COST Action FA0807 Integrated Management of Phytoplasma Epidemics in Different Crop Systems

EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY

COST Action 0807

Working group 2 – Insect vectors

Training School

"Molecular Tools to Identify Leafhopper and Planthopper Vectors of Phytoplasmas"

Ivrea-Grugliasco, Italy / 5-9 September, 2012

DIVAPRA
Università di Torino
Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali

UNIVERSITÀ DEGLI STUDI DI TORINO
ALMA UNIVERSITAS TAURINENSIS

MUSEO REGIONALE DI SCIENZE NATURALI

Organizers

University of Torino, DIVAPRA – Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali

Museo Regionale di Scienze Naturali, Torino

Supported by

COST Action 0807: Integrated Management of Phytoplasma Epidemics in Different Crop Systems

University of Torino, DIVAPRA – Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali

MIUR – PRIN Project " Identification of species and intra-specific variants of sap-sucking and parasitoid insects with molecular markers"

Organizing Committee

Prof. Domenico Bosco

Dr. Rosemarie Tedeschi

Dr. Sabrina Bertin

Dr. Luca Picciau

SCHEDULE

Monday 5th November, Theoretical course in Ivrea (Torino)

9:00-9:20	**DOMENICO BOSCO** Presentation of the course
9:20-9:50	**PAOLO PEDATA** An introduction to principles of systematics
9:50-10:20	**MAURILIA M. MONTI** DNA taxonomy
10:20-10:40	**MAURILIA M. MONTI** Integrative taxonomy
10:40-11:10	**Coffee Break**
11:10-11:40	**SABRINA BERTIN** Mitochondrial DNA
11:40-12:10	**DOMENICO BOSCO** Preparation of insect samples for molecular species/biotype identification
12:10-12:40	**SABRINA BERTIN** Nuclear DNA: ribosomal DNA and protein-coding genes
13:00-14:00	**Lunch**
14:00-14:30	**SABRINA BERTIN** Random Amplified Polymorphic DNA technique (RAPD)
14:30-15:15	**ALBERTO ACQUADRO** Microsatellite markers
15:15-16:30	**MAURILIA M. MONTI** Population and phylogenetic analysis
16:30-17:00	**Coffee Break**
17:00-17:10	**DOMENICO BOSCO** Presentation of the PRIN project "Identification of species and intra-specific variants of sap-sucking and parasitoid insects with molecular markers"
17:10-17:40	**DOMENICO BOSCO** Application of molecular markers for species identification: the Cixiidae family (Hemiptera) as a case-study

Tuesday 6th November, Theoretical course in Ivrea (Torino)

9:00-9:30	**GIUSEPPE PARRELLA** Molecular methods to detect genetic variability within the *Bemisia tabaci* cryptic species complex
9:30-9:50	**GIUSEPPE PARRELLA** Current state of knowledge on the molecular variability *of Trialeurodes vaporariorum*
9:50-10:20	**MAURILIA M. MONTI** Integration of molecular, ecological, morphological and endosymbiont data: *Pnigalio soemius* complex, a case study
10:20-10:50	**SABRINA BERTIN** Application of molecular markers for species identification: the Empoascini tribe (Hemiptera: Cicadellidae) as a case-study
10:50-11:20	**Coffee Break**
11:20-11:50	**MAURILIA M. MONTI** Taxon-specific multiplex-PCR: a case study for quick, easy, and accurate identification of parasitoid species of soft scale insects
11:50-12:50	**ELENA CARDILLO – BIO-RAD** High Resolution Melting: a new tool for insect taxonomy

13:00-14:00	**Lunch**
14:00-15:45	**LUCA PICCIAU** Morphology of Auchenorrhyncha, with particular focus on plant- and leaf-hoppers

Wednesday 7th November, Practical sessions in Grugliasco (Torino)

9:00-13:00	DNA extraction
13:00-14:30	**Lunch**
14:30-15:30	PCRs of mitochondrial and ribosomal DNA targets
15:30-18:00	Morphology-based identification of plant- and leaf-hoppers

Thursday 8th November, Practical session in Grugliasco (Torino)

9:00-11:00	Electrophoresis of mitochondrial and ribosomal PCR products
11:00-13:00	Species – specific RFLP assays on mitochondrial and ribosomal PCR products
13:00-14:30	**Lunch**
14:30-15:30	RFLP electrophoresis
15:30-18:00	DNA purification from PCR products and gel bands
evening	Visit to the city of Torino

Friday 9th November, Practical session in Grugliasco (Torino)

9:00-11:30	Computer room session: analysis of mitochondrial and ribosomal sequences, virtual RFLP assays and species-specific primer design
11:30-12:30	Discussion on strategies, troubles and pitfalls in applying molecular markers
12:30-13:00	General discussion and conclusion
13:00	**Lunch and departure**

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SHORT COURSE ON "Insect Molecular Taxonomy"

Presentation 1.
An introduction to principles of systematics
by Paolo A. Pedata, Istituto per la Protezione delle Piante, CNR, Portici (NA), Italy

Presentation 2.
DNA taxonomy
By Maurilia M. Monti, Istituto per la Protezione delle Piante, CNR, Portici (NA), Italy

Presentation 3.
Integrative taxonomy
By Maurilia M. Monti, Istituto per la Protezione delle Piante, CNR, Portici (NA), Italy

Presentation 4.
Mitochondrial DNA
By Sabrina Bertin, DISAFA - Entomology and Zoology, Università degli Studi di Torino, Italy

Presentation 5.
Preparation of insect samples for molecular species/biotype identification: collection, storage, DNA extraction and main PCR target sequences
By Domenico Bosco, DISAFA - Entomology and Zoology, Università degli Studi di Torino, Italy

Presentation 6.
Nuclear DNA: ribosomal DNA and protein-coding genes
By Sabrina Bertin, DISAFA - Entomology and Zoology, Università degli Studi di Torino, Italy

Presentation 7.
Random Amplified Polymorphic DNA technique (RAPD)
By Sabrina Bertin, DISAFA - Entomology and Zoology, Università degli Studi di Torino, Italy

Presentation 8.
Microsatellite markers
By Alberto Acquadro, DISAFA - Plant Genetics and Breeding, Università degli Studi di Torino, Italy

Presentation 9.
Population and phylogenetic analysis
By Maurilia M. Monti, Istituto per la Protezione delle Piante, CNR, Portici (NA), Italy

Presentation 10.
Application of molecular markers for species identification: the Cixiidae family (Hemiptera) as a case-study
By Domenico Bosco, DISAFA - Entomology and Zoology, Università degli Studi di Torino, Italy

Presentation 11.
Molecular methods to detect genetic variability within the *Bemisia tabaci* cryptic species complex and current state of knowledge on the molecular variability of *Trialeurodes vaporariorum*
By Giuseppe Parrella, Istituto per la Protezione delle Piante, CNR, Portici (NA), Italy

