avoid the presence of any residual culturable intestinal bacteria. Flies were then aseptically transferred, under a laminar flow hood, into larger vials, containing a layer of Plate Count Agar on the bottom as a sterility check, and sealed with a sterilized transparent gas-permeable cellulose membrane for dialysis (Sigma-Aldrich chemical co. S. Louis MO, USA). A drop of sterile glucose solution was placed on the internal side of the membrane to allow insect feeding. The drop of solution was re-wetted, whenever necessary, by spraying sterile water onto the vials (Capuzzo *et al.*, 2005).

After this the specimens were dissected and the midgut extracted. Every midgut was analyzed as described in Mazzon *et al.* (2008) using the following techniques: physiological staining using the LIVE/DEAD BacLight bacterial viability kit, plating on different standard microbiological media in order to test the presence of culturable bacteria, and biomolecular techniques.

The midgut of two species (*T. formosa* and *T. matricariae*) where symbiotic bacteria had been detected previously (Mazzon *et al.*, 2008) were cloned using the QIAGEN pDrive cloning vector, and 20 positive colonies per ligation, were sequenced. 90% of the clones contained exclusively the previously designed bacteria "*Candidatus* Stammerula tephritidis".

Symbiont sequence analysis

Symbiont sequences were aligned following the same methodology used for the insect host sequences. In this case the rRNA secondary structure of *Escherichia coli* was used as a guide (Cannone *et al.,* 2002, http://www.rna.icmb.utexas.edu/).

Also the phylogenetic analysis were carried out with the methods reported above for the insect host (ML and BI). In this case, however, the best-fit model of sequence evolution selected by MODEL TEST v3.06 (Posada & Crandall, 1998) using Akaike Information Criterion tests (Posada & Buckley, 2004) was (TrN+I+G) and so we used it for the ML analysis.

Coevolutionary analysis

Coevolution between insect host and symbiotic bacteria was tested. Several methods are available to evaluate the congruence between host and symbionts. Three of these methods have been chosen: TreeMap (tree-base method), ParaFit (distance – based method) and SH (data-based method).

TreeMap analysis

Reconciliation analysis was performed using TreeMap v1.0 (Page, 1994) and TreeMap v2.02β (Charleston & Page, 2002).

TreeMap v1.0 allows one to estimate the level of congruence between host and symbiont by calculating the number of codivergences, duplications, sorting events and host switches.

We used the heuristic search to find the best reconstruction. A randomization test was done for the host and the parasite trees simultaneously. The maximum number of observed cospeciations was compared with the maximum number of cospeciation events obtained from randomizating 1000 trees in order to determinate whether the number of observed cospeciations recovered from the reconciliation analysis was significant.

TreeMap v2.02β (available http://www.it.usyd.edu.au/~mcharles/software/software.html) computes all optimal solution by exhaustive search represented by Jungles (Charleston, 1998). As the number of possible reconstructions for the history of a host–parasite assemblage can be very large, finding all possible solutions can be computationally prohibitive in terms of both time and memory (Charleston, 1998; Page *et al*., 2004). So, we limited the set to no more than six host switches. Default settings were used for

evolutionary events (assigning a cost of zero for codivergence events and a cost of one for host switches, duplication and losses).

The statistical significance was evaluated by testing the null hypothesis that the observed number of codivergence events was not larger than the expected number of codivergence events between the observed host tree and 1,000 randomly generated trees.

ParaFit analysis

ParaFit software (Legendre *et al.*, 2002; available http://www.bio.umontreal.ca/casgrain/en/labo/parafit.html) was used to assess the null hypothesis of independent evolution of hosts and symbionts and to test the significance of each host-symbiont link. This leads to the identification of the species involved in cospeciation (Legendre, 2001). Parafit software is a useful tool and has the advantage over tree-based methods, because it can accommodate uncertainty in tree topologies, multiple hosts per parasite lineage and, as in our case, multiple symbionts (or parasite) per host lineage (Light & Hafner, 2007).

ParaFit software can compute this statistical test using phylogenetic distances, obtained using Mega 4.0 software, with ML estimates of pairwise genetic distances. Distances matrices were transformed into a rectangular matrix by principal coordinate analysis, using DistPCoA software (Legendre & Anderson, 1998) before being used in ParaFit. These matrices allow the software to calculate the probability of host-symbiont coevolution.

SH analysis

The congruence of host and symbiont was also assessed with a Shimodaira–Hasegawa likelihood-based test (S–H; Shimodaira & Hasegawa, 1999; Goldman *et al.*, 2000) run with 10000 RELL (re-estimation of likelihoods) bootstrap replicates using PAUP* 4.0b2.

Table 3.2 – Material examined with accession number for insect host and symbiotic bacteria when present. §Sequences from Mazzon *et al.* (2008); †Sequence from Spanos *et al.* (2000); all other sequences were obtained in the current study. *Cases in which symbiont bacterial DNA has been extracted from samples of different origin; Dash (-) indicates that non symbiotic bacteria have been detected. The first number in parentheses following insect origin is the number of individuals that were tested and the second indicates number os species positive for the presence of unculturable symbiotic bacteria.

Taxon	Host Plants	Origin	GenBank Accession	
			Insect: COI,II; 16S	Symbiont: 16S
OUTGROUPS				
FAMILY PLATYSTOMATIDAE				
Platystoma sp.		ITALY, Veneto, Cogollo del Cengio	xxxxxxx	-
FAMILY TEPHRITIDAE				
Subfamily Trypetinae				
Tribe Carpomyni				
Rhagoletis cerasi L.	*Prunus avium*	ITALY, Veneto, Torreglia	xxxxxxx	-
Rhagoletis completa Cressson	*Juglans regia*	ITALY, Veneto, Este	xxxxxxx	-
Subfamily Dacinae				
Tribe Ceratitidini				
Ceratitis capitata(Widemann)	-	GREECE	AJ242872†	
Tribe Dacini				
Bactrocera oleae (Rossi)	*Olea fragrans*	ITALY, Liguria, Imperia	xxxxxxx	
INGROUP (Tephritidae)				
Subfamily Tephritinae				
Tribe Myopitini				
Myopites inulaedyssentericae Blot	*Inula crithmoides*	CROATIA, Istria, Rovigno (2/0)	xxxxxxx	-
Urophora congrua Loew	*Cirsium erisithales*	ITALY, Veneto, Belluno (1/0)	xxxxxxx	-
Urophora cuspidate (Meigen)	*Centaurea scabiosa*	ITALY, Friuli V.G, Fanna (5/0)	xxxxxxx	-
Urophora quadrifasciata (Meigen)	*Centaurea jacea*	ITALY, Friuli V.G, Fanna (3/0)	xxxxxxx	-
Urophora terebrans (Loew)	*Cirsium eriophorum*	ITALY, Piemonte (2/0)	xxxxxxx	-
Urophora stylata (Fabricius)	*Cirsium arvense*	CROATIA, Istria, Rovigno (3/0)	xxxxxxx	-
Tribe Noeetini				
Ensina sonchi (Linnaeus)	*Sonchus sp.*	ITALY, Veneto, Legnaro (3/0)	xxxxxxx	-
Noeeta bisetosa Merz	*Hieracium piloselloides*	ITALY, Friuli V.G, Fanna	xxxxxxx	EF469632§
Noeeta pupillata (Fallén)	*Hieracium umbellatum*	ITALY, Veneto, Fanna	xxxxxxx	EF469633§
	Hieracium murorum	ITALY, Veneto, Cogollo del Cengio	xxxxxxx	EF469635§
	Hieracium pilosella	ITALY, Veneto, Torreglia (3/3)	xxxxxxx	xxxxxxx
Tribe Tephritini/ Campiglossa Group				
Campiglossa doronici (Loew)	*Doronicum austriacum*	ITALY, Veneto, Cogollo del Cengio	xxxxxxx	EF469636§
Campiglossa guttella Rondani	*Hieracium murorum*	ITALY, Veneto, Asiago	xxxxxxx	EF469637§
Dioxyna bidentis (Robineau-Desvoidy)	*Bidens tripartita*	ITALY, Friuli V.G, Fanna	xxxxxxx	EF469631§
Oxyna flavipennis (Loew)	*Achillea millefolium*	SLOVENIA, Kranjska, Kranjska Gora*	xxxxxxx	EF469630§
Tribe Tephritini/Sphenella Group				
Sphenella marginata (Fallén)	*Senecio alpinum*	ITALY, Veneto, Asiago	xxxxxxx	EF469629§
Tribe Tephritini/Tephritis Group				
Acanthiophilus helianthi (Rossi)	*Centaurea jacea*	ITALY, Friuli V.G, Fanna	xxxxxxx	EF469625§
Capitites ramulosa (Loew)	*Phagnalon saxatile*	ITALY, Liguria, Imperia	xxxxxxx	EF469628§
Tephritis arnicae (Linnaeus)	*Arnica montana*	ITALY, Veneto, Asiago	xxxxxxx	EF469616§
Tephritis bardanae (Schrank)	*Arctium lappa*	ITALY, Veneto, Foza	xxxxxxx	EF469617§
Tephritis cometa (Loew)	*Cirsium arvense*	CROATIA, Istria, Rovigno	xxxxxxx	EF469615§
Tephritis conura (Loew)	*Cirsium spinosissimum*	ITALY, Veneto, Asiago*	xxxxxxx	EF469618§
Tephritis divisa Rondani	*Picris echioides*	ITALY, Liguria, Imperia	xxxxxxx	EF469619§
Tephritis fallax (Loew)	*Leontodon hispidus*	ITALY, Marche, Pesaro-Urbino	xxxxxxx	EF469622§
Tephritis formosa (Loew)	*Sonchus sp.*	ITALY, Marche, Pesaro-Urbino	xxxxxxx	EF469620§
Tephritis hendeliana Hering	*Carduus nutans*	ITALY, Veneto, Roana	xxxxxxx	EF469612§
Tephritis hyoscyami (Linnaeus)	*Carduus personata*	ITALY, Friuli V.G, Tarvisio	xxxxxxx	EF469613§
Tephritis leontodontis (De Geer)	*Leontodon autumnalis*	SLOVENIA, Kranjska, Kranjska Gora	xxxxxxx	EF469621§
Tephritis matricariae (Loew)	*Crepis vesicaria*	ITALY, Veneto, Legnaro	xxxxxxx	EF469623§
Trupanea amoena (Frauenfeld)	*Reichardia picroides*	ITALY, Liguria, Imperia*	xxxxxxx	EF469626§
Trupanea stellata (Fuessly)	*Erigeron annuus*	ITALY, Veneto, Verona	xxxxxxx	EF469627§
Tribe Terelliini				
Chaetorellia jacea (R.-D.)	*Centaurea jacea*	ITALY, Friuli V.G, Fanna (2/0)	xxxxxxx	-
Chaetostomella cylindrica (R.-D.)	*Centaurea triumfetti*	ITALY, Friuli V.G, Fanna (4/0)	xxxxxxx	-
Orellia falcaae (Scopoli)	*Tragopogon orientalis*	ITALY, Friuli V.G, Fanna (1/0)	xxxxxxx	-
Terellia colon (Meigen)	*Centaurea scabiosa*	ITALY, Friuli V.G, Fanna (6/0)	xxxxxxx	-
Terellia ruficauda (Fabricius)	*Cirsium arvense*	ITALY, Friuli V.G, Fanna (2/0)	xxxxxxx	-
Terellia serratulae (Linnaeus)	*Cirsium pannonicum*	ITALY, Friuli V.G, Fanna (2/0)	xxxxxxx	-
Terellia tussilaginis (Fabricius)	*Arctium tomentosum*	ITALY, Friuli V.G, Tarvisio (2/0)	xxxxxxx	-
Terellia virens (Loew)	*Centaurea maculosa*	ITALY, Veneto, Rovolon (1/0)	xxxxxxx	-
Tribe Xyphosiini				
Xyphosia laticauda (Meigen)	*Centaurea triumfetti*	ITALY, Friuli V.G, Fanna (2/0)	xxxxxxx	-
Xyphosia miliaria(Schrank)	*Carduus nutans*	CROATIA, Istria, Rovigno (6/0)	xxxxxxx	-

Results

Phylogenetic analysis of the insects

From DNA amplification and sequencing with the primers reported above, we obtained fragments ranging from 989 to 1036 bp for the 16S rDNA gene with an average of 1027 bp, and from 1407 to 1553 for COI-tRNALeu-COII genes with an average of 1414 bp.

Treefinder software (Jobb, 2004) accepted the possibility of combining the two data sets (P-value >5%). For the combined data set a fragment ranging from 2399 to 2582 bp with an average of 2441 bp was obtained. For the 16S rDNA gene the average proportion of T:C:A:G was 45:6:38:11, for COI-tRNALeu-COII genes was 39:14:34:12 and for the combined data set was 41:11:36:12.

The alignment of the 16S rDNA realized on the basis of the secondary structure of the of *Drosophila melanogaster,* allowed a reliable alignment, of a total of 1094 bp sites, avoiding the loss of information due to the removal of ambiguous portions of the alignment. For the COI-tRNALeu-COII data set the number of sites aligned was 1568 bp, whilst for the combined data set we obtained a total of 2662 bp.

The index for substitution saturation in all cases (16S rDNA, COI and COII, all codon positions) showed 'little saturation', with a slightly higher value for the third codon positions in the COI and COII fragments. Thus the sequences can be considered suitable for further phylogenetic analyses.

As reported above, the best-fit evolutionary model for the ML-based phylogenetic analysis (as determined by Modeltest) was a general time-reversible model (GTR+I+G), for the 16S data set, the COI-tRNALeu-COII and the combined data set. For the combined data set we used the following parameters: proportion of invariant sites = 0.4636, gamma = 0.6506.

Phylogenetic trees were studied considering the statistical support. Posterior probabilities (Pp) and bootstrap probabilities (Bp) were obtained from the molecular phylogenetic analyses using BI and ML respectively. Values of 95% for Pp and 70% for Bp were considered statistically significant for clades to be supported.

Molecular relationship among the tribes of the subfamily Tephritinae

For the 16S rDNA , the COI-tRNALeu-COII and the combined data set, the phylogenetic trees obtained using both ML and BI methods showed similar topologies with some disagreements between 16S and cytochrome phylogenies.

In particular, the 16S tree agrees completely with the molecular phylogeny obtained by Han *et al.*. (2006) from the same gene, although in our case the relationships among the tribes are not resolved (Fig. 3.1).

The phylogeny of the COI-tRNALeu-COII mainly agrees with the phylogenesis proposed by Korneyev (1999), based on morphological characters. This tree resulted fully resolved and the relationships among the tribes were statistically supported by both the clustering methods (BI and ML) (Fig. 3.2).

The phylogeny of the COI-tRNALeu-COII is similar to the phylogeny obtained from the combined data set, although the relationships among the tribes lose the statistical support value due to the effect of the 16S rDNA which provides a phylogenetic tree where the relationships among the tribes are not resolved.

In the combined data set 26 nodes are highly supported in both clustering methods (BI>95; ML>70) whereas 3 nodes are only supported by the BI and 4 nodes are only supported by the ML, but all of these nodes are at least supported topologically (Fig. 3.3).

The tree of the combined data set (Fig. 3.3), showed that all 42 analyzed species, belonging to the subfamily Tephritinae, form a strongly monophyletic group (highly supported in both analyses - 100/100). This confirms the results of the larger molecular analysis carried out with the single 16S rDNA gene by Han *et al.* (2006) which concerned 53 species coming from several zoogeographic regions.

The strict affinity among the species of this subfamily was already proposed by several authors on the basis of morphological and biological data (Foote *et al.*, 1993; Korneyev, 1999; Norrbom *et al.*, 1999; Zwölfer, 1983).

Among the five paleartic tribes analyzed in this work, 4 of them were recognized as monophyletic and highly supported clades by each of the clustering methods used (Tephritini, Terellini, Myopitini, Xyphosiini) and only one (Noeetini) was topologically, but not statistically resolved (Fig. 3.3).

The first clade (Group 1) forms a strongly supported monophyletic group (100/100) and corresponds with the large tribe of Tephritini included by Korneyev (1999) in the "Higher Tephritinae" (Norrbom *et al*., 1999; Korneyev, 1999). In the phylogenetic analysis presented by Han *et al.* (2006) species belonging to the tribe Tephritini do not form a monophyletic group, probably due to the different geographical origin of the analyzed species.

This clade is subdivided into two highly supported monophyletic subgroups: 1A (100/100) and 1B (100/100). The first one (1A group), represented by the species of genera *Tephritis*, *Capitites*, *Trupanea* and *Acanthiophilus* corresponds with the "*Tephritis* Group" defined by Merz (1999), based on a morphological approach. Genus *Trupanea* appears strongly related to *Acanthiophilus* as proposed by Merz (1999) considering the morphological characters.

