Food and Agriculture Organization of the United Nations

International Plant Protection Convention

中国農業大學 China Agricultural University

Implementation of the IPPC Global Project "Strengthening the Capacity of Developing Contracting Parties to Implement the IPPC and its Standards under FAO-China South-South Cooperation (SSC) Programme" (GCP /INT/291/CPR) in the Pilot Country of Sri Lanka (2019-2021)

Molecular Identification of Economically Important Fruit Flies

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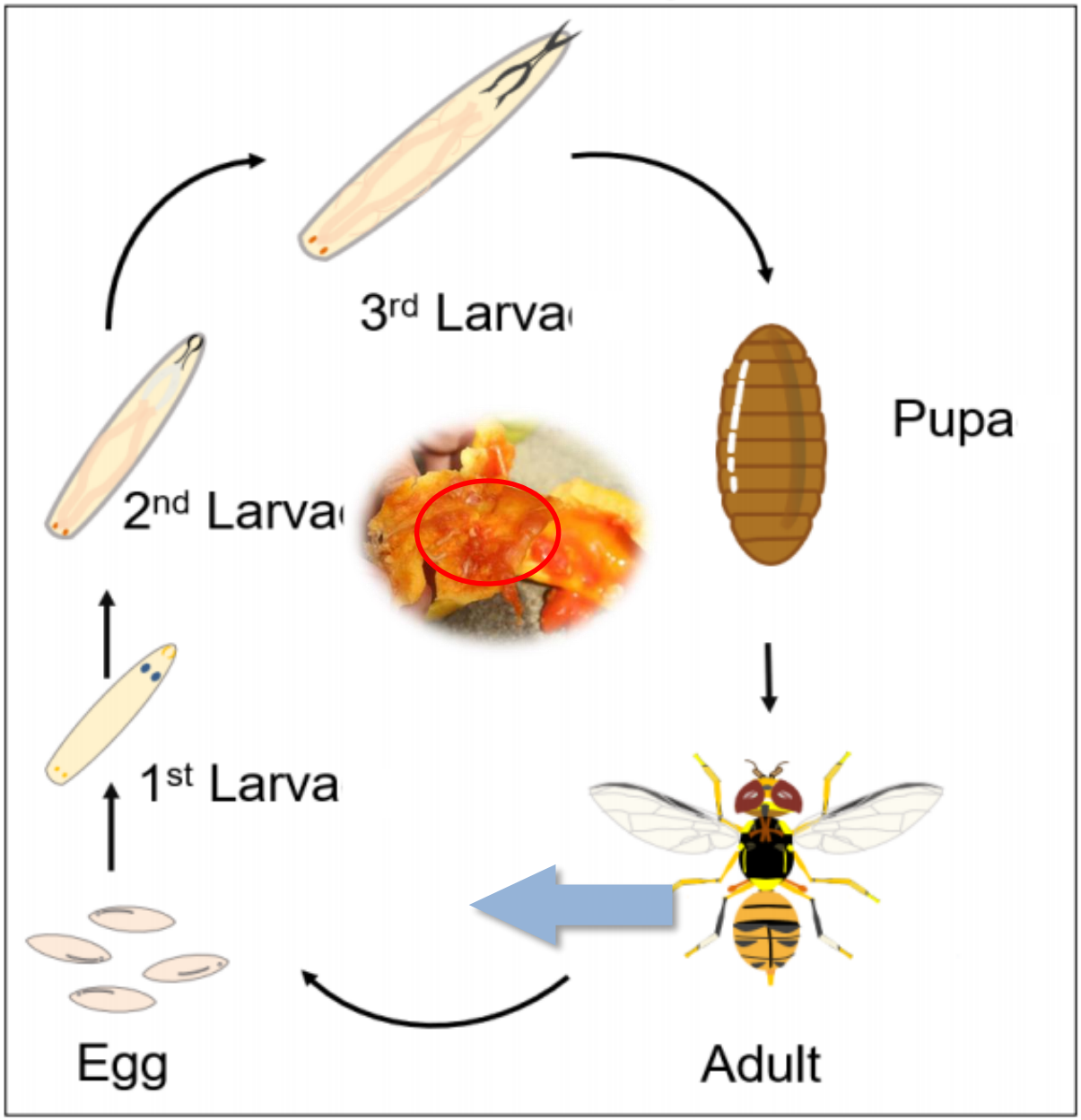
FAO-IPPC Project, Sri Lanka, Dec. 18th, 2019

Outline

- **Basic Information of Molecular Identification**
- **Principle and Operational Guidance of DNA Barcodes Technique**
- **Principle and Operational Guidance of Specificity- Primers Technique**

1. Basic Information of Molecular Identification

1.1 Life Cycle



1.2 Difficulties and Requirement for Fruit Flies Quarantine

- **Limitation of fruit flies stage**

✓ Accurate species identification based on the morphology of immature stages (i.e. egg, larva or pupa) or adult body parts, which lack distinct diagnostic features, is extremely difficult and unreliable.

✓ In quarantine work, intercepted from ports of entry involves rearing them to adults which is time-consuming and sometimes unsuccessful

- **More than 4500 species throughout the world**

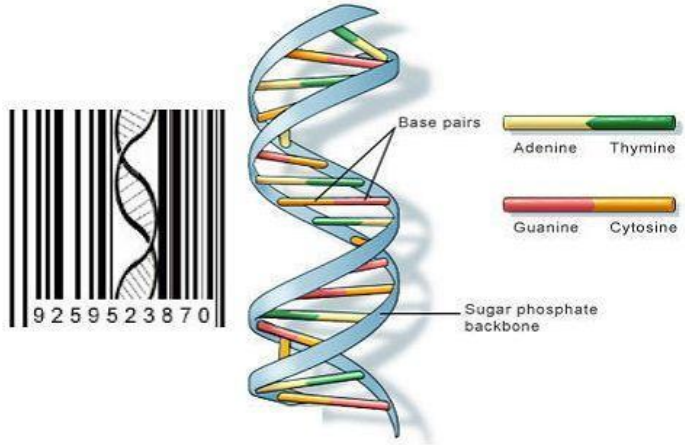
1.3 Main molecular diagnose techniques

Technique	Research Contents	References
DNA Barcodes	Diagnose effectiveness Unknown Species diagnose mirco-DNA barcodes	13 references: Armstrong *et al*., 2005~Gong *et al*., 2014
PCR	3 genera 11 species (9 *Bactrocera* spp.、1*Ceratitis* sp.、1*Carpomya vesuviana*)	7 references: Deng *et al*., 2004~Cheng *et al*., 2013
Real-time PCR	4 genera16 species (6 *Anastrepha* spp.、8 *Bactrocera* spp.、1 *Ceratitis* sp. 、1 *C. vesuviana*)	4 References: Yu *et al*., 2005~Cheng *et al*., 2014
RAPD	3 genera10 species （8 *Bactrocera* spp.、1 *Ceratitis* sp.、1 *Dacus* sp.）	4 references: Baruffi *et al*.，1995~Singn *et al*.， 2011
RFLP	4 genera55 species （25 *Bactrocera* spp.、25 *Ceratitis* spp.、4 *Rhagoletis* spp.、1 *C. vesuviana*）	12 references: Mun *et al*.，2000~Cheng *et al*., 2014
Gene Chip	3 genera30 species （2 *Anastrepha* spp.、25 *Bactrocera* spp.、3 *Ceratitis* spp.）	3 references: Yu *et al*., 2007、Jiang *et al*., 2015
LAMP	2 genera 3 speices (1 Zeugodacus spp.、 1 Ceratitis spp.、 1 Dacus spp.)	3 references: Huang *et al*., 2009 Zhong *et al*., 2019 Sinaie *et al*., 2019

