Tecniche molecolari in uso per la caratterizzazione ed identificazione di artropodi di interesse economico





Identificazione morfologica





- Identificazione morfologica:
 - uso di chiavi dicotomiche

 consultazione di esperti

Problemi identificazione morfologica

- alcuni taxa sono privi di chiavi di riconoscimento
- alcune chiavi escludono i gruppi più difficili
- chiavi sulle larve si basano spesso sull' ultimo stadio larvale
- a volte troppo complesse o poco chiare



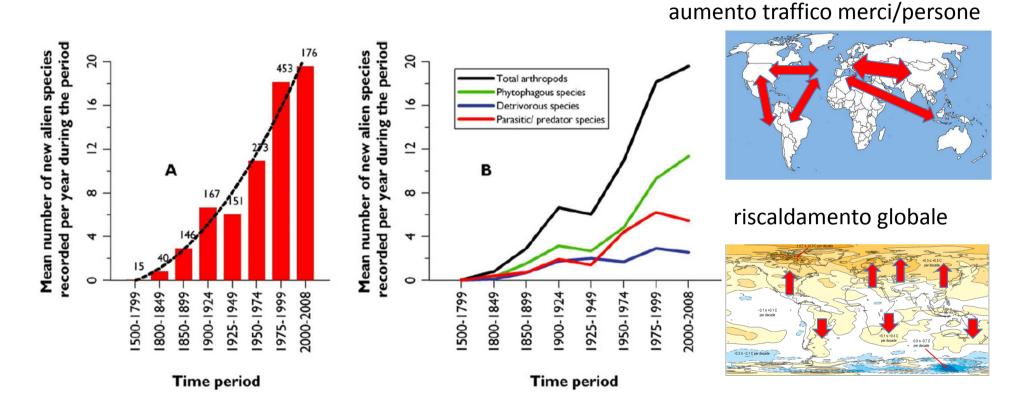


In alcuni casi (ad es. larve di insetti di nuova introduzione) per un' identificazione certa della specie bisogna ricorrere all'allevamento fino al raggiungimento dello stadio adulto.

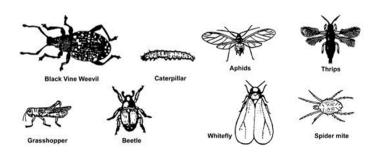


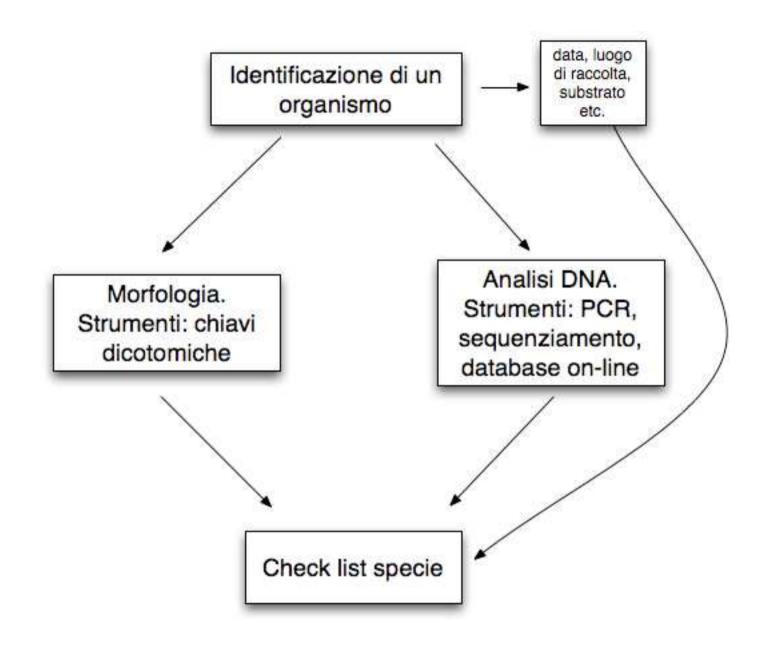


Specie invasive



Roques et al. 2010, Alien terrestrial arthropods of Europe





Tecniche di analisi genetica più utilizzate per l'identificazione degli insetti

Marcatori codominanti:

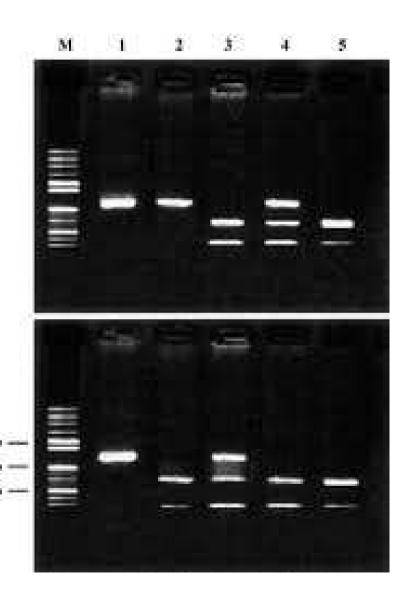
- Allozimi
- PCR-RFLP (Restriction Fragments Length Polymorphism)
- Microsatelliti
- PCR e sequenziamento diretto

Marcatori dominanti:

- RAPD (Random Amplification of Polymorphic DNA)
- AFLP

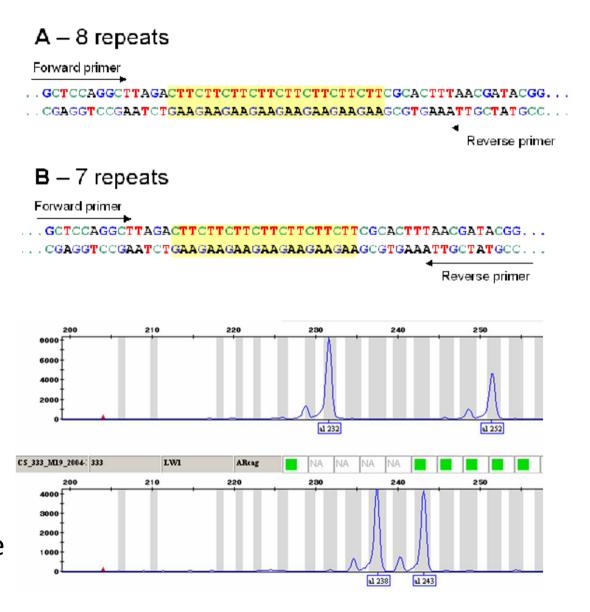
PCR-RFLP

- Amplificazione del gene da analizzare mediante PCR
- Analisi del frammento amplificato mediante restrizione con enzimi di restrizione
- Tecniche relativamente semplice e rapida
- marcatore codominante
- screening di mutazioni già note