The second monophyletic subgroup (1B) comprises species belonging to Sphenella group (*Sphenella marginata*) and Campiglossa group (genera *Campiglossa*, *Oxyna* and *Dioxyna*) as proposed by Korneyev (1999) and Norrbom *et al*. (1999). In our data set the Sphenella group is represented only by one species but in the Paleartic region this is a small group including only four genera. In the molecular analysis presented by Han *et al*. (2006) a similar group was proposed but it was not well supported.

The remaining four clades are all included in the "Lower Tephritinae" (Korneyev, 1999)

The second clade corresponds with the tribe Myopitini and represents a highly supported and monophyletic clade (100/100) formed by species showing a close affinity both to the morphological and biological approaches. The phylogenetic relationship of this tribe with the rest of tribes is unclear due to the absence of a supported statistical value. In the molecular studies of the single 16S rDNA carried out by Han, this tribe resulted in a grouping with Noeetini (except *Ensina sonchi*) and Xyphosiini in a monophyletic and supported clade. This result matches, in part, with our 16S rDNA analysis even if it is not supported (Fig. 3.1). Conversely, the phylogenetic tree inferred from the COI-tRNALeu-COII analysis suggested the tribe Myopitini as a sister group of the tribe Tephritini with a statistical support (Fig. 3.2).

Similary, the phylogeny analyses carried out on the basis of morphological characters, report that the exact relationship of the Myopitini with the rest of Tephritinae is unknown

(Freidberg and Norrbom, 1999), even though Korneyev (1999) briefly suggested morphological affinity between Myopitini and some genera of Noeetini.

The third clade 3 represents a monophyletic group and corresponds with the tribe Xyphosiini. It is a very restricted tribe made up of only 6 genera, three of which are Paleartic (Norrbom *et al.*, 1998). Our results showed that Xyphosiini are related to the clade Noeetini even if this cluster is statistically supported only by the BI analysis on COI-tRNALeu-COII (*/100) (Fig, 3,2). This result agrees with Han *et al.* (2006).

The clade 4 corresponds with the tribe Noeetini (Norrbom and Korneyev in Norrbom *et al.*, 1999). This is the only tribe that was not recovered as a monophyletic group (results not supported by a statistical value) if we consider *Ensina sonchi* as a member of this tribe. Among the inferred trees only the COI-tRNALeu-COII tree supports this clade with a very low statistical value only in the BI analysis (Fig. 3.1, 3.2, 3.3). Korneyev and Norrbom considered *E.sonchi* as a member of the Tribe Noeetini based only on a single synapormophic character (Norrbom *et al.*, 1999). In Han *et al.* (2006) biomolecular analysis, *E. sonchi* is not grouped with the rest of Noeetini. However, the authors suggest an additional molecular study as the single synapomorphic character found in *Ensina spp.* and Noeetini (sensu stricto) is considered a result of a converged evolution.

The clade 5 corresponds to the Tribe Terellini. In the analysis of the single 16S rDNA produced by Han *et al.* (2006) the tribe Terellini appears as a sister group to the "Higher Tephritinae" although this relationship is statistically poorly supported, as recovered in our 16S rDNA tree (Fig. 3.1). On the other hand, our phylogenetic tree of the COI-tRNALeu-COII agrees with the phylogenetic analysis based on a morphological approach by Korneyev (1999) which placed tribe Terellini as a sister group to the remaining Tephritinae, due to the presence of plesiomorphic characters (Fig. 3.2 and 3.3).

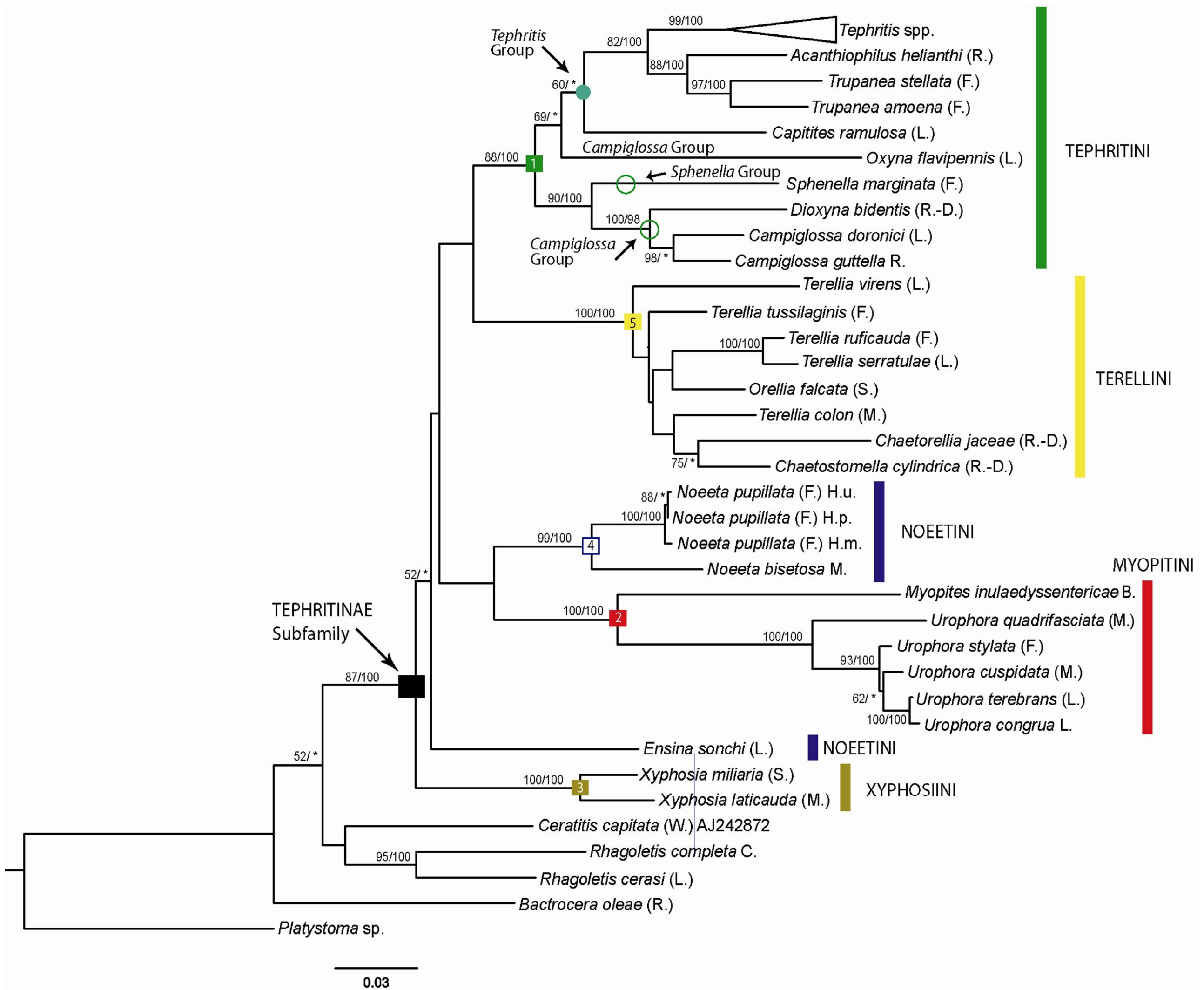


Fig. 3.1.- Phylogenetic reconstruction on the basis of the 16S rDNA. Bootstrap probabilities (ML) and posterior probabilities (BI) are reported on the nodes. Asterisks indicate bootstrap probabilities lower than 50% and posterior probabilities lower than 95%.

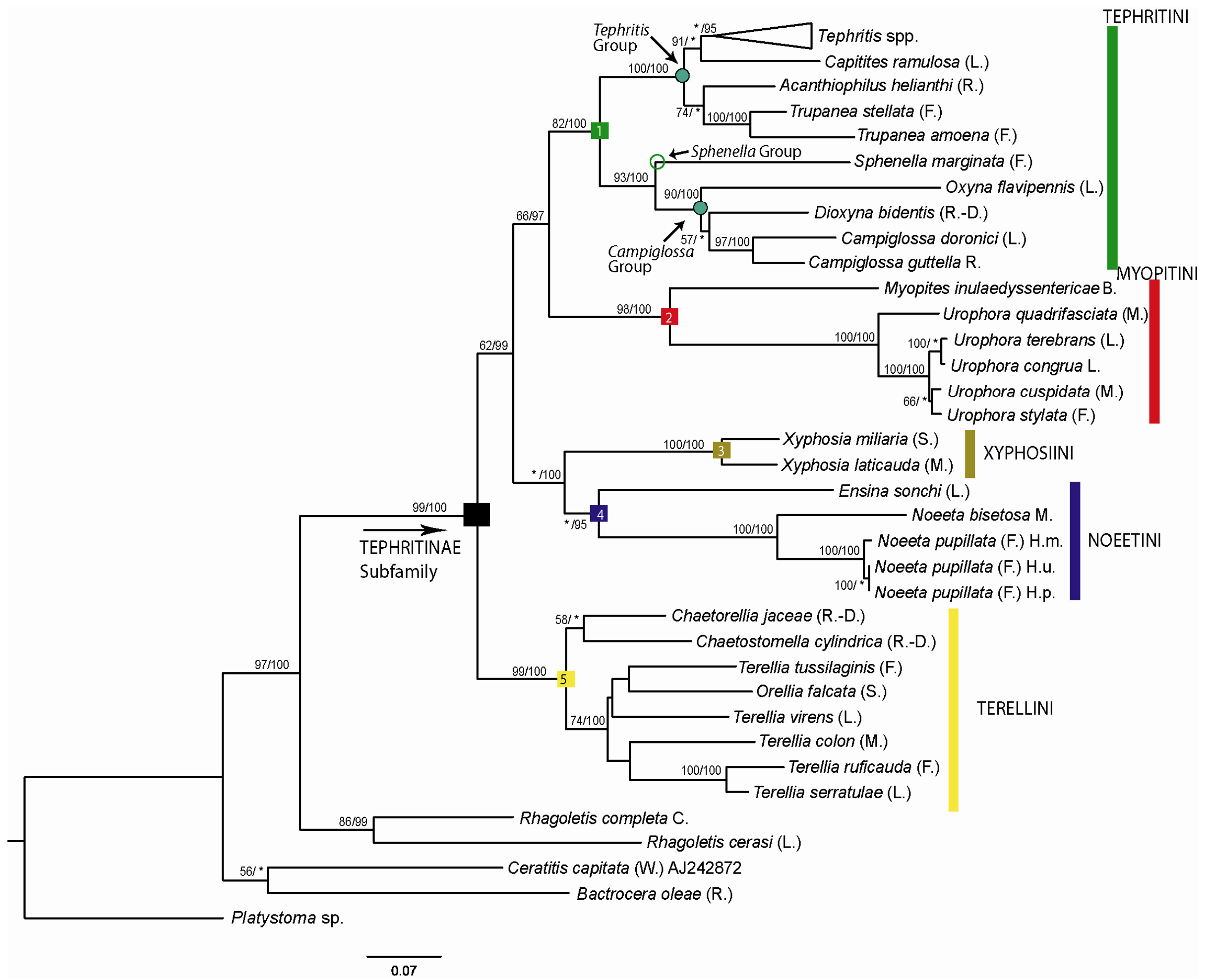


Fig. 3.2.- Phylogenetic reconstruction on the basis of the COI-tRNALeu-COII. Bootstrap probabilities (ML) and posterior probabilities (BI) are reported on the nodes. Asterisks indicate bootstrap probabilities lower than 50% and posterior probabilities lower than 95%.

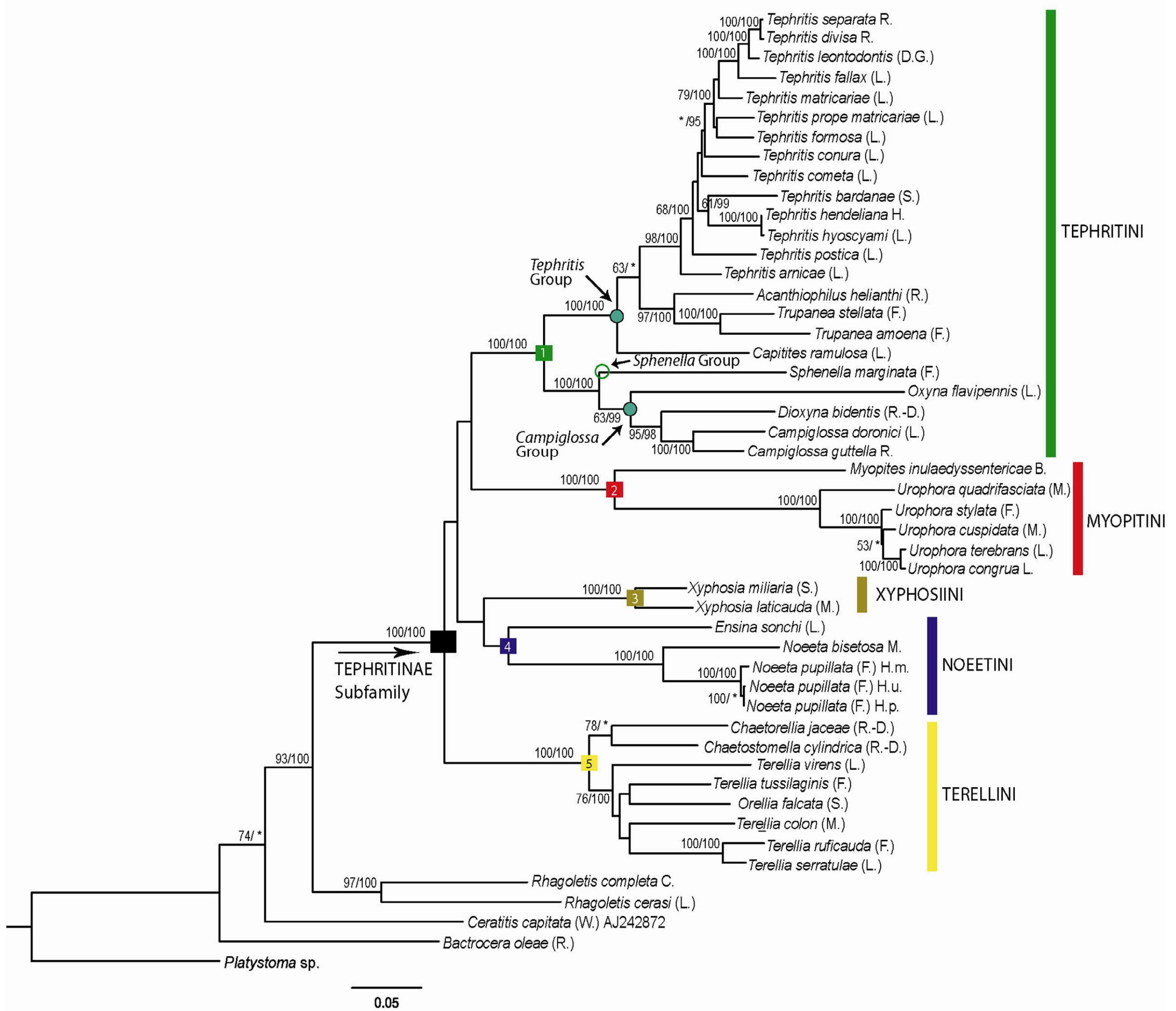


Fig. 3.3 .- Phylogenetic reconstruction of the subfamily Tephritinae on the basis of the combined data set (16S rDNA +COI-tRNALeu-COII). Bootstrap probabilities (ML) and posterior probabilities (BI) are reported on the nodes. Asterisks indicate bootstrap probabilities lower than 50% and posterior probabilities lower than 95%.

Presence of specific symbiotic bacteria in subfamily Tephritinae

The midgut of a total of 52 samples taken from sterilized pupae and reared under microbiologically controlled conditions, was analyzed (at least two samples per species). None of the species added to this analysis, showed the presence of bacteria in the midgut. Phase contrast microscopy and performance of LIVE/DAD BacLight bacterial viability test did not detect the presence of bacteria in the midgut epithelium of dissected adults. No bacterial culture was observed by plating the midgut in different media.
Moreover, the amplification of DNA extracted from the midgut, using bacterial primers to amplify a fragment of 16S rDNA gave a negative result. It emerges from this, when we consider the diverse tribes and subfamilies studied, that symbiotic bacteria are present in the 100% of the species of the Tribes Tephritini and partially in tribe Noeetini. On the other hand, in the analysis of tribes Terellini, Myopitini and Xyphosiini no positive results were apparent (Fig. 3.4). The list of species studied in which symbiotic bacteria have been found are reported in Table 3.2.