2. Principle and operational guidance of DNA Barcodes technique

2.1 What is DNA Barcoding?

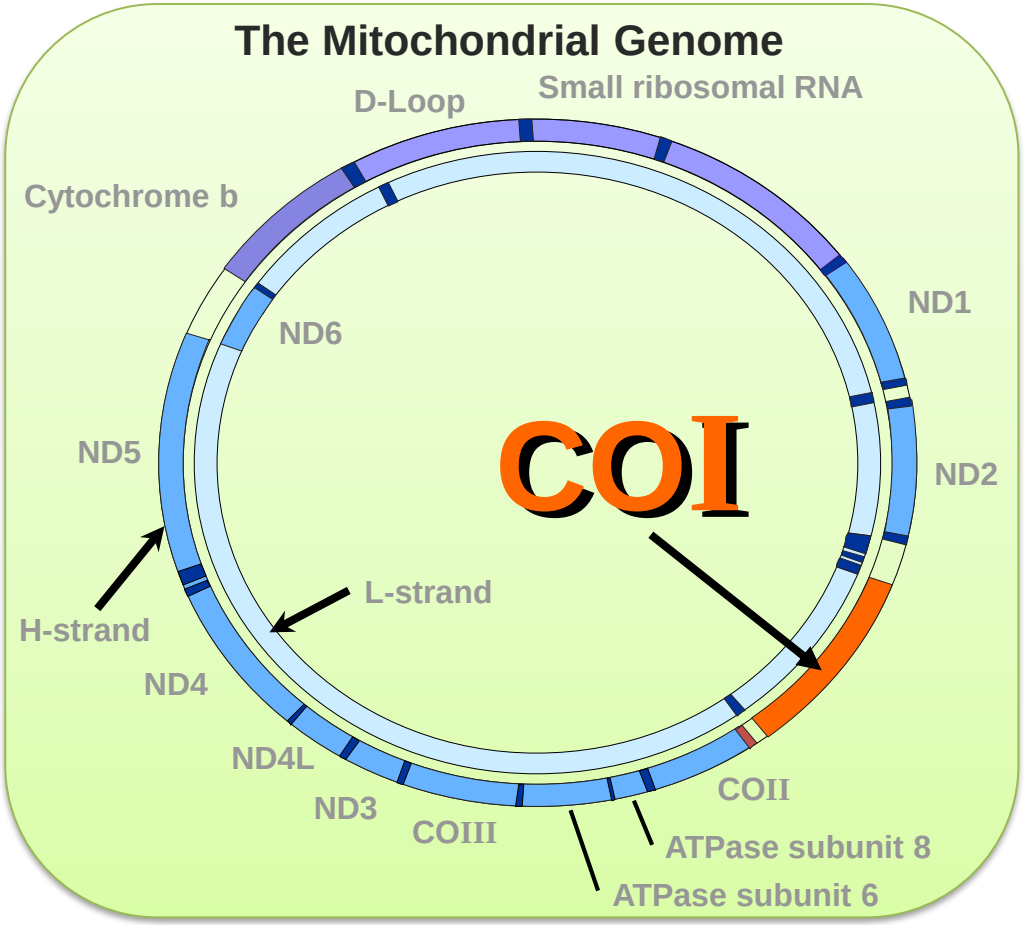
DNA barcoding is a taxonomic method, that uses one or more standardized short genetic markers in an organism's DNA to identify it as belonging to a particular species. Through this method unknown DNA samples are identified to registered species based on comparison to a reference library.



Markers that have been used for DNA barcoding in different organism groups, modified from Purty and Chatterjee.[21]

Organism group	Marker gene/locus
Animals	*COI*,[34] Cytb,[35] 12S,[36] 16S[37]
Plants	*matK*,[38] *rbcL*,[39] *psbA-trnH*,[40] *ITS*[41]
Bacteria	*COI*,[27] *rpoB*,[29] 16S,[42] *cpn60*,[28] *tuf*,[43] *RIF*,[44] *gnd*[45]
Fungi	*ITS*,[46] *RPB1* (LSU), *RPB2* (LSU), *18S* (SSU)[33]
Protists	*ITS*,[47] *COI*,[48] *rbcL*,[49] *18S*,[50] *28S*[49]

Paul Hebert



COI

DNA barcodes for animal

cytochrome c oxidase I (COI) gene 1.5kb

Barcode region 658bp

(Folmer *et al*. 1994)

2.2 Identification methods based on DNA Barcodes

- **Tree-based method — neighbour-joining (NJ) tree**

Monophyletic group √

Paraphyletic group ×

Polyphyletic group ×

- Distance-based method — barcoding gap

traspecific varation<interspecific varation

Barcoding gaps ≤2%

- **Tree-based method——MEGA 7.0**

✓ Construct a neighbour-joining (NJ) tree, which is a useful clustering method for large data sets with a Kimura-2-parameter (K2P) molecular evolution model.

✓ The branch supports were through 1000 bootstrap replications, and other parameters were set to their default settings.

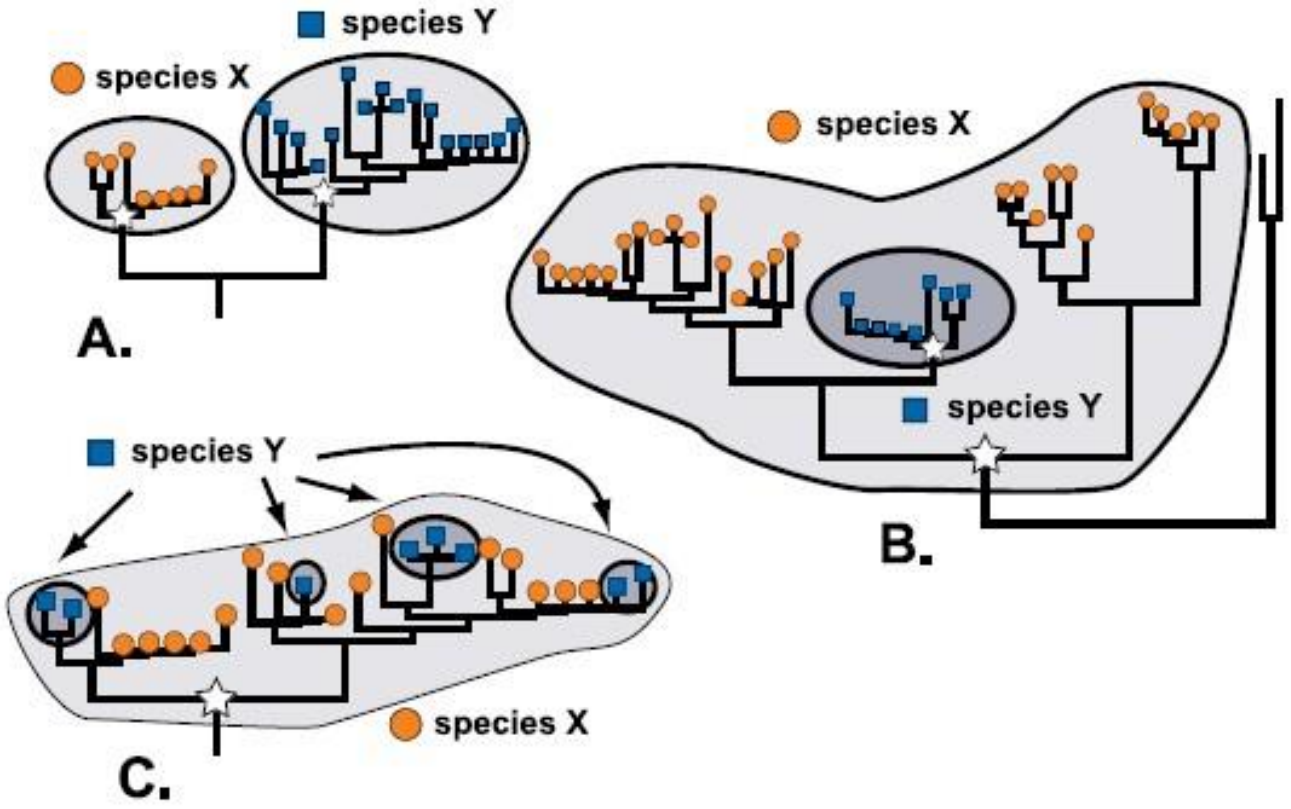


Figure 1. Phylogenetic Relationships and Terminology

(A) Reciprocal monophyly. Members of each species share a unique common ancestor. For each species, the white star represents the coalescent, the point at which all extant haplotypes share a common ancestry.