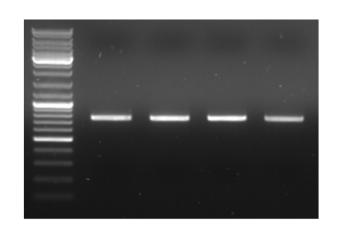


Microsatelliti (SSR: Simple Sequence Repeat)

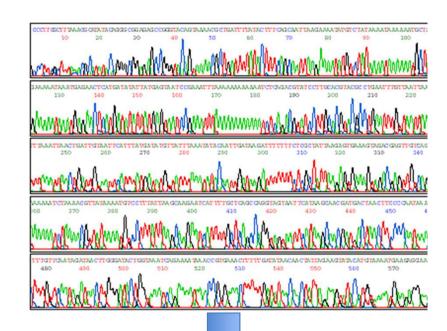
- Alto tasso di mutazione dovuto a inserzioni o delezioni
- Polimorfismo molto elevato
- Numero ripetizioni visualizzato su gel ad alta risoluzione
- Ottima riproducibilità
- Marcatori codominanti
- Messa a punto lunga e difficile
- Non esportabile ad altre specie



PCR e sequenziamento





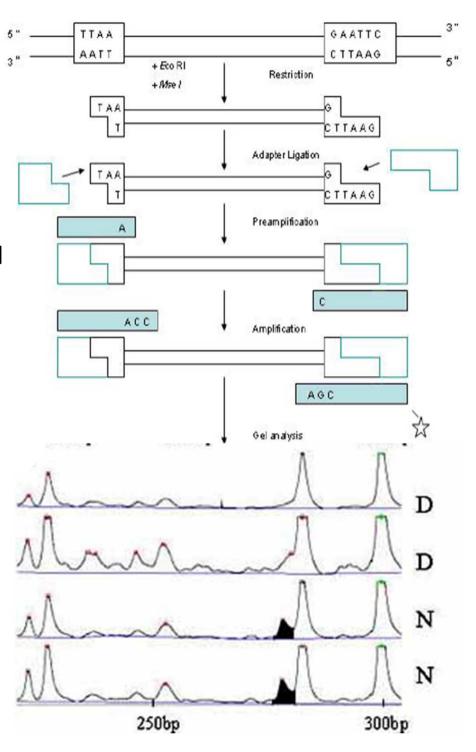


>ph_x_lco1490¶

AFLP

(Amplified Fragment Length Polymorphism)

- Digestione con enzimi di restrizione del genoma
- Ligazione con adattori
- Amplificazione con primer disegnati su adattatori con basi selettive al 3'
- Separazione su gel ad alta risoluzione
- Numero di marcatori molto elevato
- Buona riproducibilità
- Applicabile a molte specie diverse
- Marcatore dominante



Analisi classica mediante PCR in laboratorio: tempi

Estrazione DNA

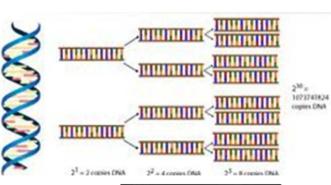
Amplificazione (PCR)
 frammento DNA target

 Visualizzazione risultati PCR

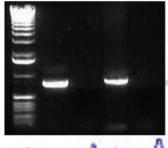
 Ulteriori analisi (restrizione/sequenziamen to)



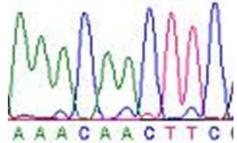
2-12 ore



2 ore



1 ora



4-48 ore

Analisi classica mediante PCR in laboratorio: strumentazione

Estrazione





PCR





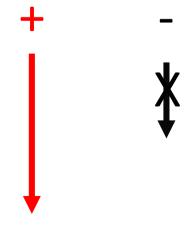


Metodi molecolari di identificazione rapida

Estrazione DNA/RNA



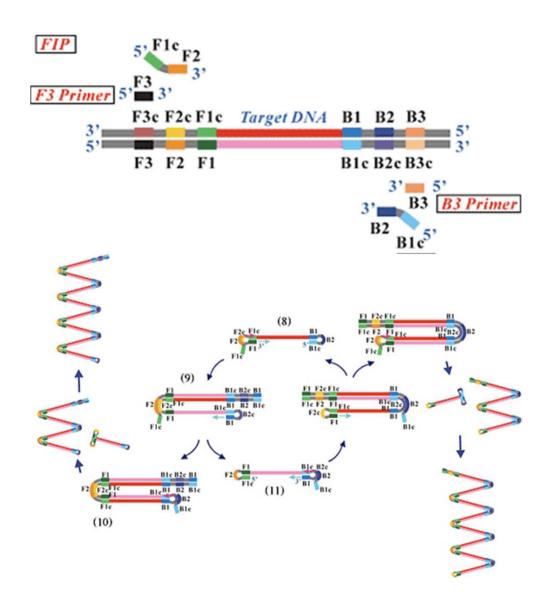
Screening rapido preliminare



Test specifici in laboratorio

LAMP – Loop mediated Isothermal Amplification (http://loopamp.eiken.co.jp/e/)

- Amplificazione a temperatura costante
- Elevata efficienza e stabilità della reazione: 109- 1010 copie del target in 10- 40 minuti
- Elevata specificità (4-6 primer invece di 2)

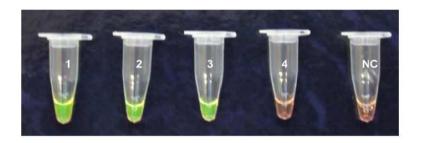


LAMP: ulteriori caratteristiche

- efficienza di amplificazione simile a PCR nested
- reazione meno soggetta ad inibizione da eventuali sostanze contaminanti
- reagenti più stabili (non è necessario frigo o freezer durante trasporto)
- non richiede utilizzo di apparecchiature di laboratorio per la visualizzazione dei risultati

Risultati rilevabili tramite fluorescenza, precipitazione o viraggio

Fluorescenza



Torbidità



Metodo colorimetrico



Realizzabile direttamente in campo grazie ad uno strumento portatile (Genie II)



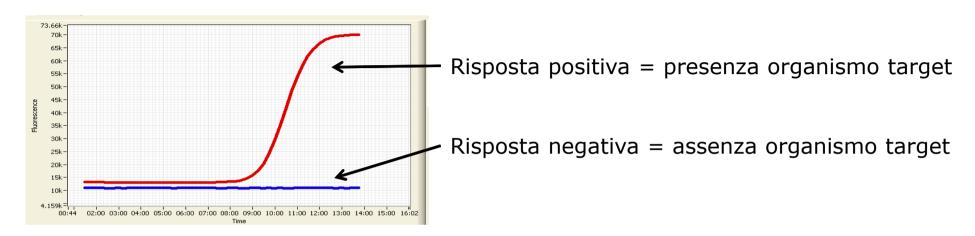
amplificazione e visualizzazione possono essere combinati in un unico strumento:

Genie II

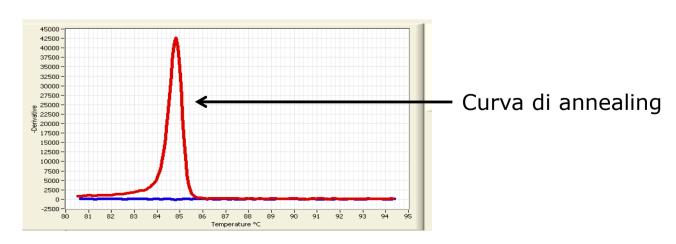


- 16 pozzetti
- touch screen e possibilità visualizzazione risultati in tempo reale
- funziona senza bisogno di PC
- batteria ricaricabile con autonomia di diverse ore

 Con un'unica reazione di ~ 30min è possibile visualizzare la presenza/assenza dell'organismo target



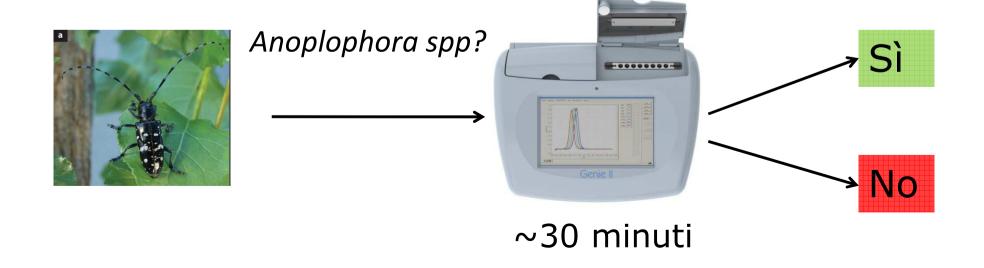
 La specificità della reazione può essere controllata confrontando la curva di annealing del campione con quella di un campione positivo di riferimento



Progetto in corso:

messa a punto e validazione delle sonde LAMP per:

• identificazione specie del genere *Anoplophora (A. glabripennis, A. chinensis)*



Anoplophora glabripennis/chinensis



larve

adulto : a) *A. glabripennis* b) *A. chinensis*

uovo

Geni utilizzati come marcatori molecolari

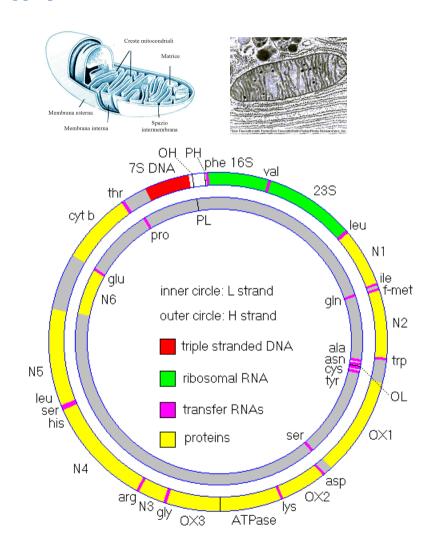
 Modalità di trasmissione DNA (in organismi diploidi a riproduzione sessuale):

- biparentale (cromosomi autosomici)
- uniparentale (mtDNA, cloroplasti, cromosoma Y)

Geni utilizzati come marcatori: mtDNA

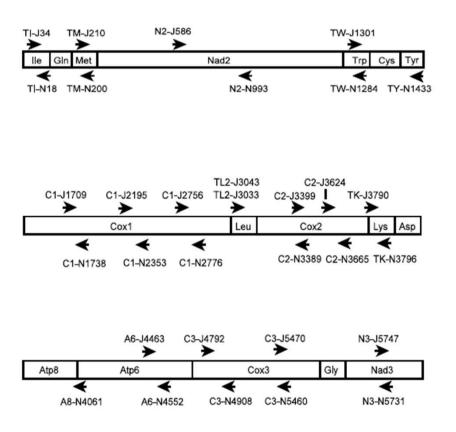
- Geni mtDNA: 16S, citocromo ossidasi 1 (cox1; regione barcode) e 2 (cox2)
 - Ereditarietà materna
 - Assenza di ricombinazione
 - Elevato numero di copie

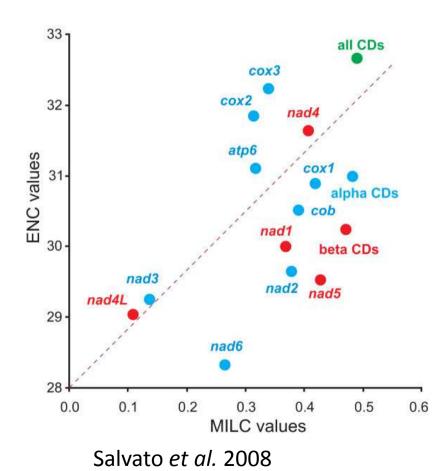
- Problemi: pseudogeni mitocondriali (numts)



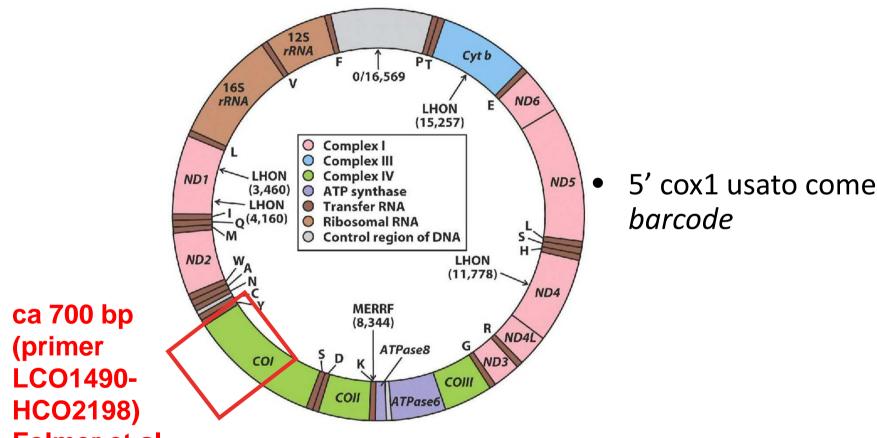
Marcatori mtDNA

- disponibili diverse coppie di primer universali per artropodi (Simon et al. 2006)
- Tasso mutazione variabile tra diversi geni mtDNA