Presentation 12.
Integration of molecular, ecological, morphological and endosymbiont data: *Pnigalio soemius* complex, a case study
By Maurilia M. Monti, Istituto per la Protezione delle Piante, CNR, Portici (NA), Italy

Presentation 13.
Application of molecular markers for species identification: the Empoascini tribe (Hemiptera: Cicadellidae) as a case-study
By Sabrina Bertin, DISAFA - Entomology and Zoology, Università degli Studi di Torino, Italy

Presentation 14.
Taxon-specific multiplex-PCR: a case study for quick, easy, and accurate identification of parasitoid species attacking soft scale insects
By Maurilia M. Monti, Istituto per la Protezione delle Piante, CNR, Portici (NA), Italy

Presentation 15.
High Resolution Melting in insect taxonomy
By Elena Cardillo, Bio-Rad Laboratories, Milano, Italy

All the pdf of the presentation of the short course on "Insect Molecular Taxonomy"are available on the COST Action FA0807 web site: (http://www.costphytoplasma.eu/WG2.htm)

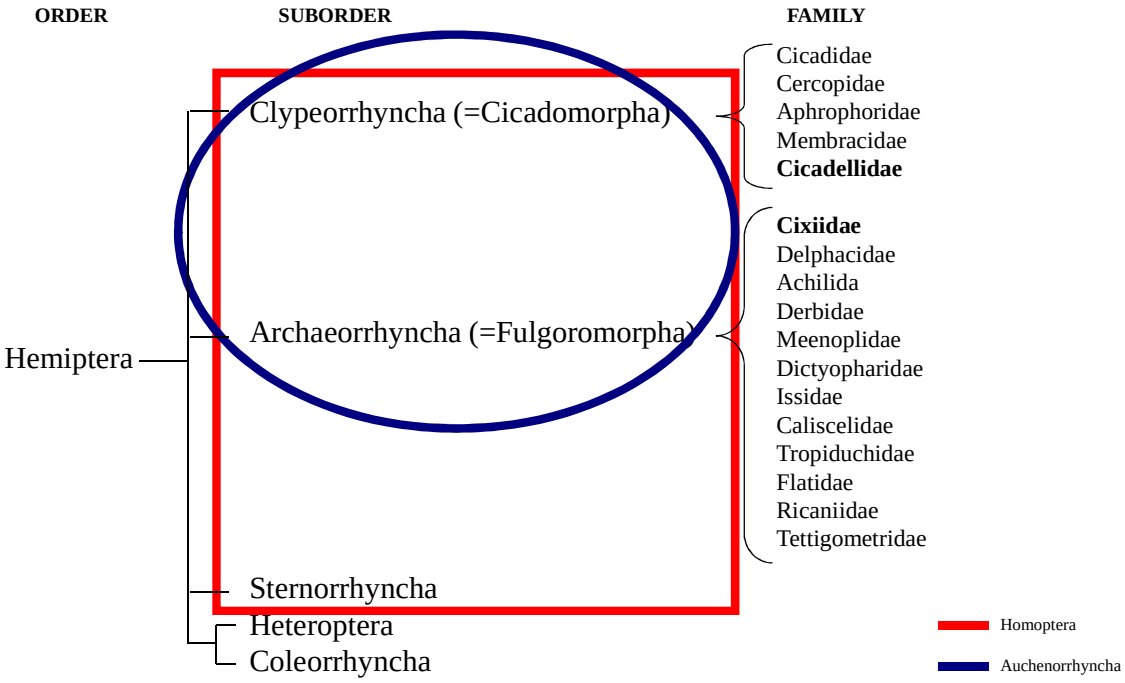
Short course on

"Insect Molecular Taxonomy"

Morphology of Auchenorrhyncha with particular focus on Cixiidae and Deltocephalinae

Luca Picciau
Entomology and Zoology, DISAFA,
Università degli Studi di Torino

Systematics of plant- and leafhoppers

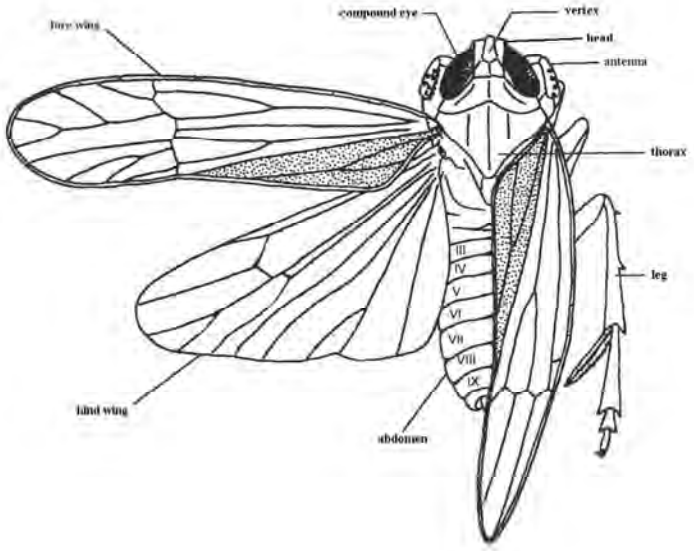


Classification of Hemiptera (According to Biedermann and Niedringhaus, 2004) with particular reference to the European families of Auchenorrhyncha

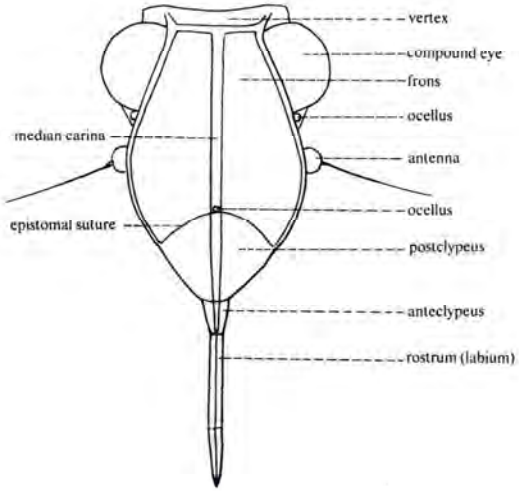
Morphological characteristics

Hemipterans differ, with few exceptions, from all other insects by their mouthparts.

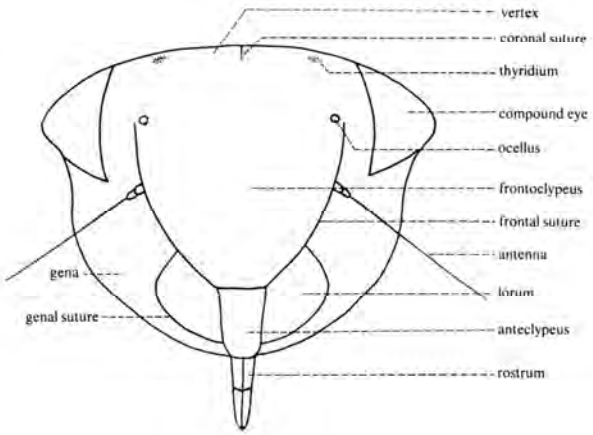
The fore wings are usually larger or at least longer than the hind wings. During flight the wings are usually held together by means of a wing-coupling apparatus.