Coevolutionary analysis

To assess the coevolution between hosts and symbionts we reduced both the host and symbiont number of sequences since the software used required too much memory for the data elaboration, and needed fully resolved trees. A total of 19 host insect species, among the most representative of each group, and their corresponding symbiotic bacteria sequences were selected for the cophylogenetic analysis.

TreeMap analysis

TreeMap software requires fully resolved trees, thus we used ML trees instead of BI trees as they are more resolved for both hosts and symbionts.
Comparing the host and the endosymbiont tanglegram, without any host-switching event, (Fig. 3.6), TreeMap v1.0 suggested eight cospeciation events, eight duplications and 37 sorting events. Adding host-switching events (with the Heuristic search), the number of possible observed cospeciation events increased to nine, with six duplications, one host switch and 25 sorting events. The number of cospeciating nodes resulted 18 which when

divided by the total number of nodes and multiplied by 100 gave a percentage nodes with cospeciation equal to 49%.

The inferred number of cospeciation events was significantly different from that obtained by the randomization of both trees which yielded 5.3±1.2 SD (*P*=0.003) cospeciation events. It was lower than the number of cospeciations obtained in our analysis (9> 5.3). The probability of obtaining the same number of cospeciations compared to the number of randomized cospeciations was significant *P*= 0.003 (Fig. 3.5). Therefore, we can reject the null hypothesis which says that the number of cospeciations obtained is no more than that which would be observed between the host and a random associated tree.

Using jungle algorism implemented in TreeMap v2.02β (Charleston & Page, 2002), 36 optimal reconstructions were found. The optimal solutions postulated a maximum of 20 codivergence events (=10 cospeciations), from 10 to 23 losses, 1-6 switches and 12-14 duplication events (Fig. 3.7). The randomization of 1000 symbiont trees, suggests that the fit between the host and the symbiont trees is statistically significant (*P*< 0.001).

ParaFit analysis

A global test of cophylogeny, was obtained using ParaFit and resulted in the rejection of the null hypothesis that the evolution of the host and symbiont was independent (*P*= 0.003 after 999 permutations). This result supports the alternative hypothesis (H1) and shows a global association between hosts and parasite.

When we assessed the significance of each host–symbiont association (using the ParaFitLink1 statistic), we found that 9 of the 19 host-symbiont links were not significant (*P*>0.05), which means that these species are most likely to have been subjected to host switching or sorting events (parasite extinction, or primary absence on daughter host lineage) (Legendre, 2002) (Tab. 3.3).

SH

The results of the SH-test indicated that there is a significant disagreement in the most likely topology supported by insects and symbionts dataset, suggesting that the observed incongruence cannot be explained by sampling error.

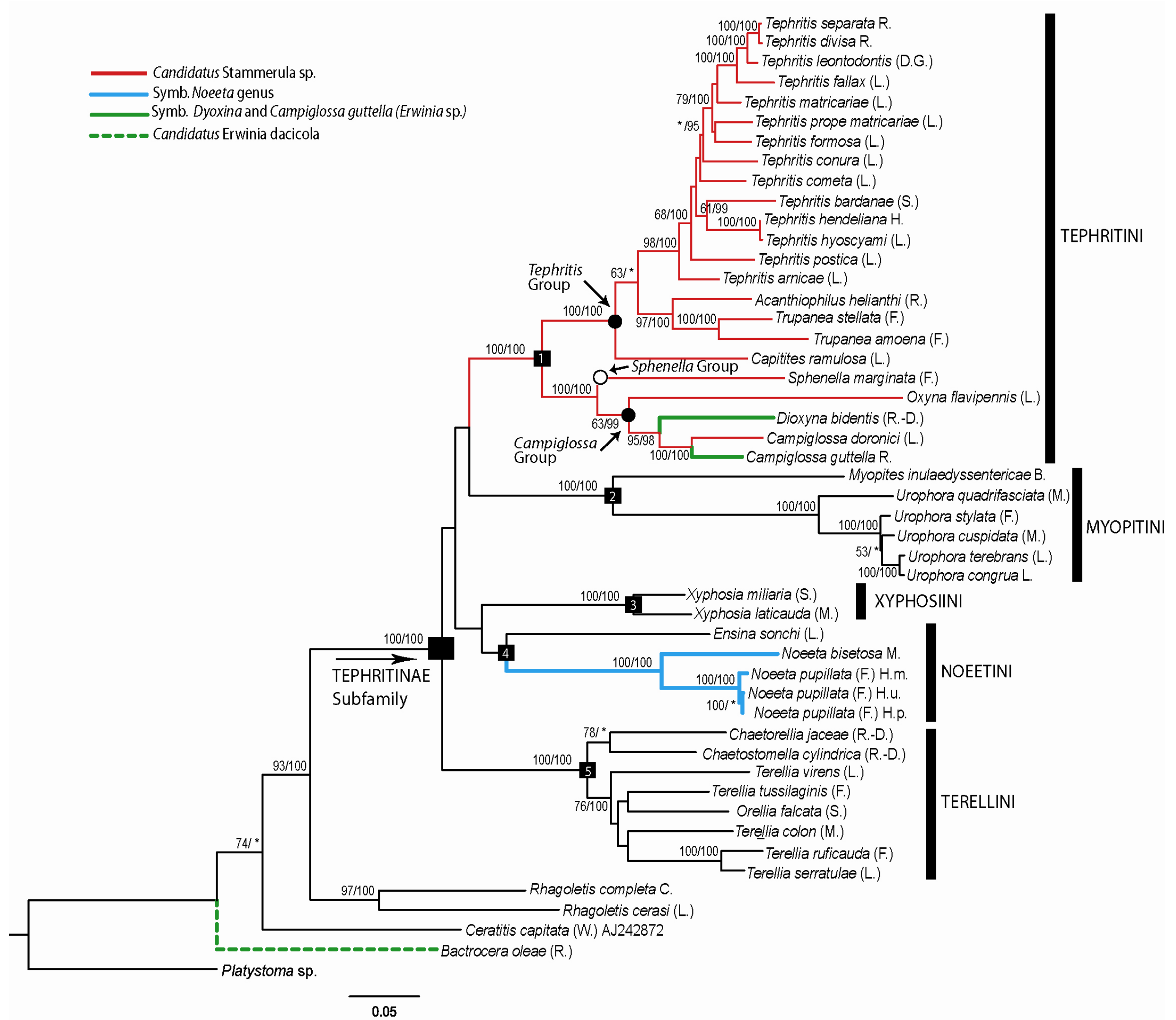


Fig. 3.4.- Phylogenetic reconstruction of the subfamily Tephritinae on the basis of the combined data set (16S rDNA +COI-tRNALeu-COII). Bootstrap probabilities (ML) and posterior probabilities (BI) are reported on the nodes. Asterisks indicate bootstrap probabilities lower than 50% and posterior probabilities lower than 95%. Colored branches corresponds to taxa where symbiotic bacteria has been detected.

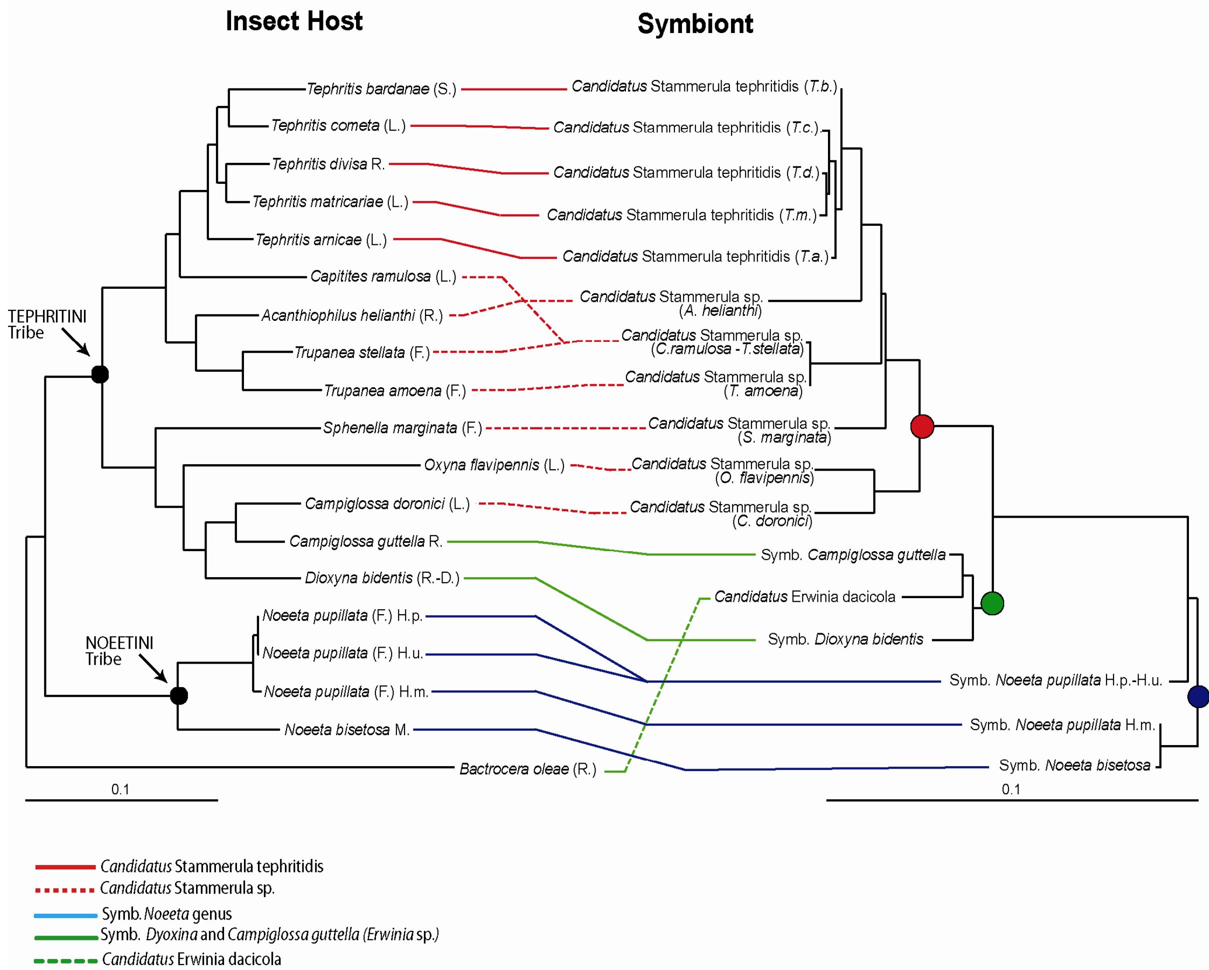


Fig. 3.6. – Pattern of hosts and symbiotic bacteria associations, with maximum-likelihood trees estimated for the hosts and parasites. Lines depict the observed host-parasite associations.

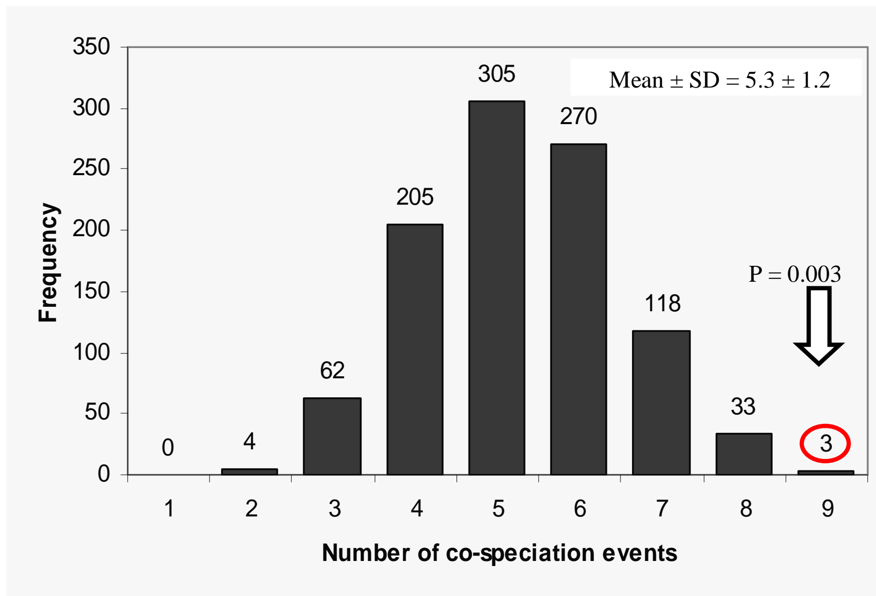
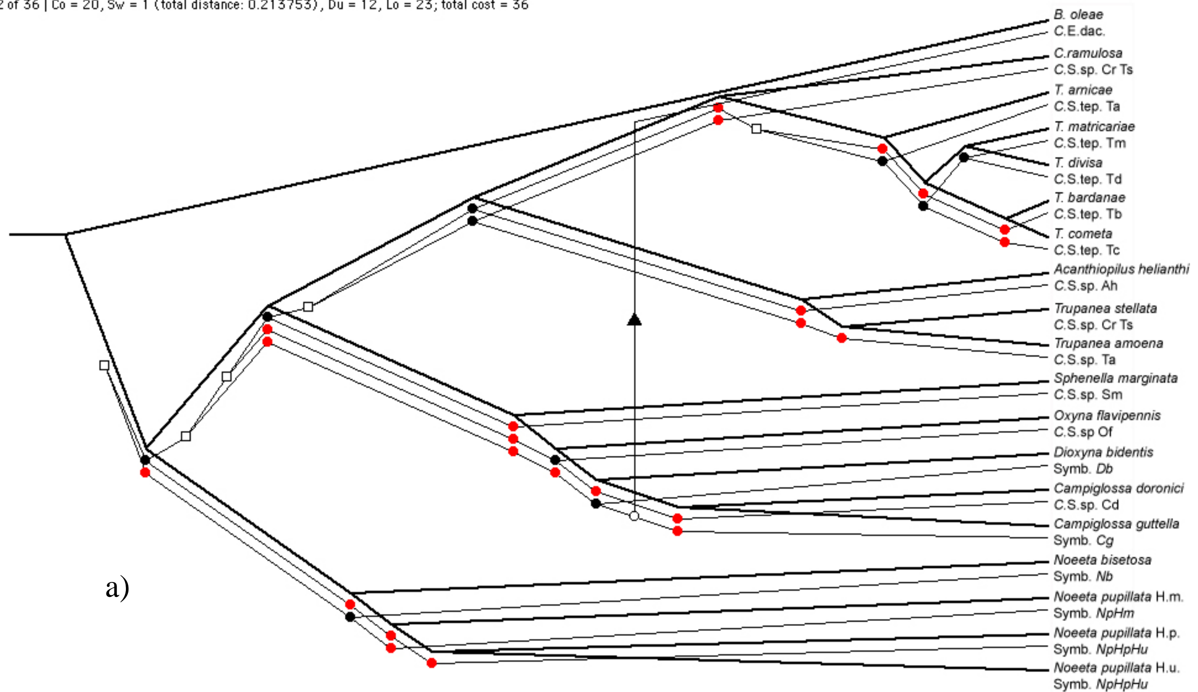
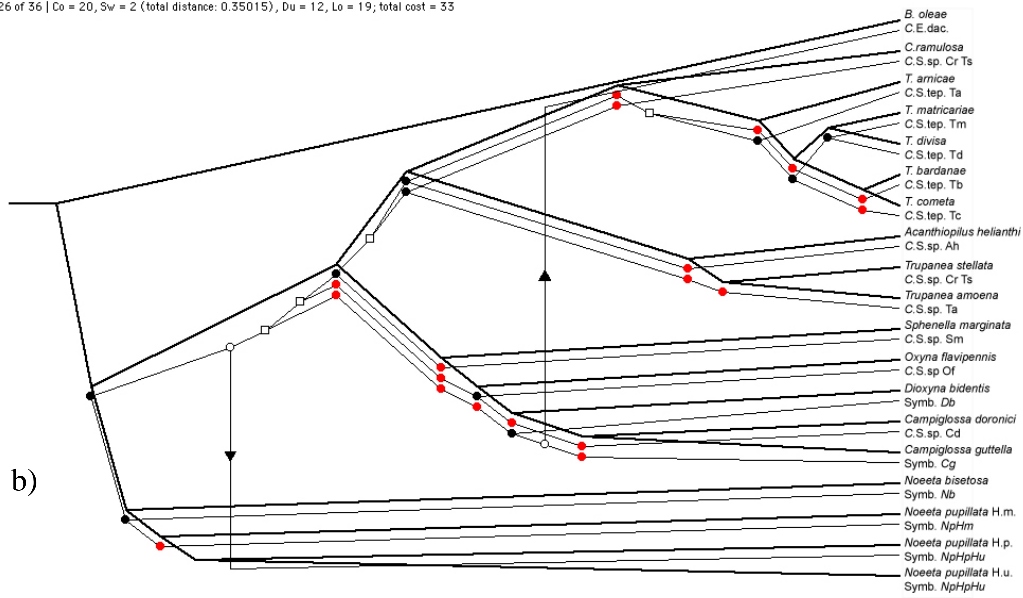
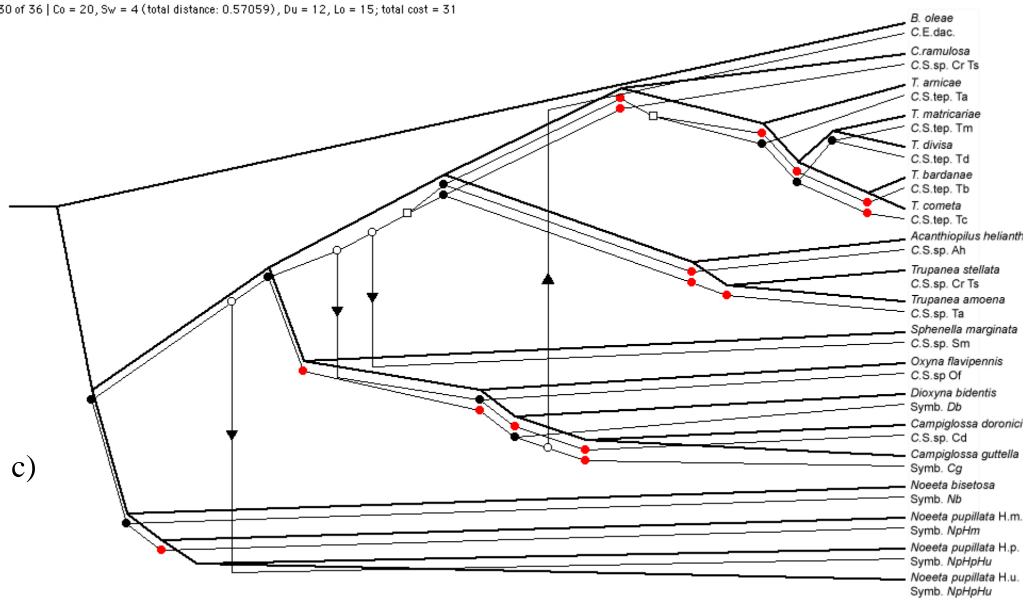