(B) Paraphyly. One species (Y), is monophyletic, but nests within another recognized species (X). Thus, the coalescent of species Y (small star) is contained within the coalescent of species X (large star).

(C) Polyphyly. Neither species X or Y are monophyletic, and both coalesce to the white star.

2.2 Identification methods based on DNA Barcodes

- Tree-based method — neighbour-joining (NJ) tree

Monophyletic group √

Paraphyletic group ×

Polyphyletic group ×

- **Distance-based method — barcoding gap**

traspecific varation < interspecific varation

Barcoding gaps ≤ 2%

- **Distance-based method——MEGA 7.0**

✓ The pairwise distances were calculated separately to determine the intraspecific and interspecific variation using with the K2P model

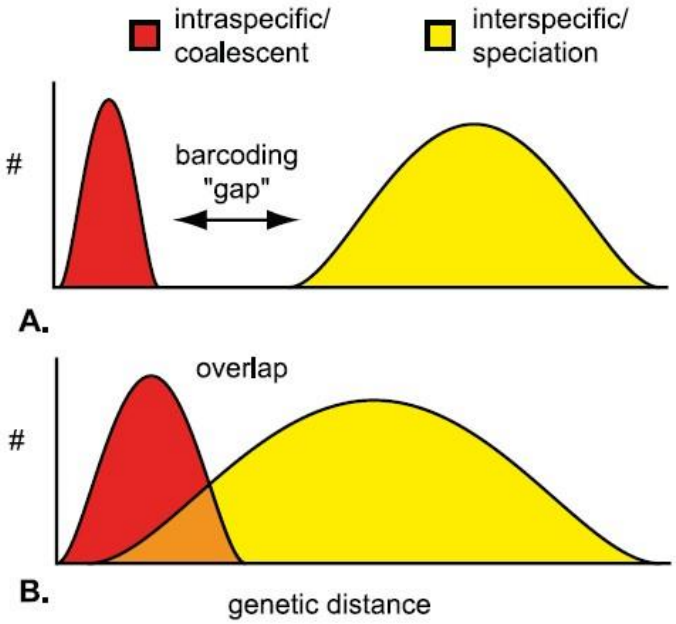
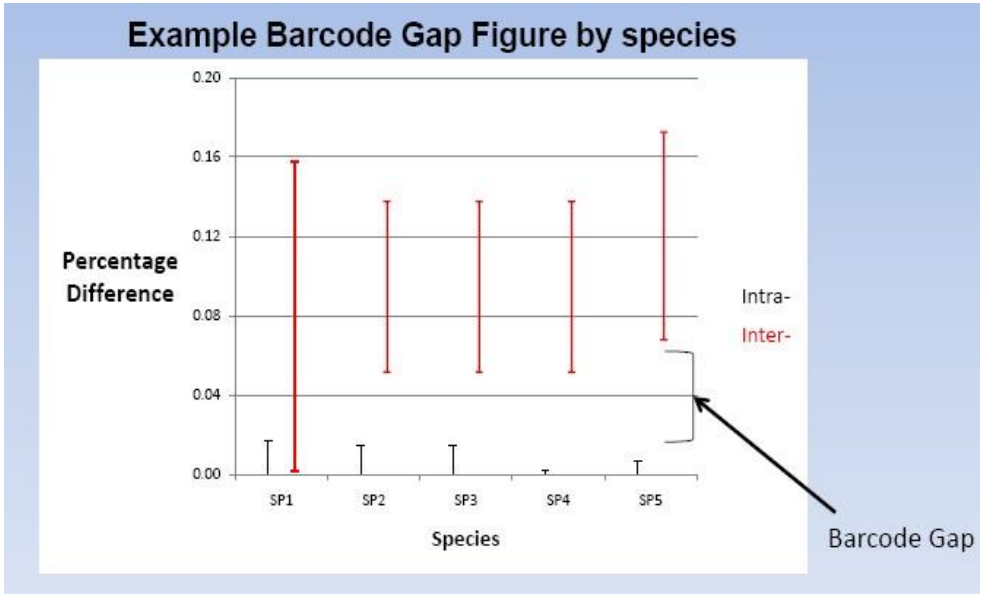
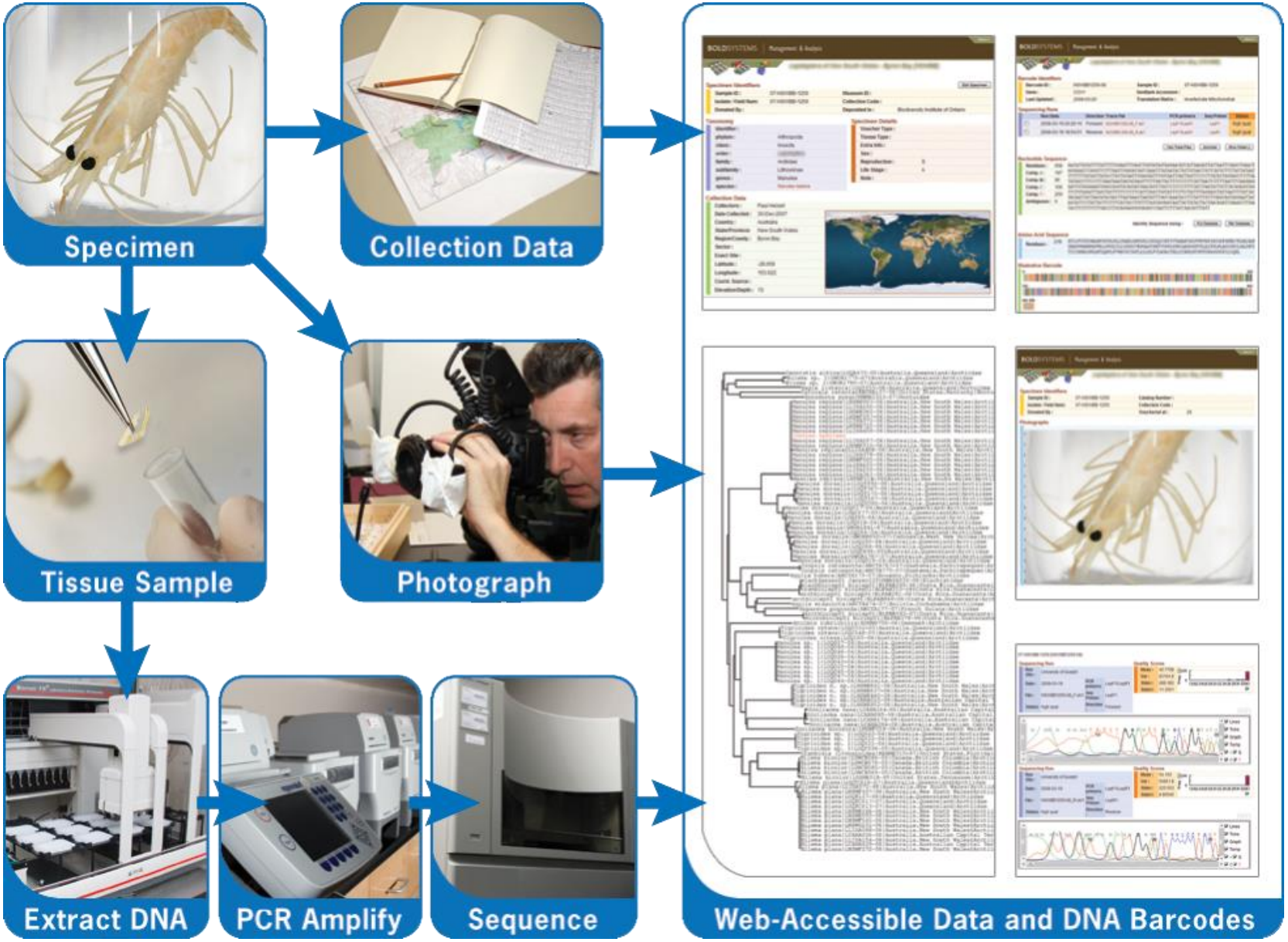