Head

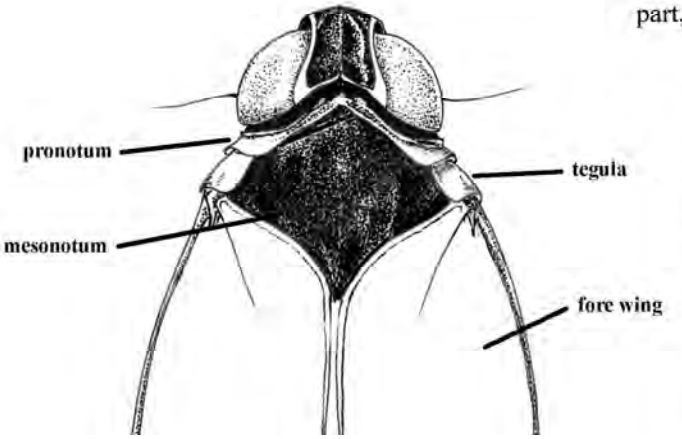


Fulgoromorpha



Cicadomorpha

Thorax

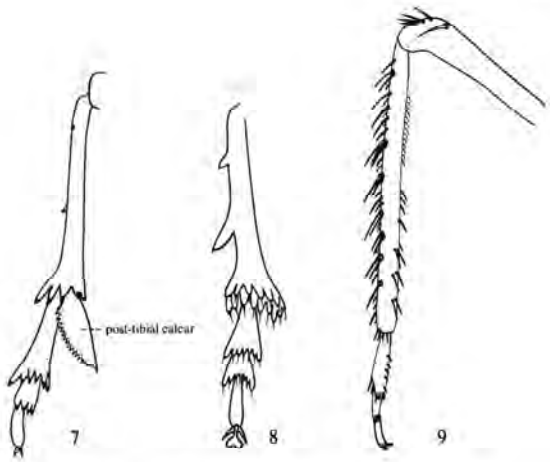


The prothorax, the foremost segment, is usually fairly short. The pronotum, its tergal part, is a transverse plate

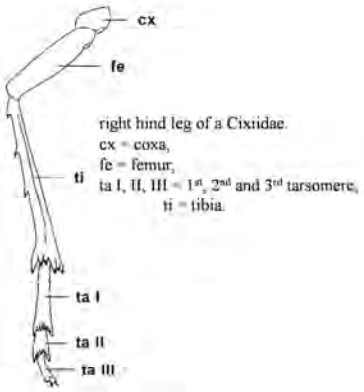
The mesothorax carries the fore wings. The mesonotum, the tergum of the mesothorax, consists of four more or less distinct parts arranged in order from the front: prescutum, scutum, scutellum, and postscutellum

Metathorax, the third thoracal segment, carries the hind wings, and metanotum, its tergal part, is entirely concealed by the wings in repose

Legs



They consist of the same elements as those of other insects.



right hind leg of a Cixiidae.
cx = coxa,
fe = femur,
ta I, II, III = 1st, 2nd and 3rd tarsomere,
ti = tibia.

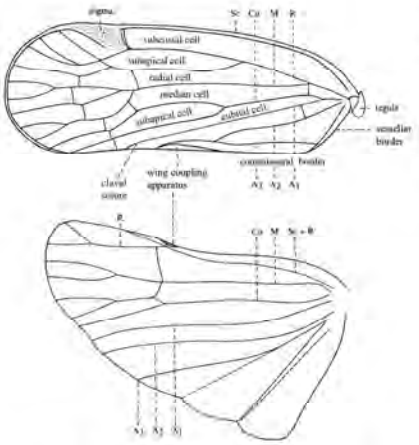
The tibiae are often armed with fixed spines and/or movable setae, the latter usually arranged in longitudinal rows

The hind femora in the Cicadellidae are apically armed with a few strong setae, the number of which is constant within the various taxa, giving a character useful in keys

Wings

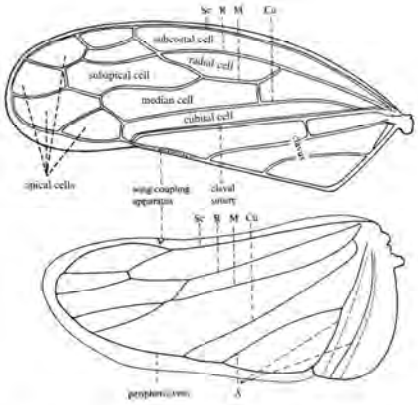
The fore wings may be leathery or membranous. Even in the latter case they are usually somewhat firmer than the hind wings.

The wing-polymorphism is usually a dimorphism.
Brachypterous: short-winged
Macropterous: long-winged



Fore and hind wing of a Cixiidae

The longitudinal veins:
costa (C)
subcosta (Sc)
radius (R)
media (M)
cubitus (Cu)
anales (A)



Fore and hind wing of a Cicadellidae

In the various groups these veins are more or less well developed and ramified, or reduced.

Abdomen

The abdomen shape in most Auchenorrhyncha is usually longish, cylindrical, conical, or with a triangular transverse section.

The first abdominal segment or segments I and II together contains a sound-producing apparatus, the so called **tymbal organ**.

the tymbals: a pair of convex plates
a pair of dorso-ventral muscles, the tymbal muscles.

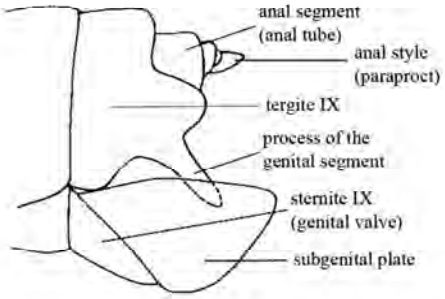
Reptalus melanochaetus: female

Many Auchenorrhyncha are equipped with **wax glands**.