Fig. 3.5. – Randomization test for the phylogenetic congruence between the Tephritinae flies and their symbionts (generated by TreeMap). Frequency distribution of the number of cospeciation events in random associations (sample size 1000).





b)



c)

Fig. 3.7. a); b); c) - Three cophylogenetic reconstructions of the subfamily Tephritinae between hosts and symbionts chosen among those with a higher number of cospeciation and a lower number of costs (duplications, losses and host-switching). Arrows indicate a host-switching event; black circles, cospeciation; grey circles, losses; and white squares, duplication events.

Symbiont	Host	***P***
Candidatus Stammerula tephritidis (T.d.)	*Tephritis divisa*	0.03*
Candidatus Stammerula tephritidis (T.m.)	*Tephritis matricariae*	0.06
Candidatus Stammerula tephritidis (T.c.)	*Tephritis cometa*	0.03*
Candidatus Stammerula tephritidis (T.a.)	*Tephritis arnicae*	0.06
Candidatus Stammerula tephritidis (T.b.)	*Tephritis bardanae*	0.02*
Symb. *Acanthiophilus helianthi*	*Acanthiophilus helianthi*	0.09
Symb. *Trupanea amoena*	*Trupanea amoena*	0.05*
Symb. *Oxyna flavipennis*	*Oxyna flavipennis*	0.03*
Symb. *Sphenella marginata*	*Sphenella marginata*	0.49
Symb. *Dioxyna bidentis*	*Dioxyna bidentis*	0.58
Symb. *Campiglossa guttella*	*Campiglossa guttella*	0.42
Symb. *Campiglossa doronici*	*Campiglossa doronici*	0.10
Symb. *Capitites ramulosa* and *Trupanea stellata*	*Capitites ramulosa*	0.07
Symb. *Capitites ramulosa* and *Trupanea stellata*	*Trupanea stellata*	0.06
Symb. *Noeeta bisetosa*	*Noeeta bisetosa*	0.002*
Symb. *Noeeta pupillata* H.p. and H.u.	*Noeeta pupillata* H.p.	0.005*
Symb. *Noeeta pupillata* H.p. and H.u.	*Noeeta pupillata* H.u.	0.004*
Symb. *Noeeta pupillata* H.m.	*Noeeta pupillata* H.m.	0.008*
Candidatus Erwinia dacicola	*Bactrocera oleae*	0.02*
Global Test		**0.003***

Tab. 3.3 - Results from ParaFit: the test was used to asses the null hypothesis of independent evolution of hosts and symbionts and to test the significance of each host-symbiont link (19 host-symbiont links). Probabilities are computed after 999 random permutations.*Significant association (P≤0.05).

Conclusion

Phylogenetic considerations

Molecular phylogenetic analysis of Paleartic species belonging to the subfamily Tephritinae confirms, in accordance with the molecular analysis of Han *et al*. (2006), the monophyly of this subfamily. Moreover, the strict affinity among species Tephitinae has been proposed by several authors on the basis of a morphological and biological approach (Foote *et al*., 1993; Korneyev, 1999; Norrbom *et al*., 1999; Zwölfer, 1983).

The molecular results obtained show five monophyletic clades corresponding to recognized tribes of subfamily Tephritinae, on the basis of morphological approaches (Norrbom *et al.*, 1999; Korneyev, 1999). Four of these tribes were also highly supported by the Pp and Bp statistical values (Fig. 3.3). The relationships among of the tribes, however, were unclear, as resulted in Han *et al*. (2006). In the phylogenetic analysis of the COI-tRNALeu-COII, the tribe Terellini appears as a sister group to the remaining Tephritinae, in agreement with the traditional classification proposed by Korneyev, and based on a morphological

approach (1999). However, it contradicts the previous molecular analysis carried out by Han *et al.* (2006) (Fig. 3.2).

The phylogeny resulting from the COI-tRNALeu-COII gene is more satisfactory than that obtained from the 16S rDNA data set, in both the clustering methods used (Fig. 3.1 and fig. 3.2), and is statistically well supported. This could be an interesting indication for future phylogenetic studies based on mitochondrial DNA, of this subfamily. These results added to that of Han *et al.* (2006), could provided an important contribution to the increase in knowledge of the phylogenetic relationships of this family.

Presence of symbiotic bacteria and concordant evolution in the subfamily Tephritinae

The analysis carried out, in the present and previous work, (Mazzon *et al*., 2008) of 40 species belonging to five of the nine Paleartic tribes have revealed the presence of symbiotic bacteria in species of the Tribe Tephritini and in species of genus *Noeeta.* On the other hand, the species of Tribes Myopitini, Xyphosiini and Terellini analyzed gave no positive results. Thus, as discovered by Stammer (1929), and confirmed by our data, many tephritinae flies seem not to harbour symbiotic bacteria. The presence of symbiotic bacteria seems clearly connected to the large tribe Tephritini; a strongly homogeneous and monophyletic group, not only from the morphologic but also from the biological point of view.

Similar to the rest of Tephritinae, tribe Tephritini feed on flower heads of the family Asteraceae and hibernate as adults, as opposed to other tribes that hibernates as larvae or pupae (Merz, 1999). Hypothetically, symbiotic bacteria could have the function of facilitating the overwintering of adults of these species. Moreover, based on the morphological approach the tribe Tephritini is included in the "Higher Tephritinae" (Korneyev, 1999) with the tribes Dithrycini, Eutretini, Schistopterini and Tephrellini. The "Higher Tephritinae" is a monophyletic complex of the advanced tribes and is consider a derived clade of the "Lower Tephritinae".

Therefore, an additional study is necessary, extending the analysis to other species included in the "Higher Tephritinae" present in other geographical regions.

Of the remainder of the tribes, included in "Lower Tephritinae", only genus *Noeeta* harbours a symbiotic bacteria, phylogenetically close to the free living bacteria species, that

denotes a different history (Fig. 3.4) (Mazzon *et al*., 2008). The Lower Tephritinae is an aggregation of tribes which retains many primitive characters and seems paraphyletic in contrast to the Higher Tephritinae. Genus *Noeeta* is included in the Tribe Noeetini whose limits have not been firmly established by morphological and molecular studies (Korneyev., 1999; Norrbom *et al*., 1999; Han *et al*., 2006). The species *Ensina sonchi* is included in the Tribe Noeetini on the basis of a single synapomorphic character (Norrbom *et al*., 1999). In our molecular phylogenetic analyses, *E. sonchi* did not appear closely related to the *Noeeta* genus nor was any symbiotic bacteria found. Also, the phylogeny of the 16S rDNA presented by Han *et al*. (2006) showed little affinity between *E. sonchi* and *Noeeta* genus, placing this species, together with *Schistopterum moebiusi* (Tribe Schistopterini).

In this case, also additional research is advisable. These results show that the study of the symbiotic bacteria in these groups can be a useful tool for the understanding of the most debated aspects of the phylogenetic relationships among the tribes in the subfamily Tephritinae.

As is also described in this study, most associations represent a combination of cospeciation and host-switching (Page & Hafner, 1996; Roy *et al*., 2001; Weiblen & Bush, 2002; Ricklefs *et al*., 2004). When studying the possibility of a coevolution between hosts and symbiotic bacteria, TreeMap 1.0 and non-timed 2.0β software suggested a significant fit between host and symbiont trees. The number of cospeciations obtained, was indeed significantly different from that obtained by randomizing symbiont taxa across Tephritinae species (Fig. 3.5). However, quite a low percentage of the nodes (49%) of both host and symbionts were congruent. ParaFit software also suggests the presence of a global coevolution between host and symbionts, although it indicates that some symbiotic bacteria species have not cospeciated with their hosts. This is evidence, that besides cospeciations, other independent events such as duplications, sorting events and host-switching have intervened.

The possibility of host switching, in particular, seems highly probably. As suggested by Stammer (1929) and subsequently described by Girolami (1973) and Mazzon *et al.* (2008), the tephritinae flies harbour symbiotic bacteria in an extracellular region between the peritrophic membrane and the midgut epithelium. Here, the chance of contacts with the

outer environment is surely more likely and they could be vulnerable to invasion and replacement by foreign microbes. In the larval stadium these chances are even higher. Bacteria are located in the intestinal caeca (Petri 1909; Stammer, 1929) without the protection of the peritrophic membrane and are therefore, in contact with free living bacteria present in the intestinal lumen. The presence of different species on the same host plants could render them vulnerable to horizontal transfer and substitutions. Another critical phase may be the still unclear mechanism of transmission of the symbiotic bacteria from the mother to the progeny, described by Petri (1909) and Capuzzo (2005) in the olive fly, *B. oleae.* All the above mentioned cases allow for possible invasion and replacement by foreign microbes and disturb the concept of strict vertical transmission.

The reconstruction chosen among those with a higher number of cospeciation and a lower number of costs, suggested the presence of two main events. The first event, probably the earliest, suggests that the common ancestor of tribe Tephritini acquired its symbiotic bacteria, coevolving over time, suffering also some losses and acquisitions (Fig. 3.6 and Fig. 3.7). These monophyletic and unculturable symbiotic bacteria have been designated "*Candidatus* Stammerula spp.", and for the genus *Tephritis spp*. "*Candidatus* Stammerula tephritidis" (Mazzon *et al.*, 2008). The biological advantages of these symbiotic bacteria are evident given that all the members of this tribe host symbiotic bacteria.

Two species belonging to the *Campiglossa* Group (*Campiglossa guttella* and *Dioxyna bidentis*) have symbiotic bacteria, different from "*Canditatus* Stammerula spp", but related to the free living bacteria *Erwinia sp*. Based on reconstructions the presence of this kind of symbiotic bacteria could result from losses or more probably recent acquisitions. Interestingly, the symbiotic bacteria of *B. oleae* "*Candidatus* Erwinia dacicola" is also closely related to the free-living bacteria *Erwinia* sp. which suggests a recent acquisition (Fig. 3.7).

The second event is represented by an independent occurrence which concerns the acquisition of a different symbiotic bacteria in flies of the genus *Noeeta,* indicating a different history. These symbiotic bacteria are phylogenetically related to the free living bacteria *Ewingella americana*. Genus *Noeeta* is a monophyletic group which includes *Noeeta bisetosa,* a monophagous species which feeds on *Hieracium pilloselloides,* and *Noeeta pupillata* an oligophagous species which feeds on several related genera of

Hieracium. Whereas *Noeeta bisetosa* contains a single symbiotic bacteria, *Noeeta pupilata* contains different symbiotic bacteria depending on the host plants on which it feeds. This fact suggests the presence of possible sibling species. Additional studies, including a more rigorous sampling of these taxa would be useful.

The present work, even given the limitations of the current experimental and statistical procedures, demonstrates an extensive and striking history of tandem diversification, made up of cospeciations and also losses, acquisitions and host-switching events.

As reported above, the ecology and biology of the tephritinae flies, is potentially susceptible to accidental horizontal transfers of the symbiont. Therefore, the presence of congruence between host and symbiont, even if not perfect, in this case of extracellular symbiotic bacteria in tephritid flies, is very interesting. Probably, strict vertical transmission is the primary basis of the tephritinae-symbiont congruence but involvement of other factors such as insect host-symbiotic bacteria physiological compatibility, should also taken into account.

Chapter 4

Morphological symbiotic arrangements and phylogenetic relationships of fruit flies (Diptera, Tephritidae)

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I contributed to experimental parts, data analysis and paper writing.

Introduction

Family Tephritidae, commonly known as “fruit flies” is a large and complex group. More than 4,200 species and subspecies of fruit flies are recognized worldwide, grouped in 471 genera (Thompson, 1999).

Within the order Diptera, the family Tephritidae belongs to the suborder Brachycera, infraorder Muscomorpha (= Cyclorrhapha), section Schizophora, and superfamily Tephritoidea (J.F. McAlpine, 1989).

Tephritid classification, have suffered several changes since the beginning. Recently, Korneyev (1999) has divided the subfamily Tephritidae into Higher Tephritinae: “Subfamily Tephritinae + Subfamily Trypetinae + Subfamily Dacinae and Lower Tephritinae: “Subfamily Tachiniscinae + Subfamily Blepharoneurinae + Subfamily Phytalmiinae”. The present work is focused on the Higher Tephritidae mainly on species of the Subfamily Tephritinae and Subfamily Trypetina present in the Paleartic region.

The varied behavior, evolution and population genetics, insect-plants interactions and biosystematics makes fruit flies a fascinating family (H. Zwölfer, 1983).

Larvae feed on different parts of the plants. Some of them are carpophagous, others feed on vegetative structures such as leaves, shoots or roots and the inflorescences of the family Asteraceae. In particular, species who feed on fruits such as *Ceratitis capitata* (Widemann), *Bactrocera oleae* (Rossi), *Rhagoletis completa* Cresson and *Rhagoletis cerasi* L. are considered notorious pests in Europe.

The biological cycle of tephritid flies varies, depending on the subfamily, genus and species. Tephritid flies can be univoltine, bivoltine or multivoltine. Diapause is a rule in Tephritinae and Trypetinae. Most of the members of the subfamily Dacinae do not hibernate. Fruit flies are distributed throughout the temperate and tropical areas of the world, being absent only from the high Arctic and Antarctic regions (Thompson, 1999).

An interesting characteristic of this family is the presence of “associated bacteria” and symbiotic bacteria.

Several authors report the presence of “associated bacteria” in Subamily Dacinae and Trypetinae, most of them belong to the genus *Enterobacter*, *Klebsiella* and *Pantoea* (Lloyd *et al.*, 1986; Drew & Lloyd, 1987; Daser & Brandl, 1992; Marchini *et al.*, 2002; Lauzon,

2003). Rossiter *et al*. (1983), for instance, described the predominance of the bacteria *Klebsiella* spp. in the fruit flies *Rhagoletis pomonella.* These associations, although they are important during the life of the insect, can be considered facultative.

These bacteria do not seem vertically transmitted from the mother to the progeny (Girolami, *pers. com*). The presence of true hereditary symbiotic bacteria was firstly reported by Petri (1909) in *B. oleae*. Thanks to the biomolecular techniques, this symbiont has been described as '*Candidatus* Erwinia dacicola' (Capuzzo *et al*., 2005).