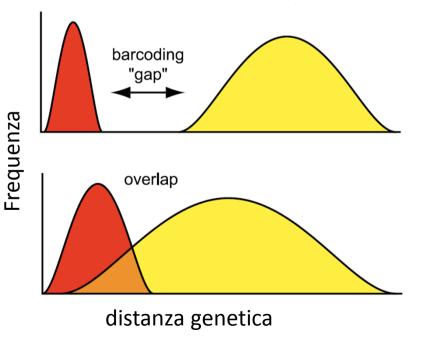
Marcatori mtDNA: regione 'barcode'



Folmer et al. 1994

Caratteristiche delle regioni di DNA 'barcode'

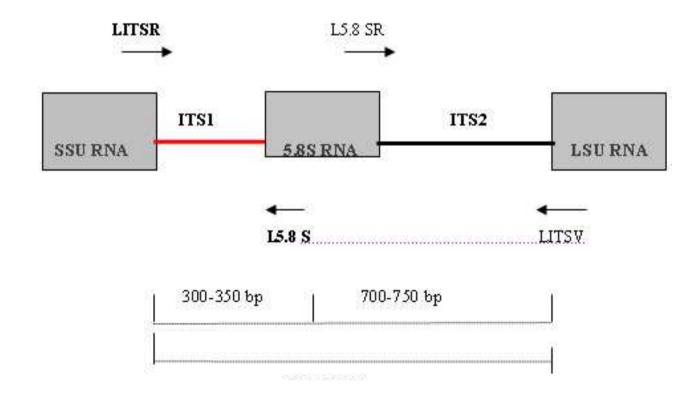
- Regioni di DNA polimorfiche
- devono essere tra regioni conservate di DNA per poter progettare primer universali
- Facilità di amplificazione/sequenziamento
- bassa variabilità <u>intra</u>specifica
- buona variabilità <u>inter</u>specifica



Geni utilizzati come marcatori: geni nucleari

-Geni DNA nucleare:

- -ITS (Internal Trascribed Spacer) 1 e 2
- -EF (Elongation Factor)

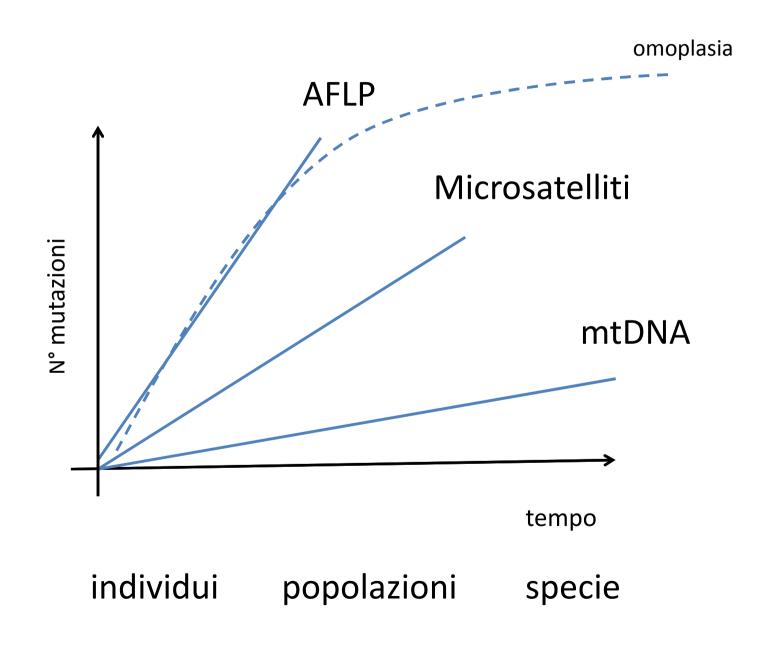


Come scegliere tecnica/marcatori adatti?

- Fondi disponibili
- Condizioni di partenza del campione (DNA + o degradato)
- Informazioni già disponibili nei database on line

- Grado di risoluzione:
 - Scala geografica
 - Scala temporale

Grado di risoluzione dei marcatori genetici



Database on-line

• NCBI (http://www.ncbi.nlm.nih.gov) GenBank



EMBL-EBI (http://www.ebi.ac.uk)



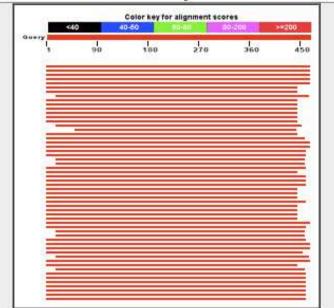
BOLD Systems (http://www.boldsystems.org)



Database BARCODE

Distribution of 100 Blast Hits on the Query Sequence

Mouse over to see the defline, click to show alignments



GenBank

Descriptions

Legend for links to other resources: UnliGene GCO Gene Structure Map Viewer PubChem BioAssay

Sequences producing significant alignments:

BLAST

(Basic Local

Search Tool)

Alignment

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AY171418.1	Diabrotica undecimpunctata COI gene, partial cds; mitochondrial gene fe	765	765	100%	0.0	97%	
AY533573.1	Diabrotica undecimpunctata howardi cytochrome oxidase subunit I (COI	760	760	100%	0.0	96%	
AY533572.1	Diabrotica undecimpunctata duodecimnotata cytochrome oxidase subun	760	760	100%	0.0	96%	
AF278555.1	Diabrotica undecimpunctata howardi cytochrome oxidase subunit I (COI	760	760	100%	0.0	96%	
F278556.1	Diabrotica undecimpunctata undecimpunctata cytochrome oxidase subu	756	756	100%	0.0	96%	
HQ582484.1	Coleoptera sp. BOLD: AAF8473 voucher 09BBCOL-1310 cytochrome oxid	744	744	95%	0.0	97%	
Q582721.1	Coleoptera sp. BOLD: AAF8473 voucher 09BBCOL-0442 cytochrome oxid	744	744	95%	0.0	97%	
Y242473.1	Diabrotica undecimpunctata howardi isolate JJG370 cytochrome oxidase	744	744	96%	0.0	97%	
Q984188.1	Coleoptera sp. BOLD: AAF8473 voucher 10BBCOL-0610 cytochrome oxid	740	740	95%	0.0	96%	
IQ582726.1	Coleoptera sp. BOLD; AAF8473 voucher 09BBCOL-0448 cytochrome oxid	740	740	95%	0.0	96%	
Q582725.1	Coleoptera sp. BOLD: AAF8473 voucher 09BBCOL-0447 cytochrome oxid	740	740	95%	0.0	96%	
HQ582722.1	Coleoptera sp. BOLD: AAF8473 voucher 09BBCOL-0444 cytochrome axid	740	740	95%	0.0	96%	
IQ582577.1	Coleoptera sp. BOLD: AAF8473 voucher 10BBCOL-0061 cytochrome oxid	740	740	95%	0.0	96%	
IQ582727.1	Coleoptera sp. BOLD:AAF8473 voucher 09BBCOL-0449 cytochrome oxid	735	735	95%	0.0	96%	
AY242451.1	Gynandrobrotica nigrofasciata isolate JJG152 cytochrome oxidase subun	733	733	93%	0.0	97%	