Female Cixiidae have the caudal end enclosed in a white waxy plate

The most important part is the apex which contains the genitalia

The specific identification mainly rely on **male genitalia**



Genital segment of a Cicadomorpha

In fulgoromorpha the tergite IX and sternite IX are melted, together with the pleurae, and form the **pygofer**

Key to the Auchenorrhyncha families of Europe (only adult specimens) from Holzinger *et al*. 2003

Fulgoromorpha
Cixiidae

1 Insertions of the median coxae widely apart from each other. Bases of the fore wings each with a tegula (**FULGOROMORPHA**).……**2**
- Insertions of the median coxae close to each other. Fore wing bases without tegulae (**CICADOMORPHA**)..……14
2 Hind tibia apically with a conspicuous movable spur……...**Delphacidae**
- Hind tibia without a conspicuous movable spur……...3
3 Fore wings large, triangular, distinctly reticulate. Second segment of the hind tarsus apically without spines……**Ricaniidae**
- Fore wings not as above. Second segment of the hind tarsus apically with at least two spines.....……....4
4 Second segment of the hind tarsus apically truncate or concave, bearing a row of spines of more or less equal size……5
- Second segment of the hind tarsus apically convex, with two lateral spines……...10
5 Fore wings strongly reduced, covering at most half of the abdomen, venation barely visible. Hind wings lacking……**Dictyopharidae: Orgeriinae**
- Fore wings covering more than half of the abdomen, often surpassing its tip. Wing venation distinct.……6
6 Head anteriorly strongly elongate (double as long as eyes). Total length usually more than 9 mm. Often green or pink……**Dictyopharidae: Dictyopharinae**
- Head not or only slightly elongate. Body colour neither green nor pink……..7
7 Dorsoventrally flattened. Clavus suture of fore wing not reaching its hind margin. Fore wings in resting position held flat over the body, their apical parts distinctly overlapping……**Achilidae**
- Clavus suture of fore wing reaching its hind margin. Fore wings in resting position held roof-like, apically not (or only slightly) overlapping……...8
8 Basis of the clavus and at least one anal vein densely covered with setiferous tubercles. Fore wings in resting position steep roof-like……..**Meenoplidae**
- Tubercles on fore wing veins evenly scattered or absent……..9
9 Fore wing tips in resting position roof-shaped. Lateral keels of frons not apically (or only laterally) produced. Head laterally without processes. Sclerotization of male genital segment complete .....**Cixiidae**
- Fore wing tips in resting position held almost vertically. Lateral keels of frons apically strongly produced. Head on both sides with subantennal process. Male genital segment laterally membranous, only dorsally and ventrally sclerotized……**Derbidae**
10 (4) Eyes relatively small, laterally not reaching hind margin of the head. Keel between eyes and frons lacking. Body dorsoventrally flattened, body shape in dorsal view more or less ovoid……**Tettigometridae**
- Eyes larger, laterally reaching hind margin of head. Eyes separated from frons by a strong keel. Body not dorso-ventrally flattened……...11
11 Mesonotum posteriorly with a transverse keel……**Tropiduchidae**
- Mesonotum without transverse keel……12
12 Fore wings in resting position steeply roof-shaped, almost vertical. Clavus with numerous setiferous pits. Living specimens covered with flaky wax particles……...**Flatidae**
- Fore wings in resting position less steep. Clavus without setiferous pits.....13
13 Outline in dorsal view rounded to ovoid. Body colouration usually brownishyellow, plain greyish or blackish-brown; markings, if present, more or less irregular. Fore legs not dilatated……...**Issidae**
- Body elongate. Usually with reddish, whitish or dark longitudinal stripes; if longitudinal stripes absent, then fore legs dilatated……**Caliscelidae**

Cicadomorpha Cicadellidae

14 (1) Vertex with three ocelli. Body length usually exceeding 15 mm. Fore legs with thickened femur, ventrally usually with two stiff spines……**Cicadidae**
- No more than two ocelli. Body length usually less than 15 mm. Femur of fore legs not thickened……15

15 Pronotum enlarged, with conspicuous posteriorly directed process……**Membracidae**
- Pronotum usually not enlarged; if enlarged, then without posterior process.....16

16 Hind tibiae in cross section angular, without fixed spines, but with numerous setae. ……..... **Cicadellidae**
- Hind tibiae in cross section rounded, without ridges or keels, more or less evenly pubescent, outer edge with 1-2 strong fixed spines (Cercopoidea)……...17

17 Fore wings red and black (rarely completely black). Head (incl. eyes) clearly narrower than pronotum, black .....**Cereopidae**
- Fore wings without red markings. Head usually not black, as broad as the pronotum……...**Aphrophoridae**

Key to subfamilies of Cicadellidae

1 Lateral carina of hind tibia strongly widened into a thin and broad plate, in European genera armed with a number of teeth on its outside. Ocel\i dorsaI. Pronotum in European genera with two large lobiform processes.…….....….....**Ledriinae**
- Lateral carina of hind tibia not especially broad. Pronotum without processes.....2

2 (1) Apex of hind femur without setae. Genae elevated, well delimited from adjacent lateral parts of face……..**Ulopinae**
- Apex of hind femur with a group of setae.Genae not sharply delimited from adjacent lateral parts of face…….…….3

3 (2) Longitudinal veins of corium distinct even basally. Fore wing with transverse veins also proximally of apical part .....….....4
- Longitudinal veins of corium basally indistinct. Fore wing with transverse veins in apical part only...……..……..**Typhlocybinae**

4 (3) Frontal and epicranial sutures marked by broad carinae..……..…**Megophthalminae**
- Frontal sutures not carinate……..……5

5 (4) Frontoclypeus inflated, its upper part visible from above as a part ofthe upper side ofthe head…….**Cicadellinae**
- Frontoclypeus not partly included in the upper side of the head…….6

6 (5) Face and vertex medially with a strong longitudinal carina. Pronotum on each side with two longitudinal carinae……..….**Dorycephalinae**
- No median carina on vertex, nor on the face……..…7

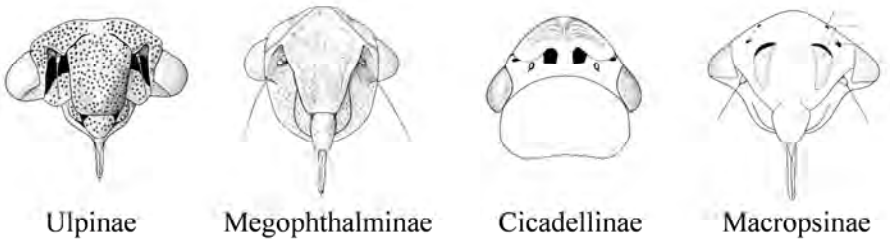
7 (6) Ocelli situated on the face……..…8
- Ocelli situated on the fore border of the head or immediately over or under it…...11

8 (7) Muscle traces on frontoclypeus appearing as two reniform or semilunar spots……..…**Macropsinae**
- Muscle traces on frontoclypeus different……..…….9