Other bacterial symbionts in tephritid flies have been described by H. J. Stammer (Stammer, 1929) and occur in several genera of the subfamily Tephritinae. Recently, these symbionts have been designated as "*Candidatus* Stammerula spp." (Mazzon *et al*., 2008) and are hosted in flies of the genus *Tephritis*, *Acanthiophilus*, *Sphenella*, *Trupanea*, *Oxyna* and *Capitites*. Recently, the presence of symbiotic bacteria, different from "*Candidatus* Stammerula spp." and close to the free living bacterium *Ewingella americana* have been observed for the first time in fruit flies of genus *Noeeta* (Mazzon *et al.*, 2008). Probably, Stammer did not detect them because of the small size of these bacteria present in the midgut, due to the difficulty in finding them with the old microscope techniques.

Up to the present no other hereditary symbiotic bacteria have been described in the family Tephritidae, but as reported above, several "associated bacteria" are present in species of this family.

In the insect head of fruit flies a specialized organ related to the symbiosis called oesophageal bulb is present. As reported in the general introduction, the oesophageal bulb, firstly described for *B. oleae* by Petri (1909), is morphologically different depending on the species. Girolami (1973) described four different types of oesophageal bulb in tephritid flies (Fig. 1.8). In *B. oleae* obligate endosymbionts multiply in a membranous secretions of the oesophageal bulb (*Dacus* type in Girolami, 1973) and are subsequently discharged into the intestine where they are eventually digested (Petri, 1909). In the rest of Dacinae as well as in subfamily Trypetinae, "membranous masses" containing culturable bacteria (non symbiotic) are also produced in their characteristic oesophageal bulb (*Ceratitis* type in Girolami, 1973) and discharged into the midgut. Species of Tribe Terellini (subfamily Tephritinae) posses a special type of oesophageal bulb (*Chaetorellia* type in Girolami, 1973), where membranous masses containing free living bacteria multiply and, as in

Trypetinae and Dacinae (except *B. oleae*), are subsequently discharged into the midgut (Girolami, 1973). The rest of species of the subfamily Tephritinae present a small oesophageal bulb (*Ensina* type in Girolami 1973) devoid of bacteria. No membranous masses are present in the midgut of this species. However, in the first tract of the midgut of species belonging to the Tribes Tephritini and Noeetini symbiotic bacteria appear (Stammer, 1929; Mazzon *et al.*, 2008). Females of these species, as well as *B. oleae*, during oviposition smear the surface of their eggs with bacteria contained in special glands located in the ovipositor, in order to ensure the vertical transmission of the bacteria to the progeny. This mechanism was firstly described by Petri (1909) in *B. oleae*.

The classification and the phylogeny of this Family, have been poorly resolved in the past. Recent studies carried out using molecular techniques, based on mitochondrial DNA sequences, have improved Tephritid higher classification, suggesting several previously unknown relationships and offering new possibilities (Han & McPheron, 1997; 1999).

In the present work, two regions of the mitochondrial DNA have been analyzed in order to study the phylogeny of these tephritid flies. Also an attempt to combine phylogenetic results with biological characteristics and symbiosis arrangements has been carried out in order to give a general picture. For these purpose, due to the needing of health and fresh specimens for the symbiosis and biological studies only species present in Europe have been analyzed. It should be interesting for future analysis extending the analysis to “Lower Tephritinae” mainly present in Afrotropical and Neotropical regions.

Material and methods

Origin and identification of biological material.

In the present work, 55 species belonging to the Family Tephritidae common of the European region has been analyzed (Tab. 4.1). Family Platystomatidae has been considered as an outgroup. All the specimens have been collected mainly in the North of Italy, in the same locations as the preceding researches (Mazzon *et al.*, 2008). They were collected in

field or in laboratory from the flower heads of plants or from fruits following the methodology described in Mazzon *et al.* (2008). Samples were treated as described by Mazzon *et al.* (2008) and routinely preserved in 95% ethanol and stored at -80°C until processed.

Tephritid higher-level classification and specific names follow Norrbom *et al.* (1999), Korneyev (1999) and Merz (1994; 1999).

All the voucher specimens were dried, pinned and deposited in the Department of Environmental Agronomy and Crop Sciences – Entomology (DAAPV), University of Padova, Italy.

DNA extraction

DNA of the whole insect body (or a part of it) was extracted as described in the salting-out protocol (Patwary, 1994). In order to asses the quality of the DNA, extract products were separated in a 1% agarose gel and viewed under UV after staining with ethidium bromide or SYBR Safe (Invitrogen).

Amplification and sequencing

Two regions of the mitochondrial DNA, a fragment of the 16S rDNA and a fragment including the 3' region of cytochrome oxidase sub-unit I, tRNA Leu- and the 5' region of cytochrome oxydase sub-unit II, were amplified.

PCR amplification was carried out in a 20µl volume containing 2µl from the nucleic acid extract, PCR Buffer 10X, 25 mM MgCl, 2mM dNTPs, 10 µM of each primer and Taq (5U/µl).

A combination of universal and specific primers were used for PCR amplification and sequencing (Tab. 4.2) and some specific primers were also designed.

The cycling program was carried out in an Eppendorf Mastercycler Gradient consisted of 96°C 5min step followed by 35 cycles at 96 °C for 1min., 52-62°C for 1min., 72°C for 1-2min. and a final extension at 72°C for 5min.

The amplified products were separated on a 1% agarose gel and viewed under UV following staining with ethidium bromide or SYBR Safe (Invitrogen). PCR products were

purified with the ExoSAP-IT kit (Amersham Biosciences) and directly sequenced at the BMR Genomics service (Padova, Italy).

Target gene	Primer gene	Sequenze	Source
16S	LR-J -12883	(5'- CTCCGGTTTGAACTCAGATC – 3')	(Xiong and Kocher, 1991)
	TV-N-14202	(5' – AGCATTTCATTTACATTGAA – 3')	(Han and McPheron, 1996)
	DFI	(5' – CATTGGGCAGGTYARACT – 3')	(this study)
	DFI2	(5' – GATTTATAGGGTCTTCTCGTC – 3')	(this study)
	DR	(5' – GATGTACCGGAAGGTGTATCT – 3') *	(this study)
	DRI	(5' – GTTATTCGTTTATAAAGRTATC – 3') *	(this study)
	LR-N-13398	(5' - CGCCTGTTTAACAAAAACAT – 3')	(Simon *et al.*, 1994,2006)
	SR – N14220	(5' – ATATG(CT)ACA(CT)ATTGCCCGTC – 3')	(Simon *et al.*, 1994,2006)
	N1 – J12261m	(5' – TACTTCGTAAGAAATTGTTTGAGC – 3')	(Simon *et al.*, 1994,2006)
	SR-J-13342	(5' – CCTTTGTAC (AG)GT CAA AAT AC(CT) GC – 3')	(Simon *et al.*, 1994,2006)
	SR-N14745	(5' – GTGCCAGCAG(CT)(CT)GCGGTTA(AGCT)AC – 3')*	(Simon *et al.*, 1994,2006)
	SR-N14588	(5' AAACTAGGATTAGATACCCTATTAT – 3') *	(Simon *et al.*, 1994,2006)
COI-tRNALeu-COII	C1-J-2195	(5'- TTGATTTTTTGGTCATCCAGAAGT– 3')	(Simon *et al.*, 1994,2006)
	TK-N-3796	(5' – ACTATAAAATGGTTTAAGAG – 3')	(Simon *et al.*, 1994,2006)
	LCO1490	(5' – GGTCAACAAATCATAAAGATATTGG – 3')	(Simon *et al.*, 1994,2006)
	C1-J-2183	(5' – CAACATTTATTTTGATTTTTTGG – 3')	(Simon *et al.*, 1994,2006)
	C1 -J -2792	(5' – ATACCTCGACGTTATTCAGA – 3')	(Simon *et al.*, 1994,2006)
	TL2-N-3014	(5' – TCCATTGCACTAATCTGCCATATTA – 3')	(Simon *et al.*, 1994,2006)
	C1-J-2441	(5' – CCTACAGGAATTAAAATTTTTAGATGATTA – 3')	(Simon *et al.*, 1994,2006)

Tab. 4.2 – Oligonucleotide primers sequences used in polymerase chain and sequencing reactions for 16S rDNA and COI-tRNALeu-COII. Primer indicate with asterisk (*) were only used in polymerase chain reaction.

Sequences alignment

Sequences of 16S rDNA and COI-tRNALeu-COII were inspected and corrected using MEGA 4.0 (Tamura *et al*., 2007).

64 available sequences of the 16S rDNA of some Tephritidae coming from other zoogeographic regions were taken from the NCBI database (accession numbers table 1) and added to our 16S rDNA alignment of 61 sequences. So, a total amount of 125 sequences of the same fragment of the 16S rDNA were aligned. The 16S sequences presented some ambiguous portions which were difficult to align. In order to resolve this problem we decided to align our sequences considering the rDNA secondary structure, thus avoiding the deletion of these ambiguous portions.

Considering the secondary structure of rRNA in the alignment, we took into account the correlation between nucleotide sites in the stem regions; this information, when ignored, could lead to a bias in the supporting confidence of the clades. Moreover, a phylogenetic study based on the secondary structure seems also to have advantage with closely related species (Buckley *et al*., 2000).

Secondary structures of LSU 16S rRNA were aligned using the rRNA secondary structure of *Drosophila melanogaster* as a guide (Cannone *et al.* 2002, http://www.rna.icmb.utexas.edu/).

For the stems we considered both the Watson-Crick and the GU-UG pairs. Stems regions were carefully checked to allow at least 70% of the sequences to match the secondary structure guide. When the 70% of sequences did not follow the secondary structure guide a consensus secondary structure was created for that stem using the "secondary struct consensus" option implemented in the software *PHASE* Version 2.0. (Jow *et al*., 2005).

Phylogenetic analysis

For the 16S fragment and for the COI-COII coding fragments, substitution saturation was checked with the index of Xia *et al*. (2003), calculated in DAMBE version 4.2.13 (Xia & Xie, 2001).

Some sequences of 16S rDNA of flies of Family Tephritidae are available in GenBank. A phylogenetic analysis of the 16S data rDNA set completed with these sequences from Han *et al*., (2006), using approximate maximum-likelihood (ML) method and Bayesian

inference (BI) analysis is still in progress. Phylogenetic relationships among sequences of European insects, for the 16S data set, the COI-tRNALeu-COII data set and the combined data sets (16S and COI-tRNALeu-COII), were estimated using two methods: approximate maximum-likelihood (ML) and Bayesian inference (BI) analysis.

For ML analysis, the best-fit model of sequence evolution was selected by MODEL TEST v3.06 (Posada & Crandall, 1998) using Akaike Information Criterion tests (Posada & Buckley, 2004) for both 16S dataset and COI-tRNALeu-COII dataset. The best model found, was used for approximate ML, using PHYML_v2.4.4 software (Guindon & Gascuel, 2003), with neighbour-joining starting trees and 100 bootstrap replications.

For the BI analysis of the host data set we used MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003).

For the 16S rRNA dataset we applied a doublet model to the stem regions and a standard 4by4 nucleotide model for the loop regions**;** for the COI-tRNALeu-COII dataset we used a codon site partioning scheme, with a 4by4 nucleotide model for each codon position. Two independent iterations were run for 10,000000 generations and sampled every 100 generations. The 50% majority rule consensus tree and Bayesian posterior probability of support were obtained discarding the first 25% of trees (25000).

Taxon	Host Plants	Origin	GenBank Accession
			Insect: COI,II; 16S
OUTGROUPS			
FAMILY PLATYSTOMATIDAE			
Platystoma sp.		ITALY, Veneto, Cogollo del Cengio	
INGROUPS			
FAMILY TEPHRITIDAE			
Subfamily Trypetinae			
Tribe Trypetini/Subtribe Trypetina			
Euleia heraclei (Linnaeus)	*Apium graveolens*	ITALY, Legnaro	
Hemilea pulchella (Fabricius)	*Lactuca sp.*	ITALY, Legnaro	
Trypeta artemisiae (Fabricius)	*Artemisia vulgaris*	ITALY, Valcellina	
Tribe Trypetini/Subtribe Chetostomatina			
Anomoia permunda (Harris)	*Crataegus monogyna*	ITALY	
Myoleja lucida (Fallén)	*Lonicera sp.*	ITALY, Valcellina	
Tribe Ad ramini			
Euphranta connexa (Fabricius)	*Vincetoxicum hirundinaria*	ITALY, Fanna	
Tribe Carpomyini			
Goniglossum wiedemanni (Meigen)	*Bryonia dioica*	ITALY, Legnaro	
Carpomya schineri (Loew)	*Rosa sp.*	ITALY, Fanna	
Carpomya vesuviana A. Costa	*Ziziphus spp.*	ITALY, Imperia	
Rhagoletis cerasi L.	*Prunus avium*	ITALY, Veneto, Torreglia	
Rhagoletis completa Cressson	*Juglans regia*	ITALY, Veneto, Este	
Rhagoletis meigeni (Loew)	*Berberis vulgaris*	ITALY, Valcellina	
Subfamily Dacinae			
Tribe Ceratitidini			
Capparimyia savastani (Martelli)	*Capparis spinosa*	ITALY, Messina	
Ceratitis capitata (Widemann)	*Ficus europea*	ITALY, Imperia	AJ242872†
Tribe Dacini			
Bactrocera (Daculus) oleae (Rossi)	*Oleae europea*	ITALY, Imperia	
Subfamily Tephritinae		ITALY, Liguria, Imperia	
Tribe Myopitini			
Myopites inulaedyssentericae Blot	*Inula crithmoides*	CROATIA, Istria, Rovigno	
Urophora congrua Loew	*Cirsium erisithales*	ITALY, Veneto, Belluno	
Urophora cuspidata (Meigen)	*Centaurea scabiosa*	ITALY, Friuli V.G, Fanna	
Urophora quadrifasciata (Meigen)	*Centaurea jacea*	ITALY, Friuli V.G, Fanna	
Urophora terebrans (Loew)	*Cirsium eriophorum*	ITALY, Piemonte	
Urophora stylata (Fabricius)	*Cirsium arvense*	CROATIA, Istria, Rovigno	
Tribe Noeetini			
Ensina sonchi (Linnaeus)	*Sonchus sp.*	ITALY, Veneto, Legnaro	
Noeeta bisetosa Merz	*Hieracium piloselloides*	ITALY, Friuli V.G, Fanna	
Noeeta pupillata (Fallén)	*Hieracium umbellatum*	ITALY, Veneto, Fanna	
	Hieracium murorum	ITALY, Veneto, Cogollo del Cengio	
	Hieracium pilosella	ITALY, Veneto, Torreglia	
Tribe Tephritini/ Campiglossa Group			
Campiglossa doronici (Loew)	*Doronicum austriacum*	ITALY, Veneto, Cogollo del Cengio	
Campiglossa guttella Rondani	*Hieracium murorum*	ITALY, Veneto, Asiago	
Dioxyna bidentis (Robineau-Desvoidy)	*Bidens tripartita*	ITALY, Friuli V.G, Fanna	
Oxyna flavipennis (Loew)	*Achillea millefolium*	SLOVENIA, Kranjska, Kranjska Gora*	

Tribe Tephritini/Sphenella Group		
Sphenella marginata (Fallén)	*Senecio alpinum*	ITALY, Veneto, Asiago
Tribe Tephritini/Tephritis Group		
Acanthiophilus helianthi (Rossi)	*Centaurea jacea*	ITALY, Friuli V.G, Fanna
Capitites ramulosa (Loew)	*Phagnalon saxatile*	ITALY, Liguria, Imperia
Tephritis arnicae (Linnaeus)	*Arnica montana*	ITALY, Veneto, Asiago
Tephritis bardanae (Schrank)	*Arctium lappa*	ITALY, Veneto, Foza
Tephritis cometa (Loew)	*Cirsium arvense*	CROATIA, Istria, Rovigno
Tephritis conura (Loew)	*Cirsium spinosissimum*	ITALY, Veneto, Asiago*
Tephritis divisa Rondani	*Picris echioides*	ITALY, Liguria, Imperia
Tephritis fallax (Loew)	*Leontodon hispidus*	ITALY, Marche, Pesaro-Urbino
Tephritis formosa (Loew)	*Sonchus sp.*	ITALY, Marche, Pesaro-Urbino
Tephritis hendeliana Hering	*Carduus nutans*	ITALY, Veneto, Roana
Tephritis hyoscyami (Linnaeus)	*Carduus personata*	ITALY, Friuli V.G, Tarvisio
Tephritis leontodontis (De Geer)	*Leontodon autumnalis*	SLOVENIA, Kranjska, Kranjska Gora
Tephritis matricariae (Loew)	*Crepis vesicaria*	ITALY, Veneto, Legnaro
Trupanea amoena (Frauenfeld)	*Reichardia picroides*	ITALY, Liguria, Imperia*
Trupanea stellata (Fuessly)	*Erigeron annuus*	ITALY, Veneto, Verona
Tribe Terellini		
Chaetorellia jacea (Robineau-Desvoidy)	*Centaurea jacea*	ITALY, Friuli V.G, Fanna
Chaetostomella cylindrica (Robineau-Desvoidy)	*Centaurea triumfetti*	ITALY, Friuli V.G, Fanna
Orellia falcata (Scopoli)	*Tragopogon orientalis*	ITALY, Friuli V.G, Fanna
Terellia colon (Meigen)	*Centaurea scabiosa*	ITALY, Friuli V.G, Fanna
Terellia ruficauda (Fabricius)	*Cirsium arvense*	ITALY, Friuli V.G, Fanna
Terellia serratulae (Linnaeus)	*Cirsium pannonicum*	ITALY, Friuli V.G, Fanna
Terellia tussilaginis (Fabricius)	*Arctium tomentosum*	ITALY, Friuli V.G, Tarvisio
Terellia virens (Loew)	*Centaurea maculosa*	ITALY, Veneto, Rovolon
Tribe Xyphosiini		
Xyphosia laticauda (Meigen)	*Centaurea triumfetti*	ITALY, Friuli V.G, Fanna
Xyphosia miliaria (Schrank)	*Carduus nutans*	CROATIA, Istria, Rovigno

Tab. 4.1 – Material examined with accession number for insect host; †Sequence from Spanos *et al*. (2000).