Diabrotica undecimpunctata COI gene, partial cds; mitochondrial gene for mitochondrial product

301 qttqttatta caqcaqtttt actactatta tcattacctq ttcttqccqq qqcaattaca 361 atattattaa cagategtaa tetaaatace tetttttttq ateetgetgg aggtggtgat

421 cetattttat ateaacattt attttgattt tteggacate et

77

GenBank: AY171418.1

GenBank FASTA Graphics Go to: ☑ linear INV 29-JAN-2003 LOCUS AY171418 DNA 462 bp DEFINITION Diabrotica undecimpunctata COI gene, partial cds; mitochondrial gene for mitochondrial product. ACCESSION AY171418 VERSION AY171418.1 GI:28133439 KEYWORDS SOURCE mitochondrion Diabrotica undecimpunctata ORGANISM Diabrotica undecimpunctata Eukarvota: Metazoa: Arthropoda: Hexapoda: Insecta: Ptervgota: Neoptera; Endopterygota; Coleoptera; Polyphaga; Cucujiformia; Chrysomeloidea; Chrysomelidae; Galerucinae; Diabroticina; Diabroticites: Diabrotica. REFERENCE 1 (bases 1 to 462) Articolo scientifico in cui AUTHORS Kim, J., Kjer, K.M. and Duckett, C.N. TITLE Comparison between molecular and morphological-based phylogenies of galerucine/alticine leaf beetles (Coleoptera:Chrysomelidae) questa sequenza è stata JOURNAL Insect Syst. Evol. (2003) In press REFERENCE 2 (bases 1 to 462) AUTHORS Kim, J., Kjer, K.M. and Duckett, C.N. pubblicata TITLE Direct Submission JOURNAL. Submitted (31-0CT-2002) Entomology, Rutgers, Cook College, 93 Lipman Dr., Blake Hall, New Brunswick, NJ 08901-8524, USA FEATURES Location/Oualifiers 1..462 source /organism="Diabrotica undecimpunctata" /organelle="mitochondrion" /mol type="genomic DNA" /db xref="taxon:50387" <1...>462 gene (località in cui è stato /gene="COI" <1..>462 CDS /gene="COI" raccolto il campione) /codon_start=1 /transl_table=5 /product="cytochrome c oxidase subunit I" /protein id="AA026246.1" /db xref="GI:28133440" /translation="GAPDMAFPRMNNMSFWLLPPSLLLLLLSSMVENGVGTGWTVYPP Sequenza proteica LSSNIAHGGSSVDLAIFSLHLAGISSILGAINFITTVINMRPMGMTFDRMPLFVWAVV ITAVLLLISLEVLAGA ITMLLTDRNINTSFFDPAGGGDP ILYOHLFWFFGHP" ORIGIN 1 gadacyccha systagcatt ccctcqaata aataatataa qattttqact tttaccaccc 61 tetttattat tattattatt aagaagaata gtagaaaatg gagttggtac eggatgaaca Sequenza DNA 121 atttateece etttateate eastattget eaeggaggtt eateagttga tttagetatt 181 tttagtttae aettggetgg aatttettea attttaggag caattaattt tattaetaea 241 qtaattaata tacqtccaat aqqaataact tttqaccqaa taccattatt tqtatqaqca

Databases

BOLD System http://www.boldsystems.org



Molecular Ecology Notes (2007)

doi: 10.1111/j.1471-8286.2006.01678.x

BARCODING

BOLD: The Barcode of Life Data System (www.barcodinglife.org)

SUJEEVAN RATNASINGHAM and PAUL D. N. HEBERT Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, University of Guelph, Guelph, ON, Canada N1G 2W1

BOLD system (http://www.boldsystems.org)



BOLD system (http://www.boldsystems.org)

BOLDSYSTEMS v2.5

Management & Analysis

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Project Options

Merge Projects Search All Records View All Primers Bibliography Submission

Campaigns

ACG Parasitoids
All Birds Barcoding Initiative
All Caddis DNA Barcoding
Anfibios Neotropicos
Ants Of The World
Arctic Life (PROBE)
Barcoding Canadian Animals
Barcoding Earthworms
Barcoding Fish (FishBOL)
Barcoding Mammals of the World
Bee Barcoding Initiative
Biocode (Moorea)
BioLep

Filter By: Project Code -

ACG Parasitoids		Specimens	Species	Species with Sequences				
ACG Parasitolas	Pub	Specimens	Species	COI-5P	28S-D2	ITS	ITS2	C
ASBA ACG Braconidae (Cheloninae)- in progress		27	12	11	-	-	-	
ASBR ACG Braconidae III- in progress		548	211	206	-	-	-	
ASBAC ACG Braconidae I- in progress		2	1	1	-	-	-	
ASMET ACG Braconidae (Meteorinae)- in progress		430	52	37	9	-	-	
ASBC ACG Braconidae (misc genera)- in progress		92	27	24	-	-	-	
ASRO ACG Braconidae (Rogadinae)- in progress		114	13	12	-	-	-	
ASCH ACG Chalcididae- in progress		604	32	32	-	-	-	
ASEN ACG Encyrtidae I- in		81	6	4	-	-	-	

BOLDSYSTEMS v2.5

Taxonomy Browser

Home | Taxonomy Browser | Request an Account | Identify Specimen | FAQs | Documentation

Kingdoms of life being barcoded

Specimen Records: 2,040,756 Specimens with Barcodes: 1,484,491 Species with Barcodes: 144,861

Animals:

- Acanthocephala [220]
- Annelida [23053]
- Arthropoda [1461594]
- Brachiopoda [131]
- Bryozoa [752]
- Chaetognatha [179]
- Chordata [263046]
- Cnidaria [4032]
- Cycliophora [295]
- Echinodermata [24063]
- Echiura [24]
- Gnathostomulida [8]
- Hemichordata [15]
- Mollusca [57803]
- Nematoda [4762]
- Onychophora [210]
- Platyhelminthes [6343]
- Porifera [1289]
- Priapulida [12]
- Rotifera [3853]
- Sipuncula [153]
- Tardigrada [785]
- Xenoturbellida [2]

Fungi:

- Ascomycota [3575]
- Basidiomycota [5665]
- Chytridiomycota [1]
- Myxomycota [10]
- Zygomycota [23]

Plants:

- Bryophyta [1570]
- Chlorophyta [7324]
- Lycopodiophyta [391]
- Magnoliophyta [114271]
- Pinophyta [3390]
- Pteridophyta [6255]
- Rhodophyta [22987]