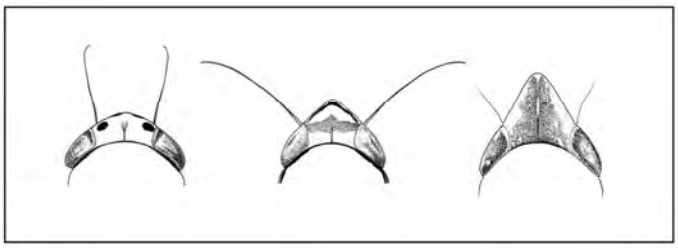
9 (8) Well-marked ridges present above antennae, extending from compound eyes to frontoclypeus. Epicranial suture weak but distinct, semicircular……..……**Iassinae**
- Antennal ridges more or less distinct, not extending from eyes to frontoclypeus...10

10 (9) Frontal sutures distinct. Aptennal ridges direct1y running into the clypeal sutures. Always macropterous…..……..…..**Idiocerinae**
- Frontal sutures absent. Antennal ridges weakly marked……..……**Agalliinae**

11 (7) 9th abdominal sternum in males laterally fused with the pygofer…...**Aphrodinae**
- 9th abdominal sternum in the male laterally touching but not fused with the pygofer, forming the so-called genital valve……..…… .**Deltocephalinae**



Ulpinae Megophthalminae Cicadellinae Macropsinae

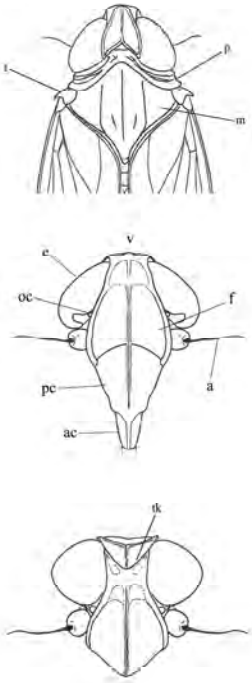


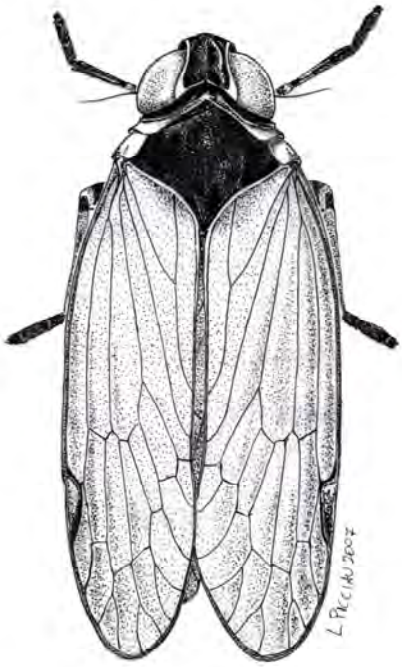
Head of three Deltocephalinae species in dorsal view

Errastunus ocellaris

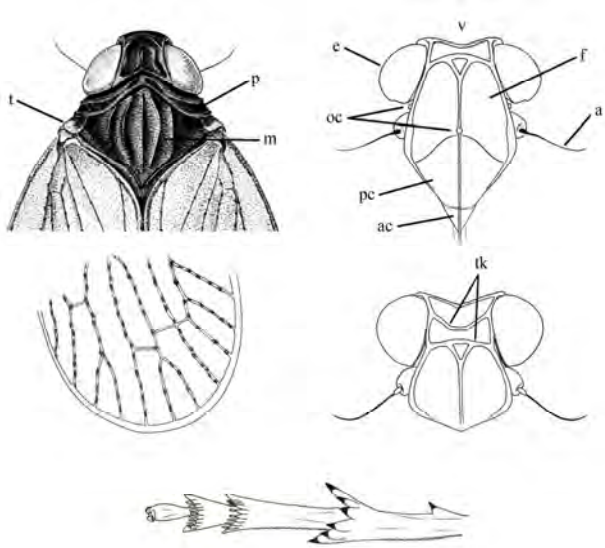
Deltocephalinae: habitus in dorsal view

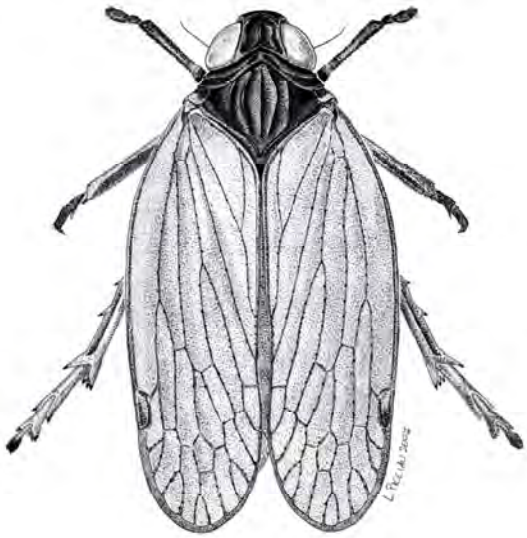
Main morphological features of the genus *Hyalesthes*



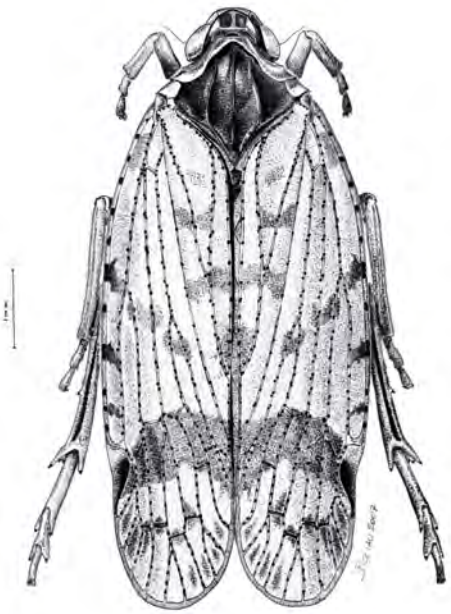


Main morphological features of the genus *Reptalus*





Differantiation between *Reptalus* and *Cixius*



Cixius cunicularius:
habitus in dorsal view

Cixius: mesonotum with 3 keels

Reptalus: mesonotum with 5 keels

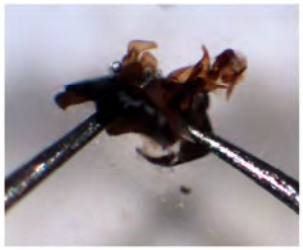
Identification of collected specimens in laboratory

Dissection of male genitalia to reach the species level

DISSECTION

Leave the genital segments macerate into a 10 % KOH solution 8-16 hours
Then wash them in distilled water and preserve them in glycerine