Results

From DNA amplification and sequencing with the primers mentioned above, I obtained fragments ranging from 989 to 1036 bp for the 16S rDNA gene sequences, and from 1407 to 1553 for COI-tRNALeu-COII genes. For the combined data set a fragment ranging from 2399 to 2582 bp was obtained. For the 16S rDNA data set, the average proportion of T:C:A:G was 45:6:38:11, for COI-tRNALeu-COII genes was 39:14:34:12 and for the combined data set was 41:11:36:12.

The alignment of the 16S rDNA based on the secondary structure of *Drosophila melanogaster,* allowed a reliable alignment of a total of 1085 bp sites, avoiding the loss of information due to the removal of ambiguous portions of the alignment. For the COI-tRNALeu-COII data set the number of sites aligned was 1573 bp, while for the combined data set a total of 2658 bp were obtained.

The index for substitution saturation in all cases (16S rDNA, COI-COII, all codon positions) showed 'little saturation', with a slightly higher value for the third codon position in the COI-COII fragments. Thus, the sequences can be considered suitable for further phylogenetic analyses.

As reported above, the best-fit evolutionary model for the ML based phylogenetic analysis (as determined by ModelTest) was a general time-reversible model (GTR+I+G) for all the data sets.

Phylogenetic trees were studied considering the statistical support. Posterior probabilities (Pp) and bootstrap probabilities (Bp) were obtained from the molecular phylogenetic analyses using BI and ML respectively for the 16S dataset, COI-tRNALeu-COII and combined data set. Values of 95% for Pp and 70% for Bp were considered statistically significant for clades to be supported.

Molecular relationships among some Paleartic tribes of the family Tephritidae

Phylogenetic analysis of the 16S rDNA data set and the COI-tRNALeu-COII data set including European sampled species was carried out. The phylogenetic trees obtained using both ML and BI methods showed similar topologies with some disagreements between 16S rDNA and COI-tRNALeu-COII phylogenies. The phylogenetic tree obtained from the analysis of the COI-tRNALeu-COII showed more highly resolved trees and the internal

nodes more highly supported than the phylogeny inferred from the 16S data set, and defined the relationships among the tribes better (Fig. 4.1 and Fig 4.3).

The phylogenetic analysis of the combined data set (16S rDNA dataset + COI-tRNALeu-COII data set) was similar to the COI-tRNALeu-COII, but in this case, the relationships among tribes were not resolved. All the 32 highly supported nodes shown in the BI tree were also recognized by the ML tree. Five of the nodes shown by the BI tree were recognized only topologically.

In order to facilitate the discussion a number for every tribe has been assigned in the combined data set. Therefore, eight monophyletic groups corresponding to eight tribes have been recognized. Among the eight tribes present in this work, six were highly supported.

Groups 1-5 correspond to the Subfamily Tephritinae previously described in Chapter 3. This subfamily was well defined and highly supported forming a monophyletic group as is well recognized today (Foote *et al*., 1993; Norrbom *et al*., 1999; Korneyev 1999; Han *et al*., 2006). Four of the five tribes, in the subfamily Tephritinae were well defined, highly supported and were recovered as monophyletic groups (Fig. 4.3). In order to avoid repetitions whilst analyzing single groups (1 to 5) of the subfamily Tephritinae, refer to chapter 3.

Groups 6 and 7 and *Euphranta connexa* are comprised of the subfamily Trypetinae, Tribe Adramini, Tribe Carpomyini and Tribe Trypetini respectively. This subfamily did not form a monophyletic group, as suggested by Korneyev (1999) in his study based on morphological characters and resulted in the phylogenetic analysis of Han & McPheron (1997). However, two tribes inside the Trypetinae (Group 6 and Group 7) were well defined and the Tribe Trypetini was also recovered as a monophyletic group (Fig. 4.3).

Group 6 corresponds to the Tribe Carpomyini (Norrbom, 1988) represented by genus *Rhagoletis*, *Carpomya* and *Goniglossum* (Fig. 4.3). Genus *Rhagoletis* appeared topologically related to *Carpomya,* but it was not supported. Nevertheless previous studies (Bush, 1965; Berlocher and Bush, 1982) suggests genus *Rhagoletis* close related to Neotropical Carpomyine.

Group 7 corresponds to the Tribe Trypetini. It forms a monophyletic highly supported group as also shown in the phylogenetic analysis of Han & McPheron (1997) and Han (2000). This group is divided into two subtribes: Chetostomatina and Trypetina. Subtribe

Chetostomatina comprises species of genera *Anomoia* and *Myoleja* and Subtribe Tripetina includes species of genera *Trypeta*, *Euleia* and *Hemilea*. Both subtribes were monophyletic and highly supported subgroups corresponding to the morphological and molecular analysis (Korneyev, 1999; Han, 2000) (Fig. 4.3).

The species *Euphranta connexa,* the single representative of the Tribe Adramini sampled for this work, appeared as a sister group to the rest of Trypetinae and Tephritinae (Fig. 4.3)

Only three species of the subfamily Dacinae, according to Korneyev (1999), were analyzed. *Bactrocera oleae* was chosen as a representative of the genus *Bactrocera* belonging to the Tribe Dacini. *Ceratitis capitata* and *Capparimyia savastani* as representative of the Tribe Ceratitidini (Group 8). The last three species did not form a monophyletic group, but species of Tribe Ceratitidini (Group 8) do form a monophyletic group (Fig 4.3). The shortage of samples is limiting and additional analysis should be done, adding new species in order to analyze the phylogeny of the subfamily Dacinae.

The placement of these species, has been largely disputed. They have been ranked as different subfamilies (one for Dacini and one for Ceratitidini) (Hancock, 1986) or even, as a in the case of *Dacus*, a separate family (Munro, 1984). Other authors, however, consider them as a tribe of the subfamily Trypetinae (Norrbom *et al.,* 1999). Recent studies based on morphological approaches such as White and Elson-Harris (1992), Foote *et al.* (1993) and Korneyev (1999) suggested subfamily status (Subfamily Dacinae) including the tribes Dacini and Ceratitidini. Phylogenetic analysis done by Han & McPheron (1997) suggested also a relationship between tribes Ceratitini and Dacini.

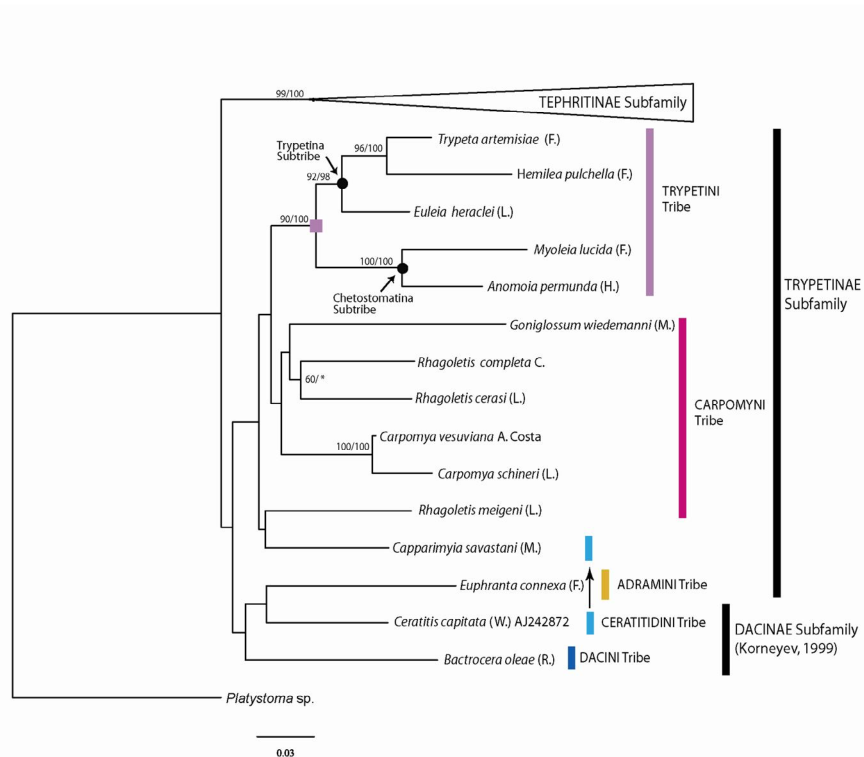

Fig. 4.1. - Phylogenetic reconstruction of "Higher Tephritidae" on the basis of the 16S rDNA data set. Bootstrap probabilities (ML) and posterior probabilities (BI) are reported on the nodes. Asterisks indicate bootstrap probabilities lower than 50% and posterior probabilities lower than 95%.

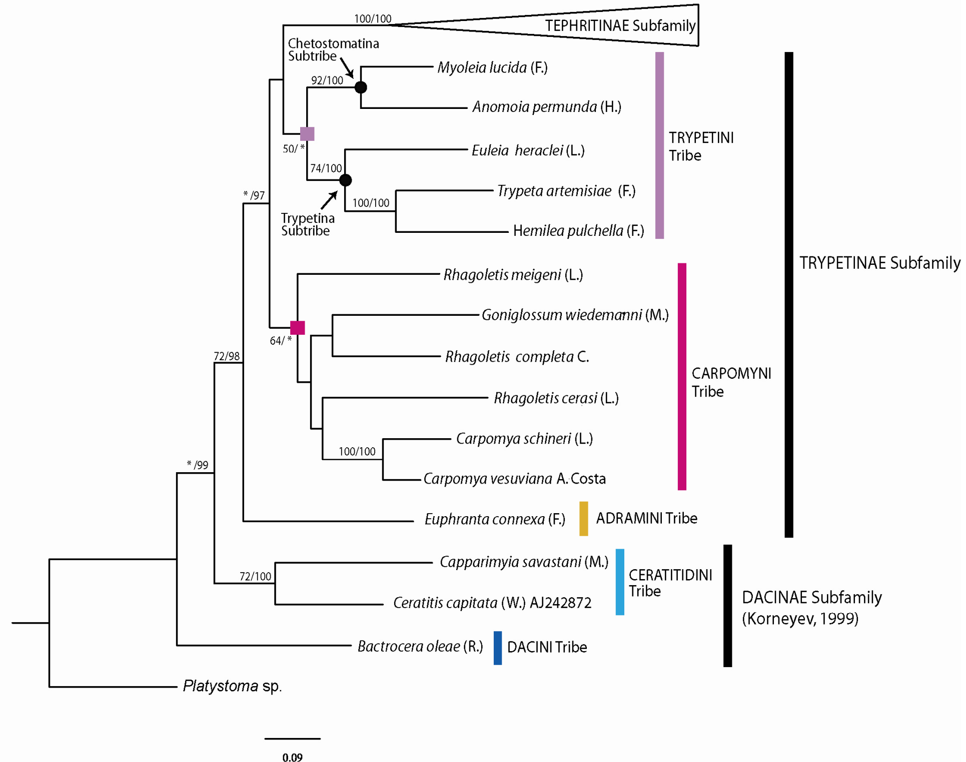

Fig. 4.2. - Phylogenetic reconstruction of "Higher Tephritidae" on the basis of the COI-tRNALeu-COII data set. Bootstrap probabilities (ML) and posterior probabilities (BI) are reported on the nodes. Asterisks indicate bootstrap probabilities lower than 50% and posterior probabilities lower than 95%.

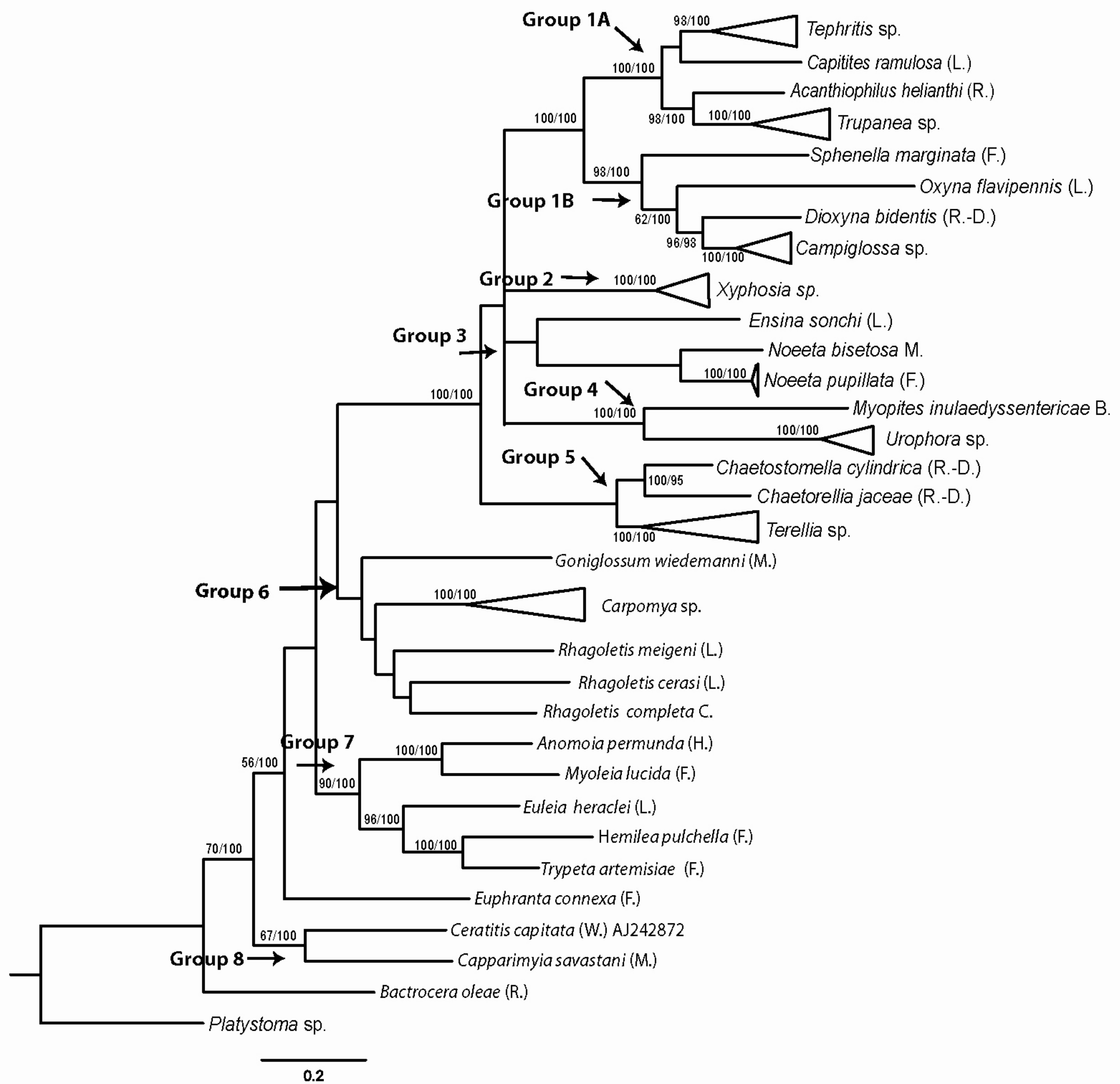

Fig. 4.3. - Phylogenetic reconstruction of "Higher Tephritidae" on the basis of the of the combined data set (16S + COI-tRNALeu-COII). Bootstrap probabilities (ML) and posterior probabilities (BI) are reported on the nodes. Asterisks indicate bootstrap probabilities lower than 50% and posterior probabilities lower than 95%.

Phylogeny and Symbiosis in Tephritid flies

I have attempted to study the phylogeny previously obtained and symbiosis arrangements as a whole (Fig. 4.4).