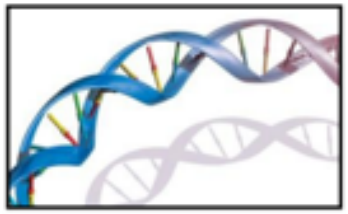
Figure 2. Schematic of the Inferred Barcoding Gap

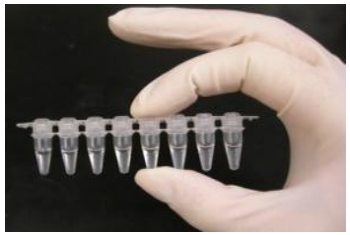
The distribution of intraspecific variation is shown in red, and interspecific divergence in yellow. (A) Ideal world for barcoding, with discrete distributions and no overlap. (B) An alternative version of the world with significant overlap and no gap.




Specimen
Collection Data
Tissue Sample
Photograph
Extract DNA
PCR Amplify
Sequence
Web-Accessible Data and DNA Barcodes

2.3 Procedure of DNA Barcoding





GenBank

BOLDSYSTEMS

1. Isolate DNA from the sample (DNA Extraction Kit)

2. PCR to amplify the DNA Barcodes (universal primers: LCO1490/HCO2198)

3. Sequencing (Sanger Sequencing)

4. Compare the resulting sequences against reference databases to find the matching species

Materials

- **Instrument and Equipment**

Dissecting microscope, Pipets, Vortexer, Microcentrifuge, Centrifugal machine (rotational speed＞12000rpm), Water Bath for heating at 56℃, PCR Amplifier, Electronic Analytical Balance, Electrophoresis Apparatus, Gel Imaging System, Horizontal Electrometer

- **Reagent**

Primers, DNA Extraction Kit, Ethanol (95%-100%), 2×Taq PCR MasterMix, D2000 DNA Marker, Gel, ddH20

- **Lab Consumable**

Pipet tips (10ul, 200 ul and 1000 ul), Microcentrifuge tubes (1.5 ml) Pestle, PCR tubes (0.2 ml), Pincette, Petri dish, Filter paper

2.3.1 Isolate DNA from the sample (DNA Extraction Kit)

- **Step 1.** Add 200 µl Buffer GA into a 1.5 ml microcentrifuge tube.
- **Step 2.** Put the legs into the tube and cut into small pieces using scissors.
- **Step 3.** Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56 ℃ for 2 h at least. Vortex occasionally during incubation to disperse the sample.
- **Step 4.** Add 200 µl Buffer GB to the sample, and mix thoroughly by vortexing.
- **Step 5.** Incubate at 70 ℃ for 10 min.
- **Step 6.** Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing.

- **Step 7.** Pipet the mixture from step 6 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at 12000 rpm) for 1 min. Discard flow-through and collection tube.
- **Step 8.** Add 500 µl Buffer GD into the DNeasy Mini spin column, and centrifuge for 1 min at 12000 rpm. Discard flow-through and collection tube.
- **Step 9.** Add 500 µl Buffer PW, and centrifuge for 1 min at 12000 rpm. Discard flow-through and collection tube.
- **Step 10.** Repeat Step 9.
- **Step 11.** Place the DNeasy Mini spin column in a clean 1.5 ml microcentrifuge tube, and pipet 50 µl Buffer TE directly onto the DNeasy membrane.
- **Step 12.** Incubate at room temperature for 5 min, and then centrifuge for 1 min at 12000 rpm to elute.

2.3.2 PCR to amplify the DNA Barcodes

- **PCR reaction system**

Template 2 ul

Forward primer (10uM) 2 ul

Reverse primer (10uM) 2 ul

2×Taq PCR Mastermix 25 ul (Taq polymerase, dNTP, reaction buffer)

ddH2O 19 ul

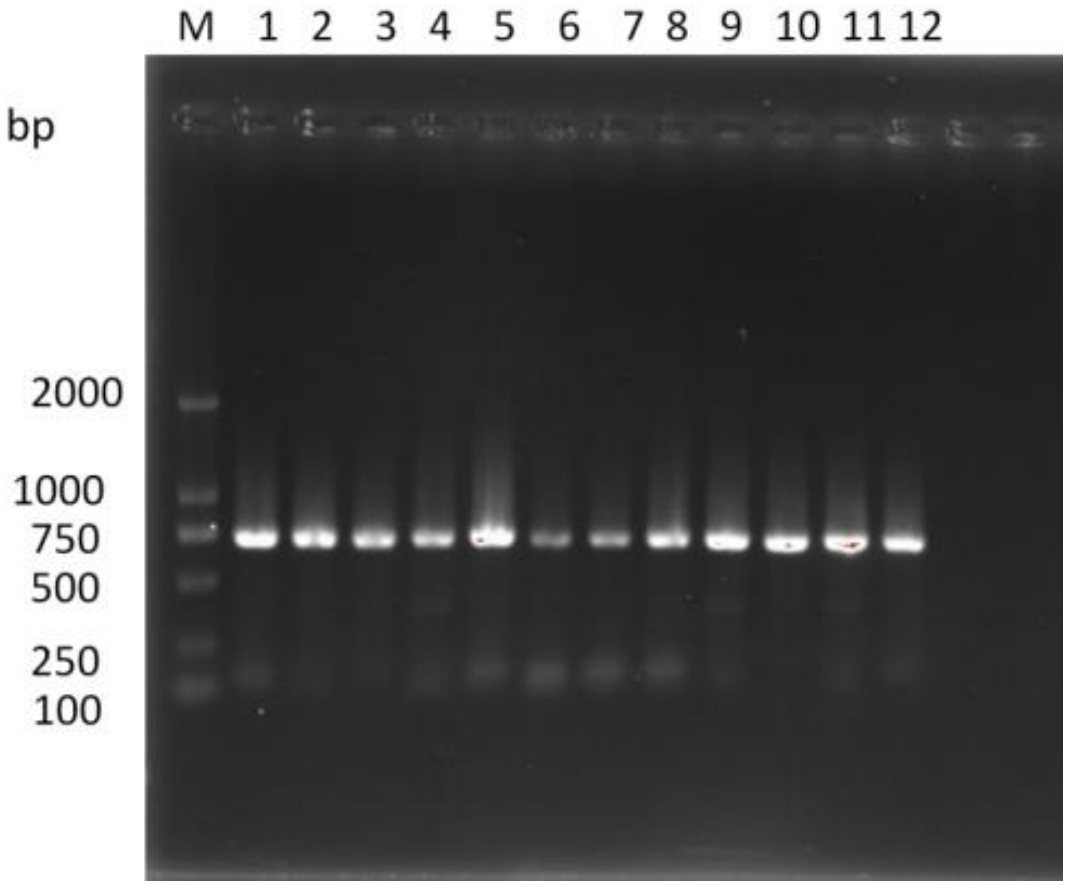
- **Reaction condition**

94℃ 3min

98℃ 30 s
50℃ 30s
72℃ 30s
} 30×

72℃ 10min

2.3.3 Detected by gel eletrophoresis and Sequencing



2.3.4 Compare the resulting sequences against reference databases to find the matching species

—— **BARCODE OF LIFE DATA SYSTEM**

(http://www.boldsystems.org)

- **The Barcode of Life Data System (BOLD)**

is an online workbench and database that supports the assembly and use of DNA barcode data. It is a collaborative hub for the scientific community and a public resource for citizens at large.