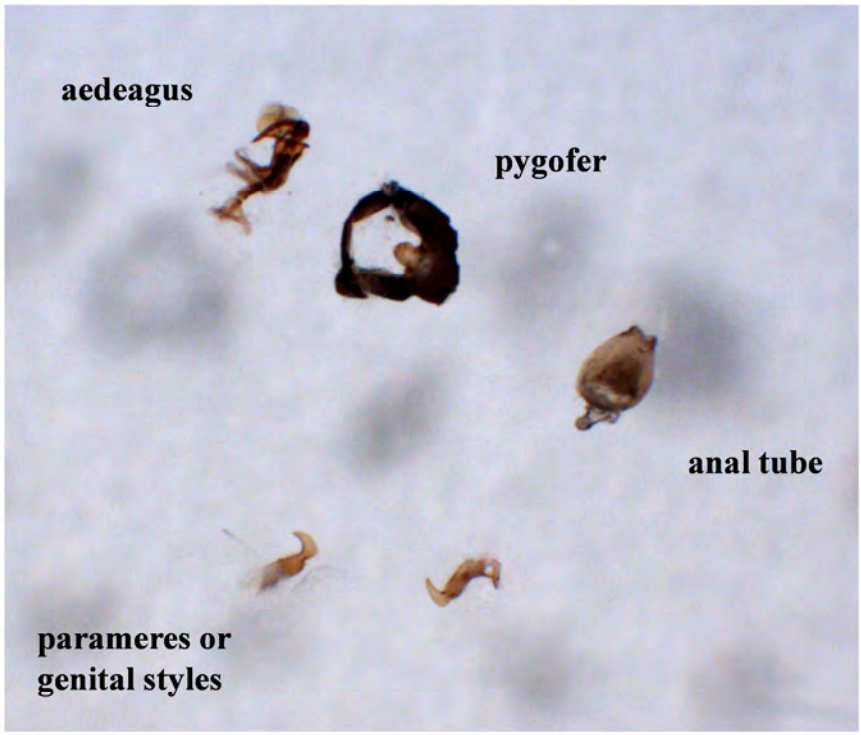
Remove gently the anal tube from the pygofer



Remove gently the aedeagus from the pygofer

Separate the two parameres from the pygofer

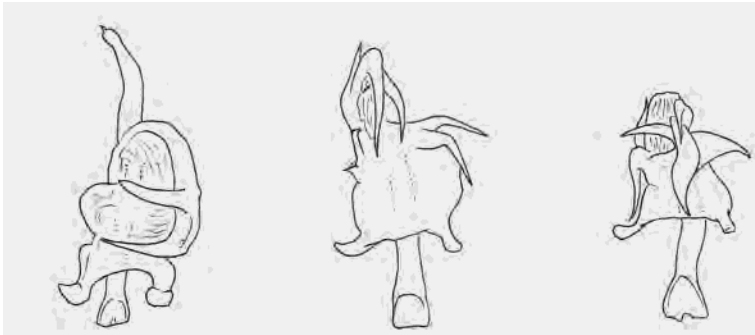
Dissected male genitalia of a cixiid



Male genitalia of three *Hyalesthes* species

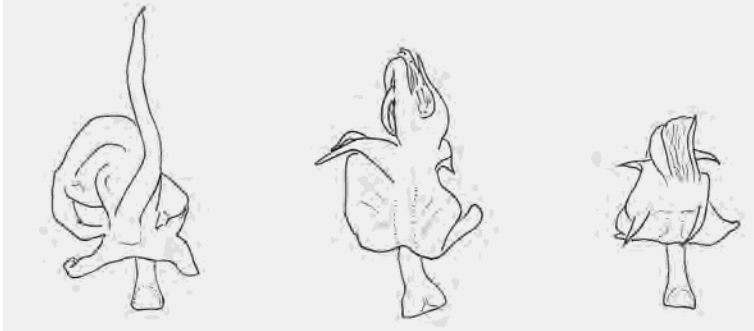
Aedeagus

Dorsal view



H. obsoletus *H. luteipes* *H. scotti*

Ventral view



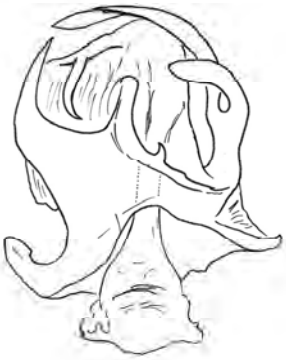
Male genitalia of four *Reptalus* species

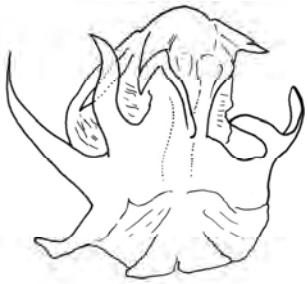
Aedeagus

R. quinquecostatus

R. cuspidatus

R. panzeri





R. melanochaetus

PROTOCOLS

DNA extraction protocol for leaf- and plant-hoppers

Doyle and Doyle protocol, modified by Marzachì et al., 1998 (C. Marzachi, F. Veratti & D. Bosco 1998. Direct PCR detection of phytoplasmas in experimentally infected insects. *Annals of Applied Biology* 133 (1): 45–54).

1) Grind single adult leafhopper in 500 µl of CTAB buffer using sterile carborundum or quartz sand and a micropestle in a 1.5-ml Eppendorf tube.
   (CTAB buffer: 2%w/v CTAB, cetyl-trimethyl-ammonium-bromide; 1.4M NaCl; 20mM EDTA pH 8.0; 100 mM Tris-HC1 pH 8.0; 0.2% mercaptoethanol).
2) Vortex the suspension and incubate it for 30 min at 60°C.
3) Centrifuge for 5 min at 12000-13000 rpm at room temperature. Transfer the supernatant in a new 1.5-ml Eppendorf tube.
4) Add 1 volume of chloroform-isoamyl alcohol (24: 1). Invert repeatedly.
5) Centrifuge for 5 min at 12000-13000 rpm at room temperature.
6) Transfer the supernatant in a new 1.5-ml Eppendorf tube. (If the supernatant contains contaminants, repeat steps 4-5). Add 1 volume of cold isopropanol (-20°C). Invert repeatedly.
7) Centrifuge for 20 min at 12000-13000 rpm at 4°C (refrigerated centrifuge). Discard the solution.
8) Wash the pellet with cold 70% ethanol (-20°C), centrifuge for 8-10 min at 12000-13000 rpm at 4°C. Discard the ethanol.
9) Dry the pellet in vacuum and re-suspend it in 30-100µl of TE or sterile water.

PCR protocol for the amplification of the mitochondrial COI gene

Primer pair:

C1-J-2195: 5'-TTGATTTTTTGGTCATCCAGAAGT-3' (anneals within COI sequence)

L2-N-3014: 5'-TCCAATGCACTAATCTGCCATATTA-3'(anneals within tRNA-leu sequence, following the COI gene).

The primers are from Simon et al., 1994 (Simon C., Frati f., Beckenbach A., Crespi B., Liu H., Flook P. 1994. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annales of Entomological Society of America* 87: 651-701).

Expected amplicon lenght: 890 – 920 bp.