As described in the last chapter some species of the "Higher Tephritidae" (Korneyev, 1999) are characterized by the presence of symbiotic bacteria. In the subfamily Tephritinae, species of the Tribe Tephritini (Group 1) and species of genus *Noeeta* (Tribe Noeetini – Group 3) contain symbiotic bacteria (Mazzon *et al.*, 2008). In subfamily Dacinae (*B. oleae* and Group 8), only *B. oleae* harbours symbiotic bacteria ("*Candidatus* Erwinia dacicola") (Capuzzo *et al.*, 2005) (Fig. 4.4).

Up to the present no hereditary symbiotic bacteria have been described in the rest of subfamilies. In the subfamily Trypetinae (Groups 6 and 7) several authors report the presence of "associated bacteria" belonging to genus *Klebsiella* and *Eneterobacter* (Lloyd *et al.*, 1986; Drew & Lloyd, 1987; Daser & Brandl, 1992; Marchini *et al.*, 2002; Lauzon, 2003).

Therefore, a supported correspondence between phylogeny and the presence of symbiotic bacteria is evident.

An interesting relationship also results from the analysis of the phylogeny of the Higher Tephritidae and some symbiosis arrangements: morphology of the oesophageal bulb, displacement of bacteria in the midgut and presence of glands filled with bacteria in the ovipositor.

As reported above, the **oesophageal bulb**, is a specialized organ in the fly head morphologically different, depending on the species (Girolami, 1983) (Fig. 4.4 and Fig. 1.8).

Groups 1 to 4, belonging to the subfamily Tephritinae all have the same oesophageal bulb (*Ensina* type): a small oesophageal bulb with an ovoidal form, and without any particular cell elongations (Fig. 4.4). Bacteria have been never found in this kind of oesophageal bulb and no "membranous masses" are produced (Girolami, 1973).

Group 5 corresponds to the Tribe Terellini (Subfamily Tephritinae) and have an oesophageal bulb (*Chaetorellia* type), with a pharyngeal outward-deflection, whose apex closely resembles that of Trypetinae (Groups 6 and 7) (Fig. 4.4). In the lumen of this

oesophageal bulb, culturable bacteria, not symbiotic, multiply (Girolami, *pers. com*) embebed in the "membranous masses".

Groups 6 and 7 (Subfamily Trypetinae), *E. connexa*, and also Group 8 (Subfamily Dacinae) with the exception of *B. oleae,* have a specific type of oesophageal bulb (*Ceratitis capitata* type). It is characterized by its spherical form having elongated cells at the apex, and the presence of culturable bacteria, that multiply close to the apex within "membranous masses" (Fig 4.4) (Girolami, 1983, Ratner & Stoffolano, 1984).

B. oleae, the only species of the subfamily Dacinae in which the presence of symbiotic bacteria is known, have a spherical oesophageal bulb, *Dacus* type according to Girolami (1973), larger than the previous one and provided with a "neck" connecting it to the pharynx. Its basal epithelial cells are elongated and symbiotic bacteria multiply next to them (Petri, 1909). It would be interesting to observe other African *Bactrocera* .

Therefore, the oesophageal bulbs of the species belonging to Subfamily Dacinae (Group 8), including *B. oleae*, Subfamily Trypetinae (Group 6 and 7) and Tribe Terellini of Subfamily Tephritinae (Group 5) produce "membranous masses" containing bacteria (Girolami, 1973). These bacteria start to multiply in the oesophageal bulb and are discharged into the gut forming the bacterial masses located inside the peritrophic membrane (Petri, 1909; Girolami, 1973).

Thus, there is an interesting correspondence between the phylogeny of the insect and the morphology and function of the oesophageal bulb.

In the **midgut** lumen of species belonging to the Groups 5, 6, 7 and 8 it is common to find, free living bacteria forming "bacterial masses" (Fig. 4.4). These bacteria as far as is known, are easily culturable. The non culturable symbiotic bacteria of *B. oleae* is also discharged from the oesophageal bulb into the midgut, forming "bacterial masses" of its symbiont "*Candidatus* Erwinia dacicola".

These "bacterial masses" are not produced in the midgut of the rest of Tephritinae (Groups 1-4) (Fig. 4.4). Species of the Tribe Tephritini (Group 1A and Group 1B) and Tribe Noeetini (Group 3) (Mazzon *et al.*, 2008), which contain symbiotic bacteria, have a specialized epithelium surrounding the first part of the midgut. Here, the symbiotic bacteria are located, in contact with the epithelium of the midgut, but outside the peritrophic

membrane. They are therefore, not in direct contact with the intestinal lumen but between the peritrophic membrane and the epithelium (Girolami, 1983).

In the species of the Tribe Xyphosiini (Group 2) and Tribe Myopitini (Group 4), in which symbiotic bacteria have not been detected, this specialized epithelium of the midgut is not present.

Again a perfect correspondence between the different monophyletic tribes and the midgut epithelium morphology appears.

Other interesting morphological structures related to the presence of symbiotic bacteria, are the **glands** present in the ovipositor of some Tephritid flies (Fig. 4.4). The ovipositor of *B. oleae* contains "anal" glands full of symbiotic bacteria (Petri, 1909). During the oviposition the egg comes into contact with these glands which release the symbiotic bacteria, covering the egg, in order to ensure the vertical transmission of the bacteria to the progeny. These kind of glands are also present in species of the Tribe Tephritini, which have symbiotic bacteria (Stammer, 1929).

However, in species of the Tribe Noeetini (Group 3), where symbiotic bacteria have been found (Mazzon *et al.*, 2008), the presence of these glands is not yet clear. Additional, histological studies have to be done in order to clarify these aspects.

These glands, however, are not present in the rest of Tephritinae (Groups 2, 4 and 5) nor in all of the Trypetinae and Dacinae, with the exception of *B. oleae* (Groups 6, 7 and 8). These species did not harbour symbiotic bacteria and demonstrate an interesting correspondence between phylogeny and morphology of the ovipositor (Fig. 4.4).

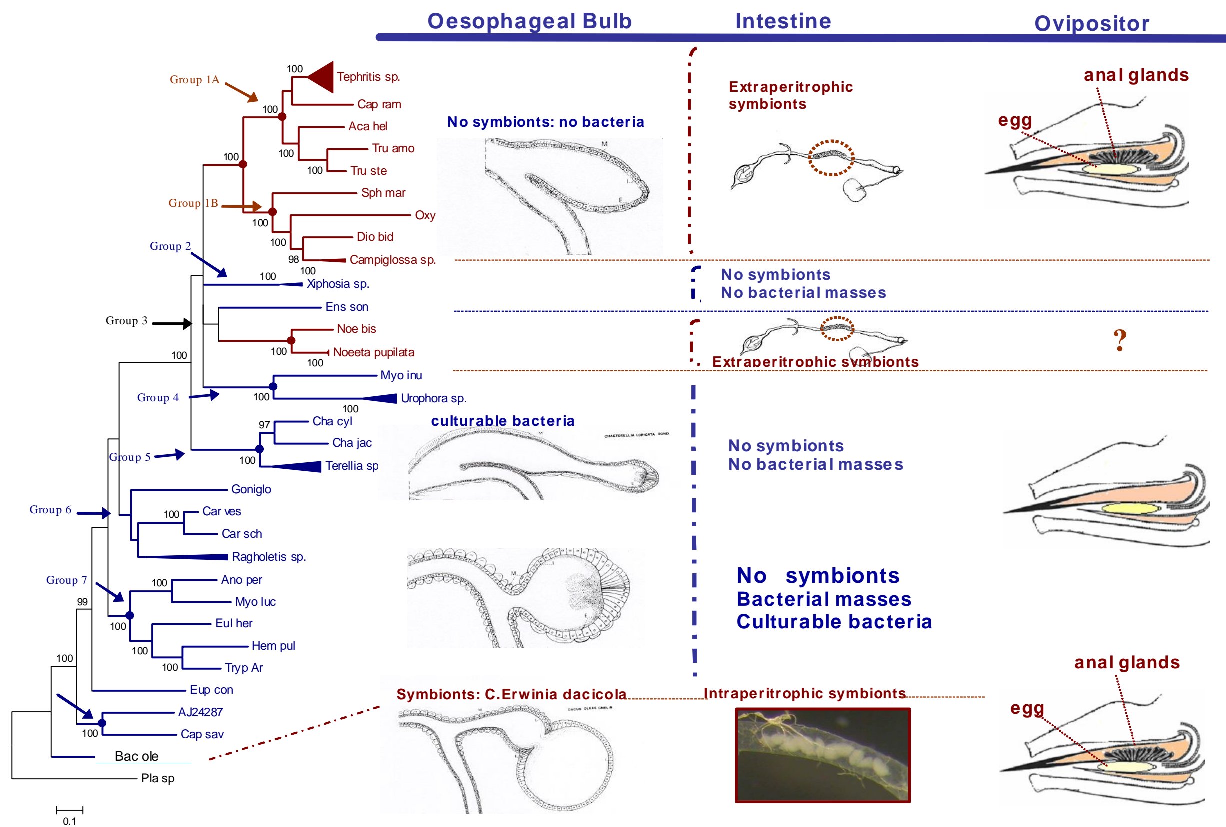

Fig. 4.4. - Summary of the relationships between phylogeny of fruit flies and morphologic symbiotic arrangements. On the left side appears phylogenetic reconstruction of "Higher Tephritidae" on the basis of the combined data set (16S rDNA + COI-tRNALeu-COII). Bootstrap probabilities (ML) and posterior probabilities (BI) are reported on the nodes. Asterisks indicate bootstrap probabilities lower than 50% and posterior probabilities lower than 95%. Brown branches correspond to taxa where symbiotic bacteria have been detected and blue branches correspond with taxa without symbiotic bacteria. On the right side, morphologic symbiotic arrangements are shown.

Phylogeny and biological characteristics

I have attempted to associate the inferred phylogeny with some biological characteristics such as voltinism, hibernation and feeding behaviour (Fig. 4.5). Our data are based on literature (White, 1988; Merz, 1994) and personal experience.

Group 1: Tribe Tephritini (Subfamily Tephritinae). All species belonging to "Tephritis group" (Group 1A) feed on the flower heads of the Asteraceae are monovoltine and hibernate as adults, with the exception of *Acanthiophilus helianthi* that is multivoltine and hibernates as pupae (Girolami, *pers. com.*).

Species belonging to the *Sphenella* and *Campiglossa* groups (Group 1B) are monovoltine and feed on the flower heads of the Asteraceae, with the exception of *Oxyna flavipennis* which develops on roots. Some of them hibernate as adults and some as pupae (Fig. 4.5).

Group 2: Tribe Xyphosiini (Subfamily Tephritinae). Species of the Tribe Xyphosiini are mainly multivoltine, feed on flower heads of the family Asteraceae where they hibernate as a pupae (Fig. 4.5).

Group 3: Tribe Noeetini (Subfamily Tephritinae). Species of the Genus *Noeeta* are bivoltine, and hibernate as pupae in the host plant, and feed on flowers heads of the genus *Hieracium* (family Asteraceae) forming galls in the capitulum. *Ensina sonchi*, however, has different biological characteristics, it is multivoltine, hibernates as a pupa or an adult, feeds on flower heads of many species the family Asteraceae, without forming galls on their capitulum (Fig. 4.5).

Group 4: Tribe Myopitini (Subfamily Tephritinae). Species of this tribe are mainly monovoltine although some of them are bivoltine such as *Urophora quadrifasciata* (White, 1988). They usually overwinter as larvae (White, 1988) and feed on flower heads of the family Asteraceae forming characteristic galls.

Group 5: Tribe Terellini (Subfamily Tephritinae). Species of this tribe are mostly bivoltine but some can be monovoltine or multivoltine. They usually overwinter as mature larvae (White, 1988) and feed on flower heads of the family Asteraceae except for *Orellia falcata* who feeds on roots and hibernates as a pupa.

To recapitulate, species of the subfamily Tephritinae (Groups 1 to 5), with some rare exceptions, feed on flower heads of the family Asteraceae. The “Higher Tepritinae” represented here by the Tribe Tephritini are mainly monovoltine hibernating as adults, whereas the rest of Tephritinae are generally bivoltine or multivoltine hibernating as larvae or pupae (Fig. 4.5).

Group 6: Tribe Carpomyini (Subfamily Trypetinae). Species of Tribe Carpomyini are specialized carpophagous, monovoltine and usually hibernate as pupae in the soil.

Group 7: Tribe Trypetini (Subfamily Trypetinae). All species of the Tribe Trypetini hibernate as pupae in the soil (White, 1988). Species belonging to the subtribe Chetostomatina are monovoltine and feed on fruits, yet others are leaf and stem-miners and mono or bivoltine (White, 1988).

E. connexa hibernates as a pupa is monovoltine and feeds on seeds of the *Vicetoxicum spp.* (Asclepiadeae). Interestingly many botanical species of this family in Africa are attacked by species of the genus Dacus subfamily Dacinae.

Group 8 and *B. oleae*: Subfamily Dacinae overwinter as adults and do not normally hibernate. They are also multivoltine and frugivorous (carpophagous) species.

A correspondence between the phylogeny, feeding strategies and hibernation behavior has been found (Fig. 4.5).

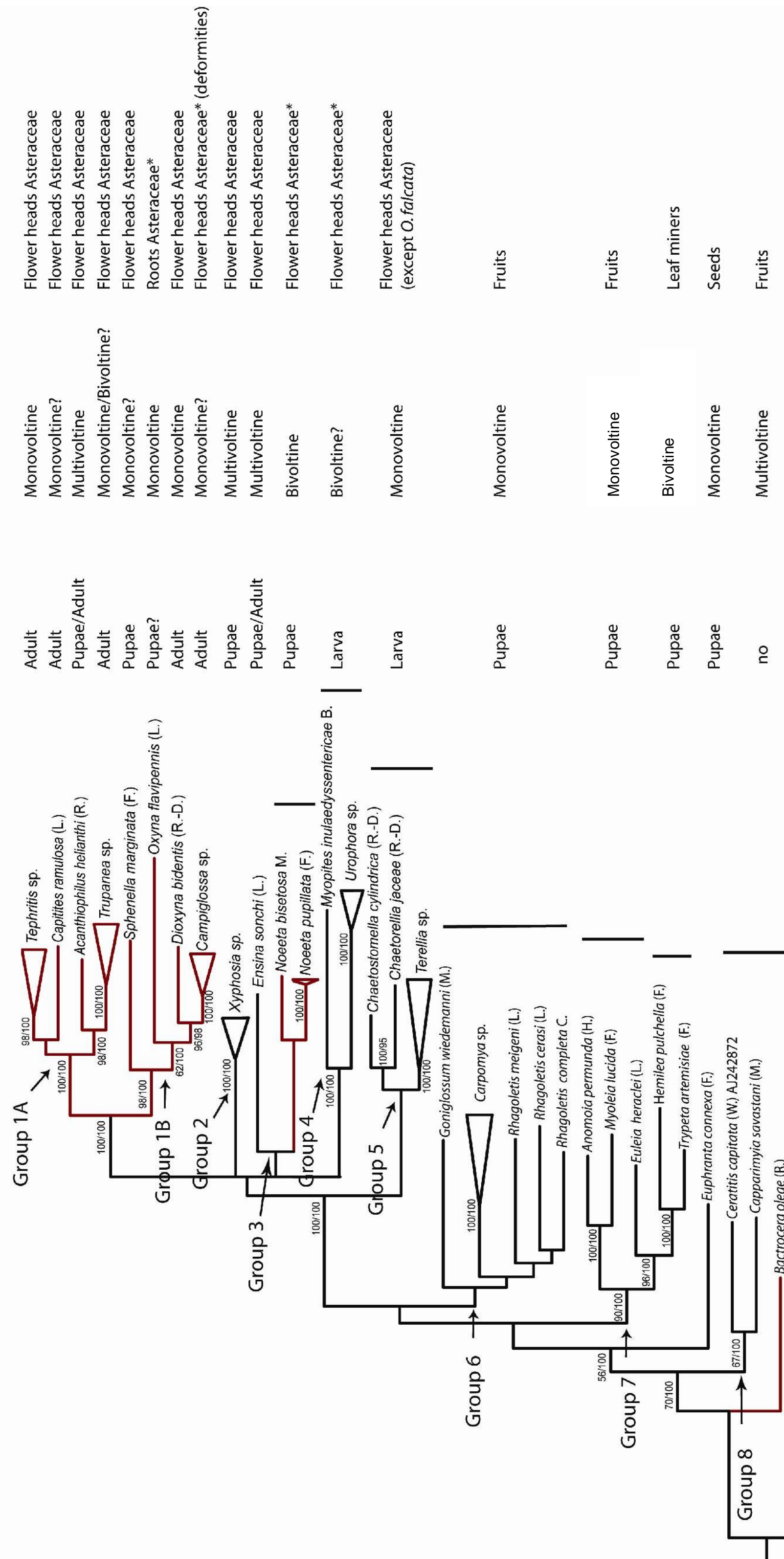

Fig. 4.5 - Summary of the relationships between phylogeny of fruit flies and biological arrangements. On the left side appears phylogenetic reconstruction of "Higher Tephritidae" on the basis of the combined data set (16S rDNA + COI-tRNALeu-COII). Bootstrap probabilities (ML) and posterior probabilities (BI) are reported on the nodes. Asterisks indicate bootstrap probabilities lower than 50% and posterior probabilities lower than 95%. Brown branches correspond to taxa where symbiotic bacteria has been detected and blue branches correspond with taxa without symbiotic bacteria. On the right side, biological symbiotic arrangements are shown.