- **Identification Methods**

✓ Tree-based method — Phylogenetics tree as monophyly

✓ Distance-based method — Similarity ≥ 98%

DESIGNED TO SUPPORT THE GENERATION & APPLICATION OF DNA BARCODE DATA

BOLD is a cloud-based data storage and analysis platform developed at the Centre for Biodiversity Genomics in Canada. It consists of four main modules, a data portal, an educational portal, a registry of BINs (putative species), and a data collection and analysis workbench.

The Barcode of Life Data Systems (BOLD) is a web platform that provides an integrated environment for the assembly and use of DNA barcode and other sequence data. It delivers an online database for the collection and management of specimen, distributional, and molecular data as well as analytical tools to support their validation. Since its launch in 2005, BOLD has been extended to provide a range of functionality including data organization, validation, visualization and publication. The most recent version of the system, version 4, launched in 2017, brings a set of improvements supporting data collection and analysis but also includes novel functionality improving data dissemination, citation, and annotation.

BOLD is freely available to any researcher with interests in DNA Barcoding. By providing specialized services, it aids in the publication of records that meet the standards needed to gain BARCODE designation in the international nucleotide sequence databases. Because of its web-based delivery and flexible data security model, it is also well positioned to support projects that involve broad research alliances.

BOLD SYSTEMS

Barcode of Life Data Systems Handbook

A web-based bioinformatics platform supporting the DNA barcoding of animal, plant, and fungal species.

2019

www.boldsystems.org
version 4.0

Animals:

- Acanthocephala [1684]
- Acoelomorpha [22]
- Annelida [88320]
- Arthropoda [8430059]
- Brachiopoda [283]
- Bryozoa [3640]
- Chaetognatha [1469]
- Chordata [776463]
- Cnidaria [26400]
- Ctenophora [473]
- Cycliophora [326]
- Echinodermata [49796]
- Entoprocta [47]
- Gastrotricha [1283]
- Gnathostomulida [24]
- Hemichordata [218]
- Kinorhyncha [715]
- Mollusca [209530]
- Nematoda [28698]
- Nematomorpha [349]
- Nemertea [4727]

Plants:

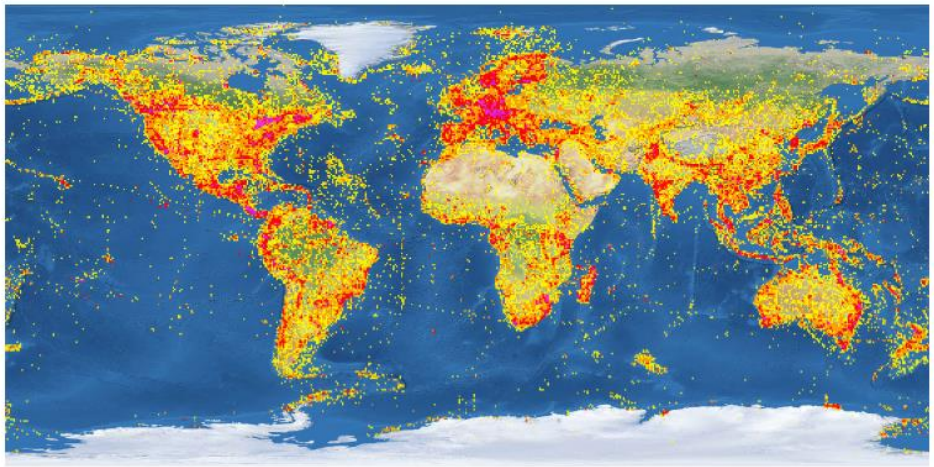
- Bryophyta [13212]
- Chlorophyta [13617]
- Lycopodiophyta [1198]
- Magnoliophyta [359343]
- Pinophyta [7016]
- Pteridophyta [11016]
- Rhodophyta [53393]

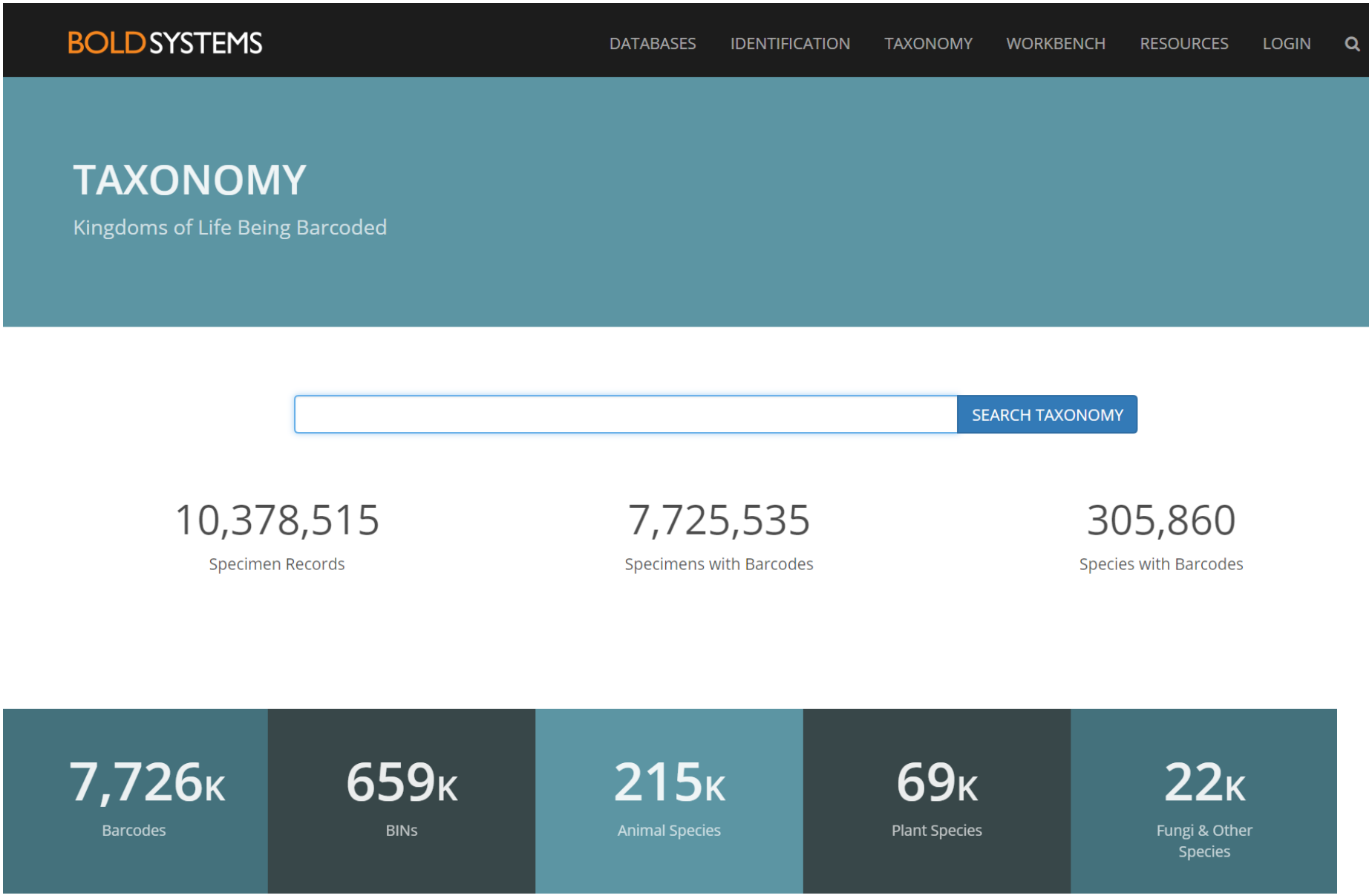
Fungi:

- Ascomycota [88771]
- Basidiomycota [61509]
- Chytridiomycota [277]
- Glomeromycota [3529]
- Myxomycota [234]
- Zygomycota [3151]

Protists:

- Chlorarachniophyta [67]
- Ciliophora [785]
- Heterokontophyta [6625]
- Pyrrophycophyta [2299]




BOLD SYSTEMS
DATABASES
IDENTIFICATION
TAXONOMY
WORKBENCH
RESOURCES
LOGIN
TAXONOMY
Kingdoms of Life Being Barcoded
SEARCH TAXONOMY
10,378,515
Specimen Records
7,725,535
Specimens with Barcodes
305,860
Species with Barcodes
7,726K
Barcodes
659K
BINs
215K
Animal Species
69K
Plant Species
22K
Fungi & Other Species

TAXONOMY BROWSER: Tephritidae

Family : Tephritidae

Arthropoda / Insecta / Diptera / Tephritidae

Image of Tephritidae

Taxon Description (Wikipedia)

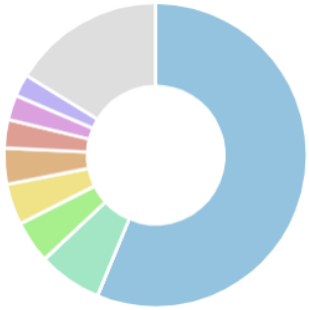
The Tephritidae are one of two fly families referred to as fruit flies, the other family being the Drosophilidae. The family Tephritidae does not include the biological model organisms of the genus Drosophila (in the family Drosophilidae), which is often called the "common fruit fly". Nearly 5,000 described species of tephritid fruit fly are categorized in almost 500 genera of the Tephritidae. Description, recategorization, and genetic analyses are constantly changing the taxonomy of this family. To distinguish them from the Drosophilidae, the Tephritidae are sometimes called peacock flies, in reference to their elaborate and colorful markings. The name comes from the Greek τεφρος, tephros, meaning "ash grey". They are found in all the ecozones. full article at Wikipedia

Statistics

Specimen Records:	27,994
Specimens with Sequences:	25,520
Specimens with Barcodes:	18,943
Species:	1,515
Species With Barcodes:	1,065
Public Records:	20,283
Public Species:	1,088
Public BINs:	846

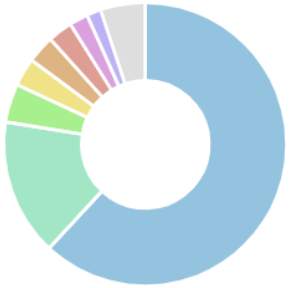
SPECIES LIST PUBLIC DATA

Specimen Depositories



- Mined from GenBank, NCBI [15078]
- Centre for Biodiversity Genomics [1818]
- University of Hawaii Insect Museum [1205]
- Naturalis Biodiversity Centre [1166]
- California State Collection of Arthropods [1023]
- Royal Museum for Central Africa [809]
- Mahasarakham University [707]
- International Centre of Insect Physiology and Ecology [630]
- 90 Others [4350]

Sequencing Labs



- Mined from GenBank, NCBI [15370]
- Biodiversity Institute of Ontario [3880]
- University of Hawaii Insect Museum [1089]
- Naturalis Biodiversity Centre [795]
- California Department for Food and Agriculture [783]
- Mahasarakham University [707]
- Royal Museum for Central Africa [523]
- Canadian Centre for DNA Barcoding [408]
- 47 Others [1271]

TAXONOMY BROWSER: Bactrocera

Genus : Bactrocera

Arthropoda / Insecta / Diptera / Tephritidae / Dacinae / Bactrocera

Image of Bactrocera strigifinis

Taxon Description (Wikipedia)

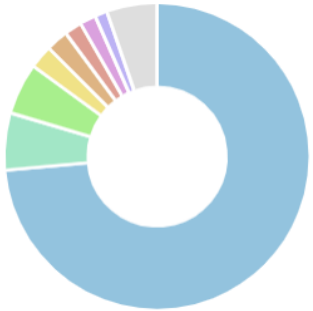
Bactrocera is a large genus of tephritid fruit flies, with close to 500 species currently described and accepted. full article at Wikipedia

Statistics

Specimen Records:	12,151
Specimens with Sequences:	11,936
Specimens with Barcodes:	8,948
Species:	293
Species With Barcodes:	231
Public Records:	10,491
Public Species:	224
Public BINs:	158

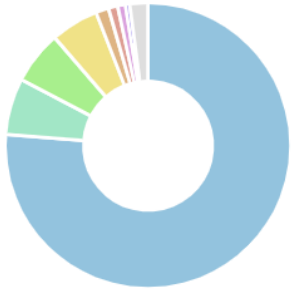
SPECIES LIST PUBLIC DATA

Specimen Depositories



- Mined from GenBank, NCBI [8725]
- University of Hawaii Insect Museum [712]
- Mahasarakham University [643]
- Centre for Biodiversity Genomics [293]
- International Centre of Insect Physiology and Ecology [277]
- Smithsonian Tropical Research Institute, Center for Tropi... [219]
- California State Collection of Arthropods [201]
- China Academy for Inspection and Quarantine [155]
- 34 Others [633]

Sequencing Labs



- Mined from GenBank, NCBI [9012]
- Biodiversity Institute of Ontario [755]
- University of Hawaii Insect Museum [710]
- Mahasarakham University [643]
- California Department for Food and Agriculture [171]
- Southern China DNA Barcoding Center [124]
- Royal Museum for Central Africa [103]
- Smithsonian Institution [59]
- 26 Others [242]

TAXONOMY BROWSER: Bactrocera dorsalis

Species : Bactrocera dorsalis

Arthropoda / Insecta / Diptera / Tephritidae / Dacinae / Bactrocera / Bactrocera dorsalis

Taxon Description (Wikipedia)

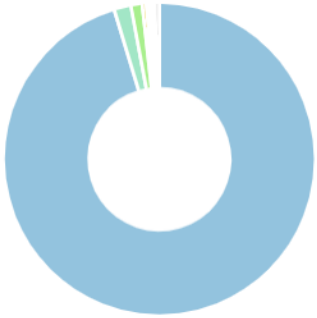
full article at Wikipedia

Statistics

Specimen Records:	5,251
Specimens with Sequences:	5,245
Specimens with Barcodes:	3,631
Subspecies:	0
Subspecies with Barcodes:	0
Public Records:	5,114
Public Subspecies:	0
Public BINs:	7