PCR cycles:

1 cycle: 94°C for 5'

35 cycles: 94°C for 30'', 52°C for 45'', 72°C for 1'

1 cycle 72°C for 10'

PCR mix:

REAGENTS			
	Initial concentration	Final concentration	Vol X 1 sample
Reaction buffer (tris-HCl; KCl)	10X	1X	2.5µl
$MgCl_2$	50mM	2.5mM	1.25µl
dNTPs	2mM	200µM	2.5µl
Primer C1-J-2195	5µM	0.5µM	2.5µl
Primer L2-N-3014	5µM	0.5µM	2.5µl
Taq Polymerase	5U/ µl	1U	0.2µl
H_2O			12.55µl
Reaction volume			24µl
DNA template			1µl
Total reaction volume:			25µl

Electrophoresis: 1% (w/v) agarose gel.

PCR protocol for the amplification of the ribosomal ITS2 region

Primer pair:

ITS2fw: 5'-TGTGAACTGCAGGACACATG-3' (anneals on 5.8S gene)

ITS2rv: 5'-ATGCTTAAATTTAGGGGGTA-3' (anneals on 28S gene).

The primers are from Collins & Paskewitz, 1996 (Collins F.H. & Paskewitz S.M. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic Anopheles species. *Insect Molecular Biology* 5: 1–9).

PCR cycles:

1 cycle: 94°C for 5'

35 cycles: 94°C for 30'', 56°C for 45'', 72°C for 1'

1 cycle 72°C for 10'

PCR mix:

REAGENTS			
	Initial concentration	Final concentration	Vol X 1 sample
Reaction buffer (tris-HCl; KCl)	10X	1X	2.5µl
$MgCl_2$	50mM	1.5mM	0.75µl
dNTPs	2mM	200µM	2.5µl
Primer ITS2fw	5µM	0.5µM	2.5µl
Primer ITS2rv	5µM	0.5µM	2.5µl
Taq Polymerase	5U/ µl	1U	0.2µl
H_2O			13.05µl
Reaction volume			24µl
DNA template			1µl
Total reaction volume:			25µl

Electrophoresis: 1% (w/v) agarose gel.

Purification of PCR products from PCR tube or gel

(PureLink™ Purification Kit - Invitrogen, Carlsbad)

Gel extraction protocol

1) Excise the area of the gel containing your desired DNA fragment using a clean, sharp razor blade. Minimize the amount of agarose surrounding the DNA fragment.

Weigh the gel slice containing the DNA fragment. Place the gel slice into a 1.5-mL microcentrifuge tube.

Add 3 volumes of Gel Solubilization Buffer (L3) for every 1 volume of gel (e.g., add 1.2 mL Gel Solubilization Buffer for a 400-mg gel slice).

Incubate the tube at 50°C (water bath or heat block) for at least 10 minutes. Invert the tube every 3 minutes to ensure complete gel dissolution.

After the gel slice appears dissolved, incubate the tube for an additional 5 minutes.

For optimal DNA yields, add 1 gel volume isopropanol to the dissolved gel slice (e.g., add 400 µL isopropanol for a 400-mg gel slice). Mix well.

PCR purification protocol

1) Add 4 volumes of Binding Buffer (B2) to 1 volume of PCR reaction (50–100 µL). Mix well.

2) Add sample to the PureLink® Spin Column. Centrifuge the PureLink® Spin Column at room temperature at 10,000 × *g* for 1 minute. Discard the flow through and replace the PureLink® Spin Column into the Wash Tube.

3) Add 650 µL Wash Buffer with ethanol to the PureLink® Spin Column. Centrifuge the PureLink® Spin Column at room temperature at 10,000 × *g* for 1 minute. Discard the flow-through from the Wash Tube and replace the PureLink® Spin Column into the tube.

4) Centrifuge the PureLink® Spin Column at maximum speed at room temperature for 2–3 minutes to remove any residual Wash Buffer. Discard the Wash Tube. Place the PureLink® Spin Column in a clean 1.7-mL PureLink® Elution Tube (supplied with the kit).

5) Add 50 µL Elution Buffer (E1) or sterile, distilled water (pH >7.0) to the center of the PureLink® Spin Column. Incubate the PureLink® Spin Column at room temperature for 1 minute. Centrifuge the PureLink® Spin Column at maximum speed for 1 minute. Remove and discard the PureLink® Spin Column. The recovered elution volume is ~48 µL.

Restriction Fragment Length Polymorphism (RFLP) analyses: enzymatic digestions

- **Enzyme *Alu*I** (Promega): incubation at 37°C for 2 h (at least).

Reaction mix:

REAGENTS			
	C_i	C_f	Vol X 1 sample
Reaction buffer (tris-HCl; KCl)	10X	1X	1.5µl
Enzyme	10U/µl	1U	0.1µl
H_2O			8.4µl
Reaction volume			10µl
+ DNA template			5µl
Total reaction volume:			15µl

- **Enzyme *Taq*I** (Promega): incubation at 65°C for 2 h (at least).

Reaction mix:

REAGENTS			
	C_i	C_f	Vol X 1 sample
Reaction buffer (tris-HCl; KCl)	10X	1X	1.5µl
Enzyme	10U/µl	1U	0.1µl
H_2O			8.4µl
Reaction volume			10µl
+ DNA template			5µl
Total reaction volume:			15µl

Electrophoresis: on 2% (w/v) 1X TBE agarose gels (MetaPhore agarose for resolution of small nucleic acids; Cambrex) at 70V.

LIST OF WEB SITES AND SOFTWARE FOR NUCLEOTIDE SEQUENCE ANALYSES, *IN SILICO* RFLP ASSAYS AND PHYLOGENETIC INFERENCES

Software displaying and managing the chromatogram files obtained from nucleotide sequencing:

ChromasLite: it opens chromatogram files form Applied Biosystem and Amershame MegaBace DNA sequencers and SCF format chromatogram files created by ALF, Li-Cor, Visible Genetics OpenGene, Beckman CEQ 2000XL and CEQ 8000, and other sequencers; it exports sequences in plaint text or FASTA format for pasting into other applications (i.e. sequence alignments); it reverse and complement the sequence and chromatogram. It can be freely downloaded at http://chromas-lite.findmysoft.com/ .

ChromasPro: it is suitable for sequencing projects up to a few megabases, and basic sequence editing and analysis. It has further features over ChromasLite: it assembles overlapping sequences into a consensus and automatically displays ambiguities for editing; it generates restriction site and fragment maps, and list cutter and non-cutters enzymes; it maps open reading frames, aided by a G+C frame plot, and translate ORFs; it performs nucleotide and protein BLAST searches through the NCBI web site; it displays translations when editing nucleotide sequences. It is a pay program.