Protists:

- Chlorarachniophyta [65]
- Ciliophora [499]
- Heterokontophyta [10470]
- Opalozoa [1]
- Pyrrophycophyta [2145]

Insecta {class} - Arthropoda;

Sub-taxa

Orders (31)

- 1. Archaeognatha [193]
- 2. Blattaria [1898]
- 3. Coleoptera [151584]
- 4. Dermaptera [572]
- 5. Diplura [16]
- 6. Diptera [329696]
- 7. Embioptera [61]
- 8. Ephemeroptera [16620]
- 9. Grylloblattodea [2]
- 10. Hemiptera [83403]
- 11. Hymenoptera [326552]
- 12. Isoptera [1704]
- 13. Lepidoptera [828457]
- 14. Mantodea [858]
- 15. Mantophasmatodea [2]
- 16. Mecoptera [147]

■ Taxon Description (Wikipedia)

Insects (from Latin, a calque of Greek [], "cut into sections") are a class of invertebrates within the chitinous exoskeleton, a three-part body (head, thorax and abdomen), three pairs of jointed legs, co antennae. They are among the most diverse groups of animals on the planet, including more than a representing more than half of all known living organisms. The number of extant species is estimate In: and potentially represent over 90% of the differing metazoan life forms on Earth. Insects may be environments, although only a small number... full article at Wikipedia

Specimen Records:	1,838,389	Public Records:
Specimens with Sequences:	1,481,193	Public Species:
Specimens with Barcodes:	1,341,162	Public BINs:
Species:	170,110	
Species With Barcodes:	130,780	

Animal Identification [COI]

Fungal Identification [ITS]

Plant Identification [rbcL & matK]

The BOLD Identification System (IDS) for COI accepts sequences from the 5' region of the mitochondrial Cytochrome c oxidase subunit I gene and returns a species-level identification when one is possible. Further validation with independent genetic markers will be desirable in some forensic applications.

Historical Databases: Jul-2011 Jul-2010 Jul-2009

Search Databases:

- All Barcode Records on BOLD (1,311,082 Sequences)
 Every COI barcode record on BOLD with a minimum sequence length of 500bp (warning: unvalidated library and includes records without species level identification). This includes many species represented by only one or two specimens as well as all species with interim taxonomy. This search only returns a list of the nearest matches and does not provide a probability of placement to a taxon.
- Species Level Barcode Records (1,084,444 Sequences/103,794 Species/44,061 Interim Species)
 Every COI barcode record with a species level identification and a minimum sequence length of 500bp. This includes many species represented by only one or two specimens as well as all species with interim taxonomy.
- Public Record Barcode Database (262,777 Sequences/35,845 Species/9,712 Interim Species)
 All published COI records from BOLD and GenBank with a minimum sequence length of 500bp. This library is a collection of records from the published projects section of BOLD.
- Full Length Record Barcode Database (893,217 Sequences/94,098 Species/38,647 Interim Species)
 Subset of the Species library with a minimum sequence length of 640bp and containing containing both public and private records. This library is intended for short sequence identification as it provides maximum overlap with short reads from the barcode region of COI.

Enter sequence	in 1	fasta '	format	t
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Specimen Identification Request



▼ Query: Ph-x_LCO1490_copy

Top Hit: Arthropoda - Coleoptera - Phloeosinus thujae (98.71%)

Search Request:

Type: COI FULL DATABASE (includes records without species designation)

Search Result:

Tree Based Identification



67

78

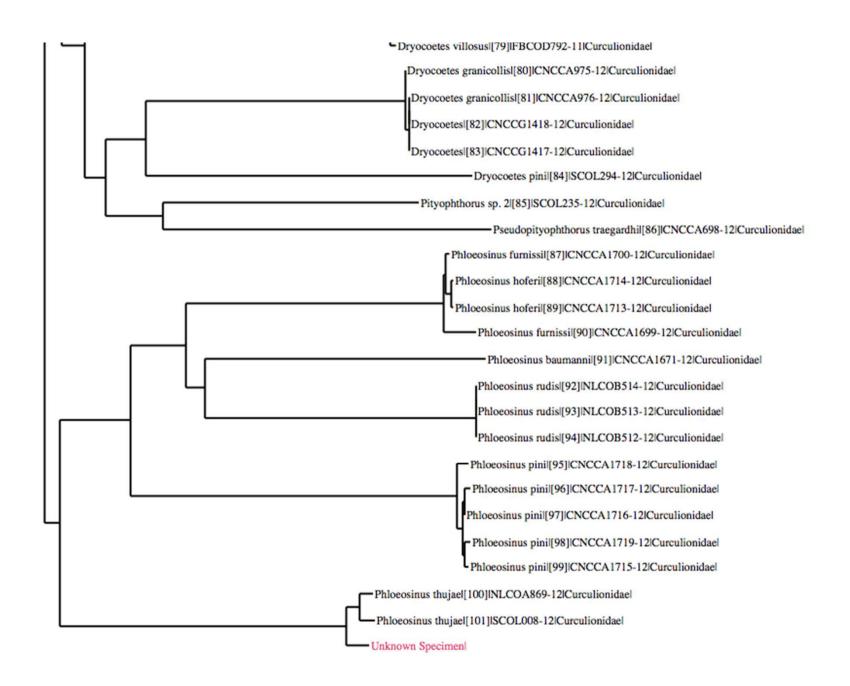
100

Similarity scores of the top 100 matches

23

12

Phylum	Class	Order	Family	Genus	Species	Specimen Similarity (%)
Arthropoda	Insecta	Coleoptera	Curculionidae	Phloeosinus	thujae	98.71
Arthropoda	Insecta	Coleoptera	Curculionidae	Phloeosinus	thujae	98.28
Arthropoda	Insecta	Coleoptera	Curculionidae			82.61
Arthropoda	Insecta	Coleoptera	Curculionidae	Phloeosinus	hoferi	82.61
Arthropoda	Insecta	Coleoptera	Curculionidae	Hylesinus	laticollis	82.58
Arthropoda	Insecta	Coleoptera	Curculionidae	Phloeosinus	furnissi	82.58
Arthropoda	Insecta	Coleoptera	Curculionidae	Larinus	planus	82.51
Arthropoda	Insecta	Coleoptera	Curculionidae			82.45
Arthropoda	Insecta	Coleoptera	Curculionidae	Phloeosinus	hoferi	82.45
Arthropoda	Insecta	Coleoptera	Curculionidae	Phloeosinus		82.37
Arthropoda	Insecta	Coleoptera	Curculionidae			82.29
						02.20



Species (26)