Discussion

An interesting and strong relationship between phylogeny, symbiosis and biological arrangements of Tephritid flies has been found.

Sufamily Tephritinae (Group 1-5)

Tribe Tephritini **(Group 1)** is a strongly supported monophyletic clade represented by species of the "Tephritis group" (Merz, 1999) (Group 1A) and *Sphenella* and *Campiglossa* group (1B).

The genus "*Tephritis*" appears as a highly supported clade. All the species of this genus have the symbiotic bacteria currently designated as "*Candidatus* Stammerula tephritidis" (Mazzon *et al*., 2008). None of them harbour bacteria in the esophageal bulb and all of them have specialized epithelium in the midgut where bacteria are located. In the ovipositor of genus *Tephritis* glands filled with bacteria are present. A common bacterial ancestor is assumed in this case. This group is also characterized and supported by their common biological characteristics. All of these species have similar feeding behavior, feeding on the flower heads of the Asteraceae, hibernating as adults and, as a rule, are monovoltine.

Genera *Tephritis*, *Capitites*, *Acanthiophilus* and *Trupanea* form the "Tephritis group" (Merz, 1999) (Group 1A). This group is characterized by the presence of the symbiotic bacteria "*Candidatus* Stammerula spp.". They have oesophageal bulbs devoid of bacteria and have specialized structures in the midgut, outside the peritrophic membrane where the symbiotic bacteria are located. All of them feed on the flower heads of the Asteraceae, are monovoltine and hibernate as adults. *A. helianthi* is the only interesting exception; bacteria are located in an evagination present only in one side of the midgut (Fig. 1.5). It also hibernates as a pupa and is probably multivoltine. Again a strong connection appears between phylogeny, symbiosis and biological characteristics.

Campiglossa group and *Sphenella* group also form a well defined phylogenetic cluster (Group 1B). As a rule species of these groups contain bacteria of the genus *Stammerula*. However, *Campiglossa guttella* and *Dioxyna bidentis* are an exception; they are characterized by the presence of symbiotic bacteria, related to the free living bacteria *Erwinia persicina*. In this group, there is no complete correlation between phylogeny and symbiosis. *C. guttella* forms a monophyletic group with *C. doronici* and could have lost its

symbiotic bacteria "*Candidatus* Stammerula spp." but has subsequently acquired new bacteria which have become its new symbiotic bacteria. On the other hand, the possibility of a horizontal transfer of the symbiotic bacteria "*Candidatus* Stammerula spp" from one *Tephritis spp*. to *C. doronici* cannot be excluded. However, none of them contain bacteria in the oesophageal bulb and all of them have specialized structures in the midgut where bacteria are located, as in *Tephritis* group. It is interesting to note that *S. marginata* has specialized bilobate evaginations in the midgut but in a separated position with respect to the midgut, where bacteria multiply (Fig 1.6) (Stammer, 1929).

Species of Group 1B generally hibernate as adults, but some hibernate as pupae. *O. flavipennis* lives on roots but the remaining species in this group feed on the flower heads of the Asteraceae.

Tribe Xyphosiini **(Group 2)** forms a strongly monophyletic group. Species of this tribe, where no symbiotic bacteria has been found, have an oesophageal bulb devoid of bacteria. In the midgut of these species, no specialized structures are present and the ovipositors possess no glands. These species hibernate as pupae and are multivoltine. A strict affinity appears in this monophlyletic group on the basis of their biological and morphological characteristics related to the absence of bacterial symbiosis.

Next group **(Group 3)** corresponds to Tribe Noeetini, and is represented by species of genus *Noeeta* and *E. sonchi*.

Noeeta genus is the next cluster where symbiotic bacteria have been discovered (Mazzon *et al.,* 2008). These kind of bacteria, are phylogenetically close to the free living bacteria *Ewingella americana*. All of the species of genus *Noeeta* show similar biological characteristics: bivoltinism, overwintering as pupae and oligophagy, feeding on species of genus *Hieracium* and forming characteristics galls in the capitulum.

Within the Tribe Noeetini the presence of *E. sonchi* has been questioned. Norrbom and Korneyev (Norrbom, 1999), based on a single synapomorhic character, considered *E. sonchi* as a member of the Tribe Noeetini. In the phylogenetic analysis of Han *et al*. (2006) *E. sonchi* was not grouped with the rest of Noeetini. Our phylogenetic analysis of the combined and COI-tRNALeu-COII data set (Fig. 4.3 and Fig. 4.4) showed *E. sonchi* grouped with the species of genus *Noeeta* but with a low statistical support. Moreover, the biological characteristics of *E. sonchi* are different from the rest of Noeetini; it is

multivoltine, polyphagous, feeding on flower heads of the family Asteraceae, without forming galls and the overwintering stadium is unknown. Therefore, *E. sonchi* appears different from the rest of Noeetini based on the phylogenetic and biological approaches and also on the absence of symbiotic bacteria. As Han *et al.,* (2006) proposes, additional studies of this tribe could be useful.

The Tribe Myopitinae **(Group 4)** is a strongly monophyletic group. Species of this tribe do not contain symbiotic bacteria, the oesophageal bulb is devoid of bacteria and the ovipositor does not have glands (Fig 4.4). Myopitini feed on flowers of family Asteraceae forming galls and, as a rule, are bivoltine, hibernating as mature larvae in the host plant. Thus, Tribe Myopitini is strongly grouped, considering the phylogeny, the absence of symbiosis and the biological characteristics.

Tribe Terellini **(Group 5)** have an intermediate oesophageal bulb (Fig 4.4). In the apex part it resembles to the bulb of Trypetinae (Group 6-7), the basal part, however, resembles the oesophageal bulb of the rest of Tephrtitinae (Group 1-4). Like Trypetinae and Dacinae (except *B. oleae*) "membranous masses" are present in the oesophageal bulb. The presence of symbiotic bacteria has not been noted in this tribe but the occurrence of free living bacteria has been described (Girolami, 1973). These bacteria multiply in the oesophageal bulb and are discharged into the midgut forming "bacterial masses", as in the Tribe Trypetinae and Dacinae. In the phylogenetic tree, Tribe Terellini seems placed in an intermediate position as well; it seems to be a sister group of the rest of Tephritinae.

Species of the Tribe Terellini feed on flower heads of family Asteraceae, with the exception of *Orellia falcata* which feeds on roots. All overwinter as mature larvae or pupae on the host plant.

Subfamily Trypetinae (Groups 6-7) does not result as a monophyletic group (Fig 4.3). To date, hereditary symbiotic bacteria in species of Subfamily Trypetinae have not been found. All species belonging to this subfamily have a similar oesophageal bulb defined as *Ceratitis* type in Girolami, 1973, where "membranous masses" impregnated with culturable bacteria are produced and subsequently discharged into the midgut (Fig. 4.4).

Subfamily Trypetinae is represented here by Paleartic and Neartic species included in two subtribes. The first one, Tribe Carpomyini (**Group 6),** is grouped for the phylogenetic

analysis, but also for its biological characteristics: monovoltinism, hibernation as a pupae and as a rule, carpophagy.

At the same time, the Tribe Trypetini **(Group 7)** is a monophyletic group divided in two subtribes (Chetostomatina and Trypetina) strongly grouped, both from the phylogenetic and biological point of view. Subtribe Chetostomatina includes carpophagous and monovoltine species which hibernate as pupae in the soil. Subtribe Trypetina comprises leaf-miner species, which are multivoltine and hibernate as pupae in the soil.

Euphranta connexa belongs to the Tribe Adramini and seems phlylogenetically different from the rest of Trypetinae, but its position is not statistically supported. Nevertheless, the symbiosis arrangements are similar to the rest of the subfamily Trypetinae. This species hibernates as a pupa, is monovoltine and feeds on seeds of the *Vicetoxicum spp.* (Asclepiadaceae). Interestingly, many botanical species of this family in Africa are attacked by species of the genus *Dacus* subfamily Dacinae.

Subfamily Dacinae (**Group 8** and *B. oleae*): unfortunately this subfamily is represented in the present work by only three species: *Bactrocera oleae*, *Ceratitis capitata* and *Capparimyia savastani*. Except for *B. oleae*, this subfamily has the same bacterial arrangements as species of Subfamily Trypetinae. These are a similar oesophageal bulb, the absence of glands in the ovipositor and presence of membranous and bacterial masses.

In this subfamily, *B. oleae* is a particularly interesting case due to the presence of symbiotic bacteria and special morphological structures related to symbiosis such as anal glands in the ovipositor and a larger oesophageal bulb unlike the rest of Dacinae. The basal epithelial cells of the oesophageal bulb are elongated and symbiotic bacteria is located next to them (Girolami, 1973).

Phylogenetically *B. oleae* is recognized as a sister group of genus *Bactrocera* (Smith *et al.*, 2005). The presence of symbiotic bacteria and a different oesophageal bulb represents an interesting difference with respect to the other *Bactrocera* already studied: *B. dorsalis*, *B. cucurbitae* (Girolami, 1983). Therefore, *B. oleae* could be considered a species quite different from the rest of *Bactrocera* and consequently Dacinae, from the symbiosis point of view. White (2006) considers some Dacinae native to the Afrotropical region (Africa), including *B. oleae*. Maybe *B. oleae* and other African *Bactrocera* spp. could form a special group, where symbiosis has evolved. So, *B. oleae* could be the representative of a group of

species having the same oesophageal bulb containing symbiotic bacteria. This group could include species related to the Oleraceae such as *Bactrocera biguttula* e *Bactrocera munroi*. These, however, are simply hypotheses. This is a complex argument and further and intensive studies have still to be done. Additional sequences of *Bactrocera* spp. have to be added to our preliminary phylogenetic analysis and the presence of symbiotic bacteria has yet to be studied in these African species. This aspect will be the object of future researches. A preliminary phylogenetic analysis, including other available sequences of the 16S rDNA of fruit flies (Han *et al*. 2006) is still in progress. This analysis will provide an interesting and comprehensive revision of the tribal and subfamily classification, and also afford useful points of study of the possible presence of symbiosis in phylogenetic groups from different zoogeographical regions.

This preliminary study suggests an interesting correspondence between phylogenetic and biological information, presence or absence of symbiosis and morphological structures related to the symbiosis, in tephritid flies.

It is interesting to note that all the species that overwinter as adults (Tephritid Group and subfamily Dacinae) contain either symbiotic bacteria, or "bacterial masses" inside the oesophageal bulb and peritrophic membrane. Conversely, none of the species of the subfamily Tephritinae, where symbiotic are not present, overwinter as adults. The presence, therefore, of these bacteria seems to be essential for the overwintering adults. Indeed, whilst the diet of larval stages includes relatively rich substrates such as flower tissue and ripening seeds; overwintering glycophagous adults have access to less resources. Thus the presence of bacteria could be more critical for their survival than in the earlier stages of development. As already pointed out, bacteria are observed to rapidly multiply once adults emerge; there is indeed a possibility that bacteria, constitute, in themselves, the renewable nutritional source that improves adult fitness for survival during overwinter.

CONCLUSION

According to Stammer, (1929) the presence of non culturable symbiotic bacteria was detected in species of genera *Tephritis*, *Campiglossa*, *Trupanea, Acanthiophilus*, *Sphenella*, and *Oxyna*. Symbiotic bacteria were also found in other genera (*Capitites, Dioxyna, Noeeta*), not studied by Stammer. Sequencing of the small subunit rDNA gene from these symbiotic bacteria indicated that they belong to the family Enterobacteriaceae and a novel candidate organism was proposed for the symbiotic bacteria of the genus *Tephritis*, under the designation '*Candidatus* Stammerula tephritidis' (Mazzon et al., 2008).

The extended analysis, using the previous methodology, of other species belonging to the Tribes Xyphosiini, Myopitini and Terellini did not point out the presence of symbiotic bacteria. Thus, in subfamily Tephritinae the presence of symbiotic bacteria is limited to the tribe Tephritini and genus *Noeeta* included in Tribe Noeetini. The symbiotic bacteria of *Noeeta spp*. was shown to be phylogenetically related to the free living bacteria *Ewingella spp.,* denoting a different history from that of symbiotic bacteria of tribe Tephritini.

The phylogenetic analysis of two different regions of the mitochondrial DNA of 42 Paleartic species of the subfamily Tephritinae suggested the presence of five monophyletic and highly supported clusters, (except for Tribe Noeetini, which presents a low statistical value), corresponding to five tribes of current classification (Norrbom et al., 1999; Korneyev, 1999) of this subfamily: Tephritini, Myopitini, Xyphosiini, Noeetini and Terellini. The monophyly of Tephritinae, was confirmed by our phylogenetic analysis according to morphological and phylogenetic approaches (Foote *et al*., 1993; Korneyev, 1999; Norrbom *et al*., 1999; Zwölfer, 1983; Han *et al*., 2006). Interestingly, the phylogeny obtained from the analysis of the COI-tRNALeu-COII resulted in fully resolved trees, with the internal nodes highly supported. On the other hand, several internal nodes of the phylogeny inferred from the 16S rDNA data set were not statistically supported. This could be an interesting indication for future phylogenetic studies of this subfamily.

Studying the possibility of a coevolution between hosts and symbiotic bacteria, cophylogenetic analyses suggested a significant fit between the host and the symbiont trees. However, the congruence between host and symbionts resulted imperfect, suggesting that some independent events such as duplications, sorting events and host-switching had

intervened. Indeed, these bacteria are extracellular symbionts and some opportunities for host-switching occurred during the biological cycle of the fly. In the larval stadium, for instance, bacteria are located in the intestinal caeca (Petri 1909; Stammer, 1929) without the protection of the peritrophic membrane and thus, in contact with free living bacteria present in the intestinal lumen. During the oviposition, females smear the surfaces of their eggs with bacteria in order to ensure the vertical transmission of the bacteria to the progeny increasing the chance of contacts with the external environment, and in particular eggs and larvae of other species. Considering all of these aspects, the presence of a congruence, even if not strict, is a particularly interesting result and a compatibility between host and symbiont seems to appear. The reconstructions obtained hypotheses the presence of two main events. The first one suggests that the common ancestor of Tephritini Tribe acquired their symbiotic bacteria ("*Candidatus* Stammerula sp."), coevolving over time with some subsequently losses and acquisitions. The second independent event, denotes a different history and concerns the acquisition of a different from *Erwinia* sp. symbiotic bacteria in flies of the genus *Noeeta.* Probably, strict vertical transmission is the primary basis of the tephritinae-symbiont congruence but the involvement of other factors such as insect host-symbiotic bacteria physiological compatibility, should also taken into account.

The phylogenetic analysis of species belonging to Subfamily Tephritinae, Trypetinae and Dacinae confirms the traditional classification based on a morphological approach but also suggested interesting relationships such as: the non-monophyly of the subfamily Trypetinae, according to the molecular analysis carried out by Han & McPheron (1997) and the morphological approach of Korneyev; sister group relationship between the tribe Terellini and the rest of Paleartic tribes of the subfamily Tephritinae present in this work, was highly supported in the analysis of the COI-tRNALeu-COII; and the questioned presence of *Ensina sonchi* in the tribe Noeetini, which appeared not statistically supported, as in Han *et al.*'s, analysis (2006).

In the association of phylogenetic analysis to biological and morphological information related to symbiosis, an interesting correspondence was pointed out. Monophyletic tribes share, as a rule, the same symbiotic arrangements and biological characteristics. In particular, it was pointed out that all the species of the subfamily Tephritinae that overwinter as adults, present symbiotic bacteria in the first tract of the midgut. Conversely,

none of the species of the subfamily Tephritinae, where symbiotic are not present, overwinter as adults. The presence of these bacteria seems to be essential for the overwintering adults. Indeed, while the diet of larval stages includes relatively rich substrates such as flower tissue and seeds, glycophagous adults have access to less resources. Thus the presence of bacteria could be more critical for their survival than that in the earlier stages. Bacteria, could constitute, in themselves, the renewable nutritional source that improves adult fitness for survival.

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