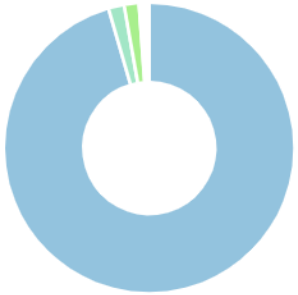
SUBSPECIES LIST PUBLIC DATA

Specimen Depositories

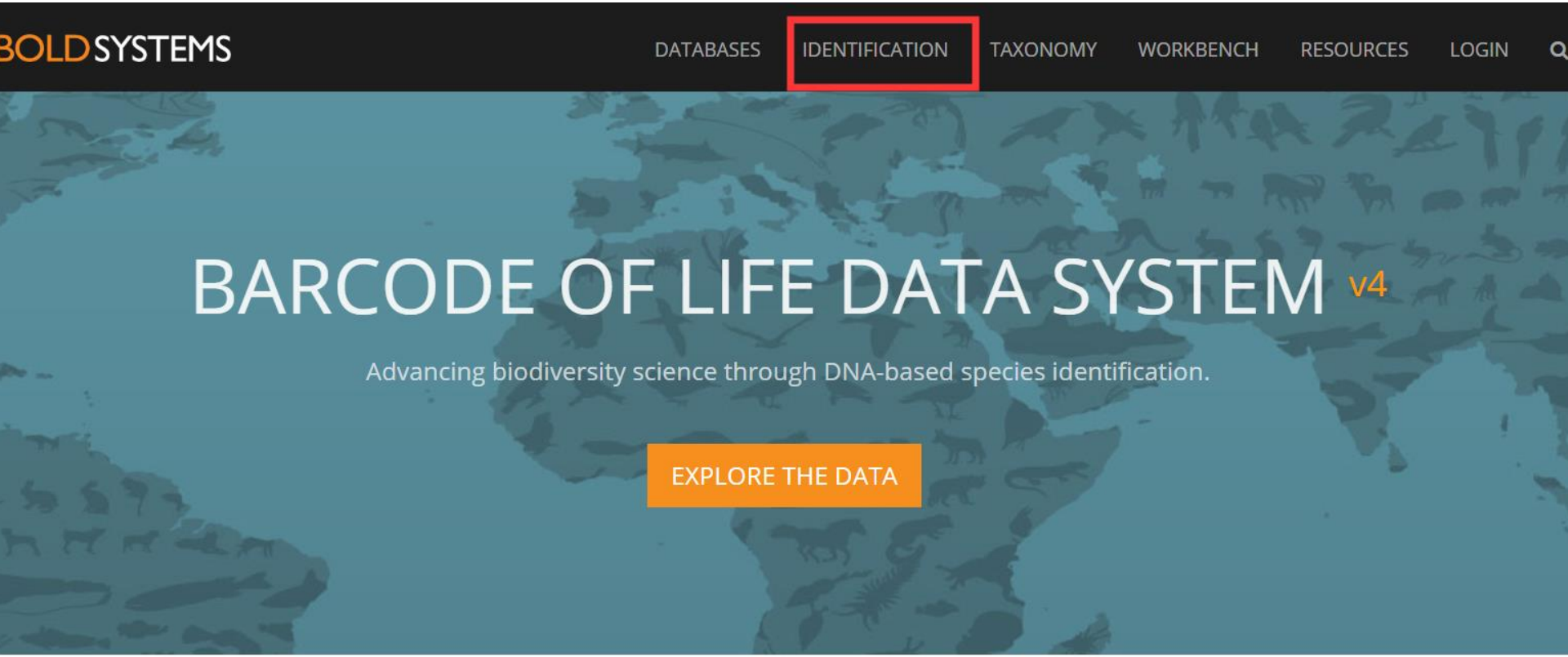


- Mined from GenBank, NCBI [4989]
- Mahasarakham University [92]
- National Plant Protection Organization, France [61]
- Royal Museum for Central Africa [22]
- Fairylake Botanical Garden [14]
- China National GeneBank [13]
- International Centre of Insect Physiology and Ecology [10]
- University of the Philippines, DNA Barcoding Laboratory [9]
- 9 Others [25]

Sequencing Labs



- Mined from GenBank, NCBI [4999]
- Mahasarakham University [92]
- Royal Museum for Central Africa [86]
- 1st Base Pte Ltd [14]
- China National GeneBank [13]
- Fairylake Botanical Garden [9]
- California Department for Food and Agriculture [8]
- Biodiversity Institute of Ontario [7]
- 5 Others [8]


BOLD SYSTEMS
DATABASES
IDENTIFICATION
TAXONOMY
WORKBENCH
RESOURCES
LOGIN
BARCODE OF LIFE DATA SYSTEM v4
Advancing biodiversity science through DNA-based species identification.
EXPLORE THE DATA

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 specie identification when one is possible. Further validation with independent genetic markers will be desirable in some forensic applications.

Historical Databases: Current | Jul-2019 | Jul-2018 | Jul-2017 | Jul-2016 | Jul-2015 | Jul-2014 | Jul-2013 | Jul-2012 | Jul-2011 | Jul-2010 | Jul-2009

Search Databases:

- **All Barcode Records on BOLD (6,902,034 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 ma 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 probabili placement to a taxon.

- **Species Level Barcode Records (3,651,651 Sequences/214,238 Species/93,912 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 a species with interim taxonomy.

- **Public Record Barcode Database (1,879,085 Sequences/130,226 Species/44,808 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 (2,338,758 Sequences/192,239 Species/75,805 Interim Species)**
  Subset of the Species library with a minimum sequence length of 640bp and containing both public and private records. This library is intended for short sequence identification as it p maximum overlap with short reads from the barcode region of COI.

- **Animal - COI**
- **Fungal - ITS**
- **Plant - RBCL & MATK**

Current Jul-2019 Jul-2018 Jul-2017 Jul-2016 Jul-2015 Jul-2014 Jul-2013 Jul-2012 Jul-2011 Jul-2010 Jul-2009

Search Databases:

- **All Barcode Records on BOLD (6,902,034 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 (3,651,651 Sequences/214,238 Species/93,912 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 (1,879,085 Sequences/130,226 Species/44,808 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 (2,338,758 Sequences/192,239 Species/75,805 Interim Species)**
  Subset of the Species library with a minimum sequence length of 640bp and 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 fasta formatted sequences in the forward orientation:

AACCCTATATTTTATTTTCGGGGCTTGAGCAGGAATAGTAGGAACTTCACTTAGAATCCTTGTTCGAGCA
GAACTGGGACACCCTGGAGCCTTAATCGGAGACGACCAAATCTATAATGTAATCGTTACTGCTCACGCCT
TCGTAATAATCTTCTTTATGGTTATACCCATCATAATTGGGGGATTCGGAAACTGATTAGTGCCCCTAAT
ACTAGGAGCCCCCGACATAGCTTTCCCACGAATAAATAATATAAGATTCTGATTACTGCCCCCATCCCTT
ACCCTATTGTTACTCAGCAGCATAGTGGAAAACGGGGCGGGCACAGGTTGAACTGTTTACCCACCGCTGT
CATCTATTATTGCCCATGGTGGAGCCTCAGTCGATCTAGCCATTTTCTCCCTTCACCTAGCAGGAATCTC
ATCAATTCTAGGAGCAGTAAATTTTATCACCACAGTAATTAATATACGCTCAACAGGAATTACATTTGAC
CGAATACCCCTCTTTGTATGAGCCGTAGTACTAACGGCCCTCCTTCTTTTACTATCCCTGCCAGTATTAG
CTGGAGCTATCACTATACTTTTAACGGACCGAAACCTAAATACATCCTTCTTTGACCCAGCGGGAGGGGG
AGACCCCATTCTATACCAACACTTATTC

SUBMIT

BOLD SYSTEMS

DATABASES IDENTIFICATION TAXONOMY WORKBENCH RESOURCES LOGIN

IDENTIFICATION ENGINE: RESULTS

PRINT

Results Summary

Download

Query ID	Best ID	Search DB	Tree	Top %	Graph	Low %
unlabeled_sequence	*Bactrocera tsuneonis*	COI SPECIES DATABASE		99.69		85.17

Query: unlabeled_sequence
Top Hit: Arthropoda Insecta - Diptera - *Bactrocera tsuneonis* (99.69%)

Search Result:

The submitted sequence has been matched to *Bactrocera tsuneonis*. This identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed. Such cases are rare.