- 1. Phloeosinus arizonicus [4]
- 2. Phloeosinus aubei [3]
- 3. Phloeosinus baumanni [5]
- 4. Phloeosinus canadensis [5]
- 5. Phioeosinus cristatus [4]
- 6. Phloeosinus cupressi [5]
- 7. Phloeosinus deleoni [3]
- 8. Phloeosinus dentatus [4]
- Phioeosinus furnissi [5]

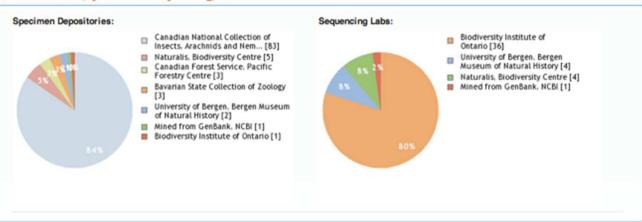
 Phioeosinus furnissi [5]
- 10. Phloeosinus hoferi [4]
- 11. Phloeosinus keeni [3]
- 12. Phloeosinus noferi [1]
- 13. Phloeosinus pini [5]
- 14. Phloeosinus punctatus [5]
- 15. Phloeosinus rudis [3]
- 16. Phloeosinus scopulorimu scopulorium [3]
- 17. Phloeosinus seguoiae [3]
- 18. Phloeosinus sequoiae [6]
- 19. Phloeosinus serratus [5]
- 20. Phloeosinus setosus [3]
- 21. Phloeosinus sinensis [2]
- 22. Phloeosinus sinesil [3]
- 23. Phloeosinus sinesis [1]
- 24. Phloeosinus thujae [6]
- 25. Phloeosinus turkestanicus [1]
- 26. Phloeosinus variolatus [3]

Specimen Records:	98	Public Records:	2
Specimens with Sequences:	45	Public Species:	2
Specimens with Barcodes:	23	Public BINs:	1
Species:	26		
Species With Barcodes:	9		

Species List - Progress

Access Published & Released Data

Contributors (Specimens & Sequencing)



Imagery

images representing subtaxa of Phloeosinus



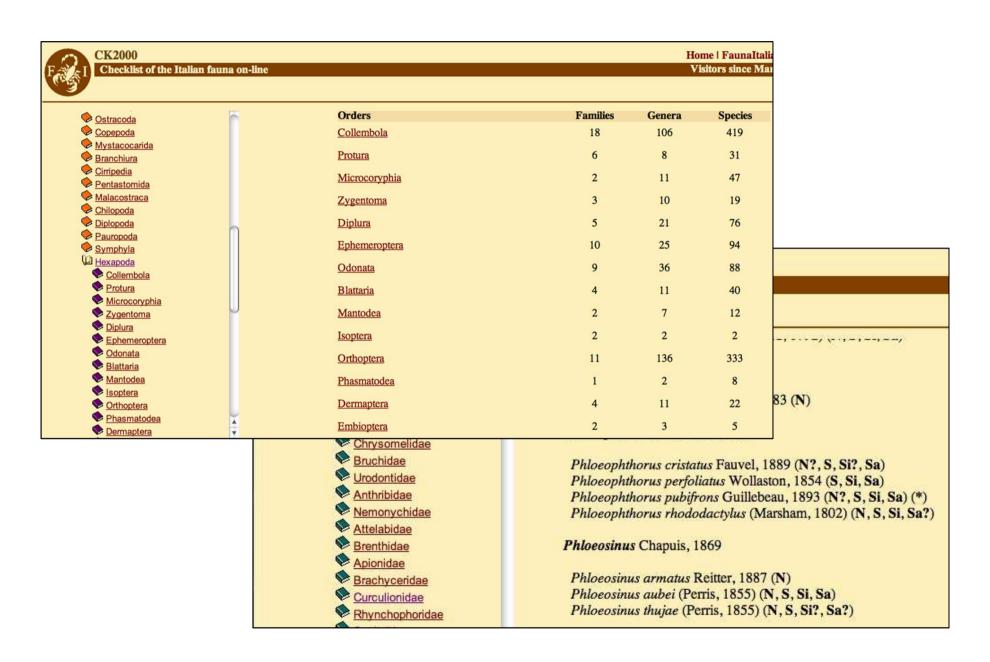


Strumenti informatici on-line

- PCR-RFLP: Webcutter (http://bio.lundberg.gu.se/cutter2)
- PCR:
 - Primer3 (http://frodo.wi.mit.edu/primer3)
 - Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast)
- Sequenze: BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
- AFLP: in silico AFLP

(http://bioinformatics.psb.ugent.be/webtools/aflpinsilico)

Check list of the Italian fauna on-line



EPPO

European and Mediterranean Plant Protection Organization





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Invasive alien plants

EPPO A1 List of pests recommended for regulation as quarantine pests

(version 2013-09)

EPPO recommends its member countries to regulate the pests listed below as quarantine pests (A1 pests are absent from the EPPO region). The EPPO A1 List is reviewed every year by the Working Party on Phytosanitary Regulations and approved by

For geographical distribution, please note that data may not be up-to-date in the datasheets, please consult the new PQR version (which now contains maps).

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EPPO A2 List of pests recommended for regulation as quarantine pests

(version 2013-09)

EPPO recommends its member countries to regulate the pests listed below as quarantine pests (A2 pests are locally present in the EPPO region). The EPPO A2 List is reviewed every year by the Working Party on Phytosanitary Regulations and approved by Council.

For geographical distribution, please note that data may not be up-to-date in the datasheets, please consult the new PQR version (which now contains maps).

Principali applicazioni dei marcatori molecolari

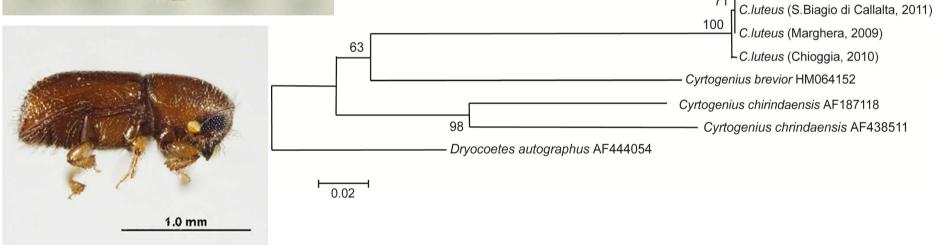
- Identificazione specie
- Caratterizzazione specie (biotipi/specie criptiche)
- Stime su rotte di migrazione/colonizzazione
- Interazioni con pianta ospite o rapporti preda/predatore/parassitoidi



Identificazione mediante barcode di specie di nuova introduzione

Cyrtogenius luteus/C. brevior

C.luteus (Chioggia, 2009)