Software and web sites for multiple nucleotide sequence alignments (inputs must be in FASTA format):

ClustalX, ClustalW and ClustalOmega: ClustalW is the command-line version whereas ClustalX is the graphical version of the Clustal programs. ClustalOmega is the latest Clustal program and it offers a significant increase in scalability over previous versions, allowing hundreds of thousands of sequences to be aligned in only a few hours. ClustalOmega is currently a command line-only tool. The three versions can be freely downloaded at http://www.clustal.org/download/current/ or run on two web servers: EBI web server (http://www.ebi.ac.uk) and Swiss Institute of Bioinformatics (http://www.isb-sib.ch/).
More recent papers for reference:

Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J., Higgins D.G. (2007). Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948.
Sievers F., Wilm A., Dineen D.G., Gibson T.J., Karplus K., Li W., Lopez R., McWilliam H., Remmert M., Söding J., Thompson J.D., Higgins D.G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular Systems Biology 7: 539.

BioEdit: BioEdit is a fairly comprehensive sequence alignment and analysis tool. BioEdit supports a wide array of file types and offers a simple interface for local BLAST searches. Free at http://www.mbio.ncsu.edu/BioEdit/bioedit.html
Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Opal: it can be freely downloaded at http://opal.cs.arizona.edu .
Wheeler T.J. and Kececioglu J.D. (2007). Multiple alignment by aligning alignments. Proceedings of the 15th ISCB Conference on Intelligent Systems for Molecular Biology, Bioinformatics 23: i559-i568.

Muscle: it can be freely downloaded or run on the web servers: EBI web server (http://www.ebi.ac.uk) and Swiss Institute of Bioinformatics (http://www.isb-sib.ch/).
Edgar R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5): 1792-1797.

T-coffee: it runs on the web server: http://tcoffee.crg.cat/ .
Notredame, Higgins, Heringa (2000). T-Coffee: A novel method for multiple sequence alignments. Journal of molecular Biology 302: 205-217.

Software and web sites simulating *in silico* the enzyme digestion of DNA sequences and virtually reproducing RFLP profiles:

Webcutter: it runs on the web server http://bio.lundberg.gu.se/cutter2/ . It generates restriction site and fragment maps, and list cutter and non-cutters enzymes.

NEBcutter: it runs on the web server http://tools.neb.com/NEBcutter2/ . It generates restriction site and fragment maps, and list cutter and non-cutters enzymes.
Vincze T., Posfai J. and Roberts R.J. (2003). NEBcutter: A program to cleave DNA with restriction enzymes. Nucleic Acids Research 31: 3688-3691.

WatCut: it runs on the web server http://watcut.uwaterloo.ca/ . It generates restriction site and fragment maps, and list cutter and non-cutters enzymes.

Restriction Site Analysis: it runs on the web server http://biotools.umassmed.edu. It generates restriction site and fragment maps, and list cutter and non-cutters enzymes. It provides also a considerable choice output format, including pseudo gel maps.

pDRAW32: it can be freely downloaded at http://www.acaclone.com/. It provides also pseudo-gel maps.

Software for phylogenetic and population analyses:

DnaSP 5: a software package freely distributed at http://www.ub.es/dnasp/. For the analysis of nucleotide polymorphism from aligned DNA sequence data. DnaSP can estimate several measures of DNA sequence variation within and between populations (in noncoding, synonymous or nonsynonymous sites), as well as linkage disequilibrium, recombination, gene flow and gene conversion parameters.
Librado P, Rozas J (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.

MEGA 5: it is freely provided at http://www.megasoftware.net/mega.php. MEGA is an integrated tool for conducting automatic and manual sequence alignment, inferring phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses.
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731-2739.

PAUP: Phylogenetic Analysis Using Parsimony. PAUP is a program for inferring phylogenies from discrete-character data under the principle of maximum parsimony.
Swofford, D. L. (2003). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

BEAST: (Bayesian Evolutionary Analysis by Sampling Trees). **It** is freely downloadable at http://beast.bio.ed.ac.uk/Main_Page#Downloads . BEAST is a program for Bayesian Markov chain Monte Carlo (MCMC) analysis of molecular sequences. It is oriented towards inferring rooted, time-measured phylogenies using strict or relaxed molecular clock models. It can be used as a method of reconstructing phylogenies but is also a framework for testing evolutionary hypotheses without conditioning on a single tree topology.
Suchard MA & Rambaut A (2009). Many-Core Algorithms for Statistical Phylogenetics. Bioinformatics 25: 1370-1376.

MrBayes: **it** is freely downloadable at http://mrbayes.sourceforge.net/manual.php. It is a program for the Bayesian estimation of phylogeny. Bayesian inference of phylogeny is based upon a quantity called the posterior probability distribution of trees, which is the probability of a tree conditioned on the observations. MrBayes uses a simulation technique called Markov chain Monte Carlo (MCMC) to approximate the posterior probabilities of trees.

jModeltest: it is freely downloadable at http://darwin.uvigo.es/software/jmodeltest.html . It carries out statistical selection of best-fit models of nucleotide substitution. It implements different model selection strategies: hierarchical and dynamical likelihood ratio tests (hLRT and dLRT), Akaike and Bayesian information criteria (AIC and BIC).
Posada D. (2008). jModelTest: Phylogenetic Model Averaging. Molecular Biology and Evolution 25: 1253-1256.

RAxML: a program for faster reconstruction of phylogenies by maximum likelihood. It provides faster heuristic search, use of parallel processing, and a simulated annealing algorithm. RAxML can also carry out parsimony, bootstrapping, and consensus tree methods. The programs are available as C source code, Windows executables, and Mac OS X executables at the Exelexis Lab software web page at http://sco.h-its.org/exelixis/software.html
Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690.

PHYLIP: PHYLogeny Inference Package. It is a free package of programs for inferring phylogenies. The site is http://evolution.gs.washington.edu/phylip.html and it includes programs to carry out parsimony, distance matrix methods, maximum likelihood, and other methods on a variety of types of data, including DNA and RNA sequences, protein sequences, restriction sites, 0/1 discrete characters data, gene frequencies, continuous characters and distance matrices
Felsenstein, J. (1989). PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166.

TreeView: provides a simple way to view the contents of a NEXUS, PHYLIP, or other format tree file. It can draw rooted and unrooted trees, display bootstrap values, and edit trees by moving branches, collapsing them, and rerooting. The program is free, and can be obtained from http://taxonomy.zoology.gla.ac.uk/rod/treeview.html .

Arlequin: free at http://cmpg.unibe.ch/software/arlequin35/Arl35Downloads.html .
It works in population genetics with quite a large set of basic methods and statistical tests, in order to extract information on genetic and demographic features of a collection of population samples.
Excoffier, L. and H.E. L. Lischer (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources. 10: 564-567.

A more complete list of molecular biology software can be found at: http://evolution.genetics.washington.edu/phylip/software.html .