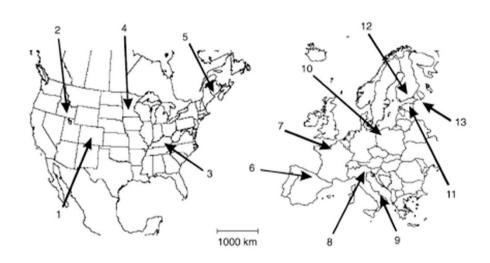
Faccoli et al. 2012

mtDNA/AFLP:

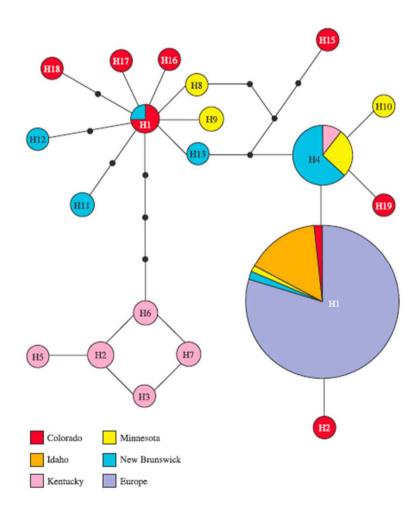
Leptinotarsa decemlineata



 mtDNA: frammento di 577 bp (3' COI -5' COII)



Grapputo et al. 2005



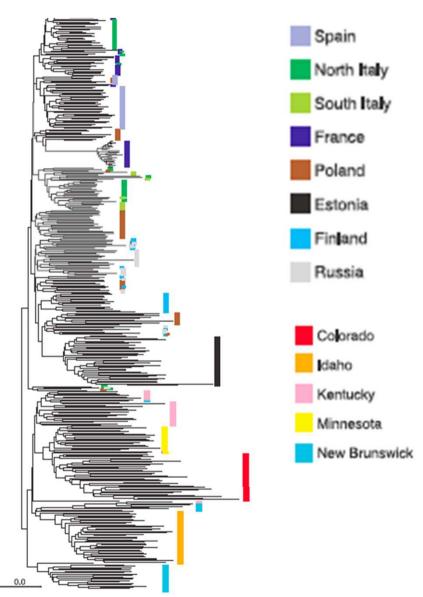
mtDNA/AFLP:

Leptinotarsa decemlineata



AFLP:

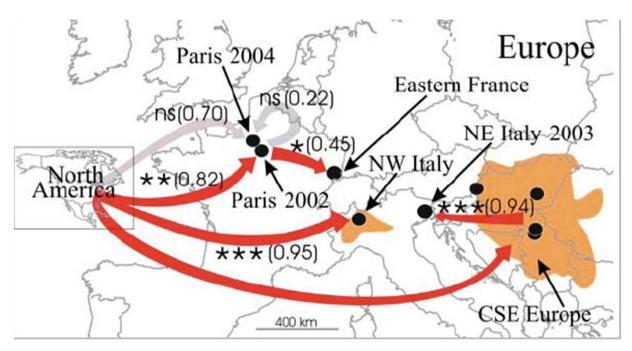
297 loci



Grapputo et al. 2005

Microsatelliti: Diabrotica virgifera





 analisi DNA nucleare (variabilità in 8 loci microsatelliti)

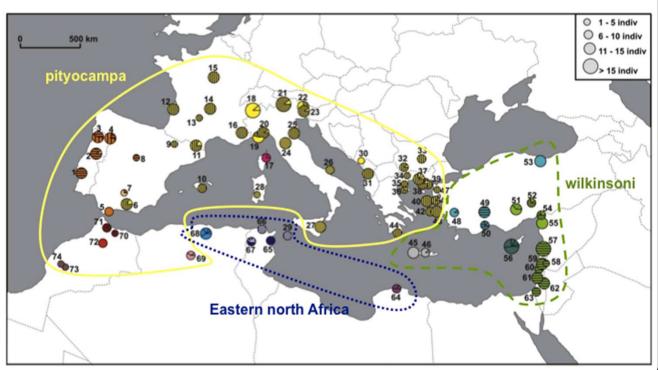
mtDNA:

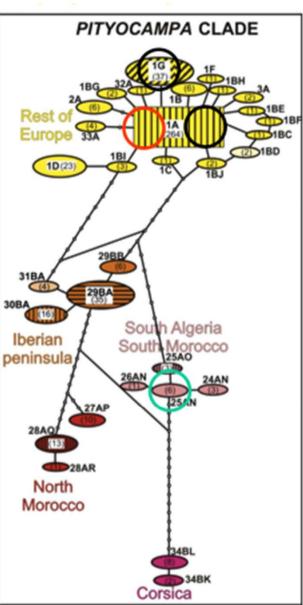
Thaumetopoea pityocampa



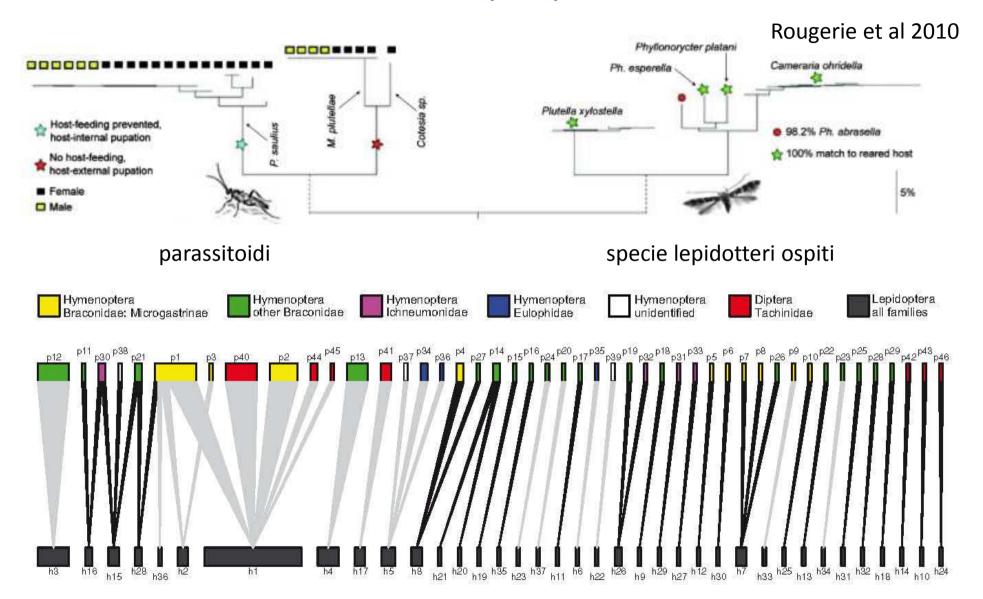
mtDNA COI, COII, 604 bp

Kerdelhué et al. 2009





Interazioni ospiti/parassitoidi



Hrcek et al. 2011