A species page is available for this taxon: SPECIES PAGE

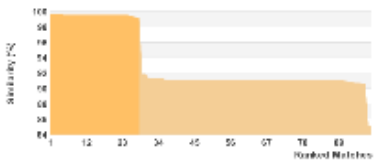
Closest matching BIN (within 3%): BIN PAGE

For a hierarchical placement - a neighbor-joining tree is provided: TREE BASED IDENTIFICATION

Identification Summary

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Arthropoda	100
Class	Insecta	100
Order	Diptera	100
Family	Tephritidae	100
Genus	*Bactrocera*	100
Species	*Bactrocera tsuneonis*	99.7

Similarity Scores of Top 99 Matches

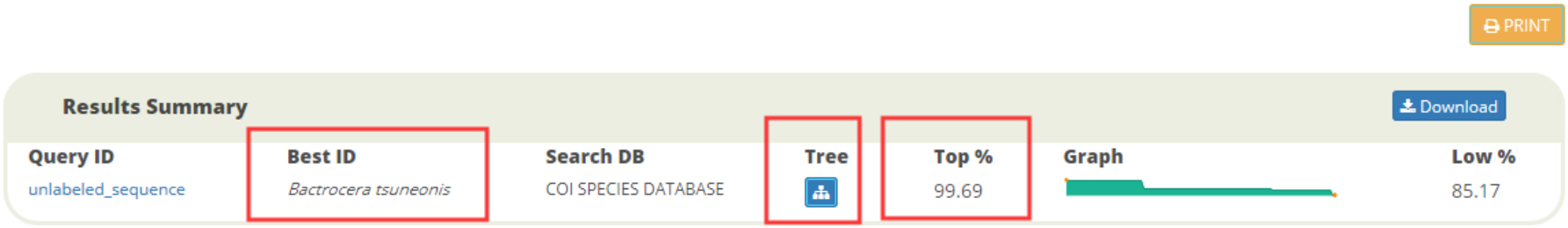


Top 20 Matches

Display: Top 20

Phylum	Class	Order	Family	Genus	Species	Subspecies	Similarity (%)	Status
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.69	Published
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.69	Published
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.69	Published
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.69	Published
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54	Published
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54	Published
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54	Published
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54	Published

IDENTIFICATION ENGINE: RESULTS



Search Result:

The submitted sequence has been matched to *Bactrocera tsuneonis*. This identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed. Such cases are rare.

A species page is available for this taxon: SPECIES PAGE

Closest matching BIN (within 3%): BIN PAGE

For a hierarchical placement - a neighbor-joining tree is provided: TREE BASED IDENTIFICATION

Identification Summary

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Arthropoda	100
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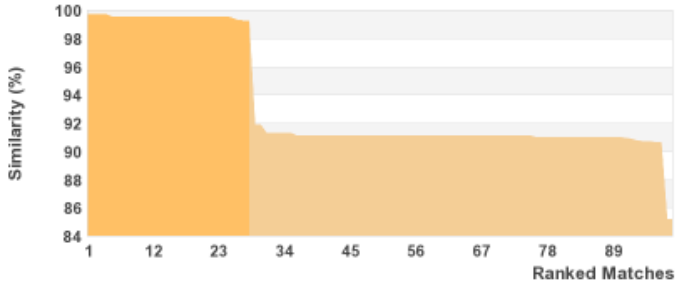
Family	Tephritidae	100
Genus	*Bactrocera*	100
Species	*Bactrocera tsuneonis*	99.7

Similarity Scores of Top 99 Matches



Display:

Top 20 Matches

Phylum	Class	Order	Family	Genus	Species	Subspecies	Similarity (%)
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.69
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.69
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.69
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.69
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54
Arthropoda	Insecta	Diptera	Tephritidae	*Bactrocera*	*tsuneonis*		99.54

IDENTIFICATION ENGINE: RESULTS

Results Summary

Query ID	Best ID	Search DB	Tree	Top %	Graph
unlabeled_sequence	*Bactrocera tsuneonis*	COI SPECIES DATABASE		99.69	

Taxon ID Tree Result | Project Console | BOLDSYSTEMS

B

Tree Result

PDF tree : View Tree Download Tree

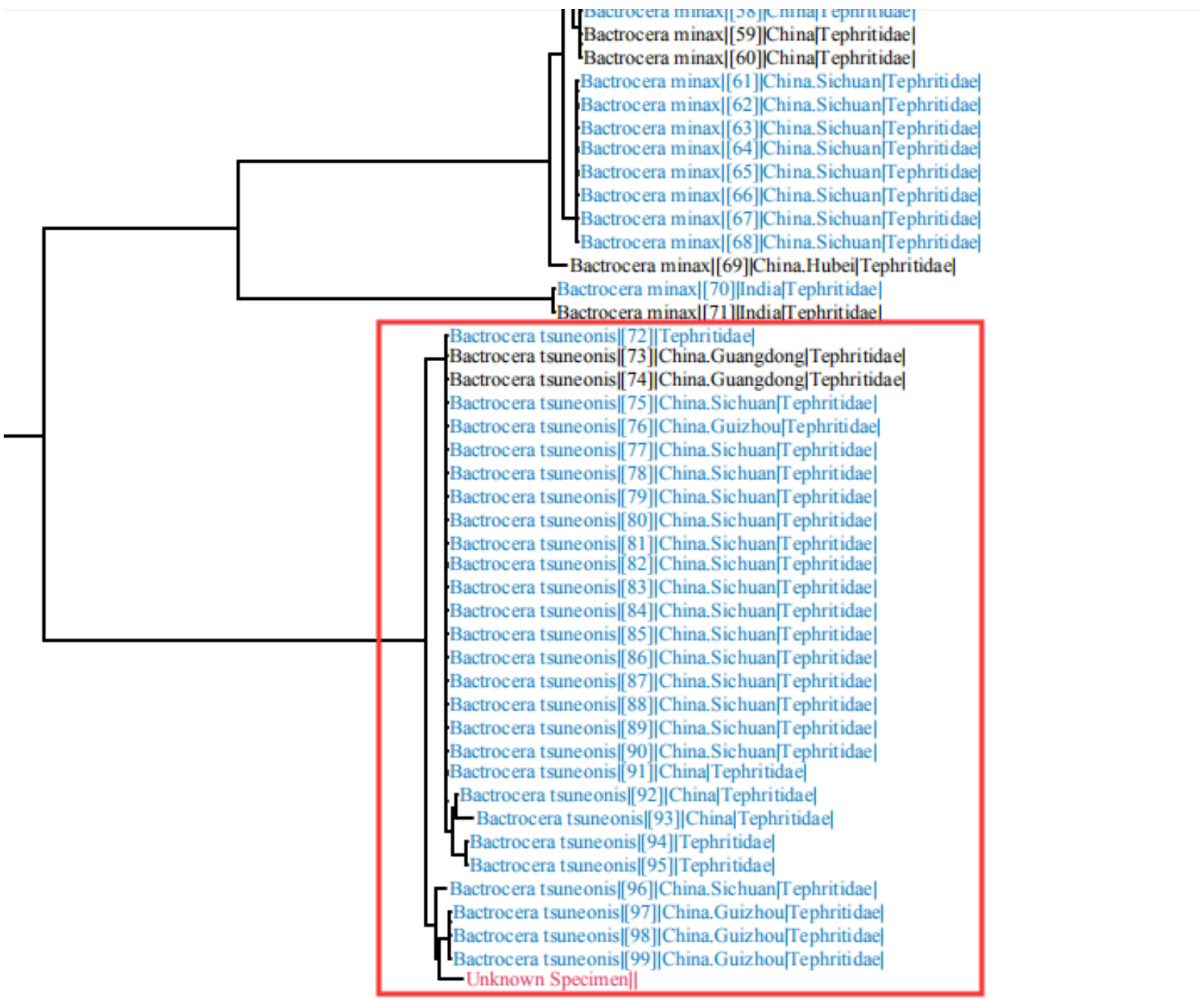
Export Tree to Newick Format : Download File

Taxonomy Report : View Report

Image List : View Image List

Spreadsheet : View Spreadsheet

NOTE : Query sequence will be marked red on the tree with BOLD sequences in black. GenB
sequence accuracy and taxonomic identification and will be marked in blue.


Bactrocera minax|[59]|China|Tephritidae|
Bactrocera minax|[60]|China|Tephritidae|
Bactrocera minax|[61]|China.Sichuan|Tephritidae|
Bactrocera minax|[62]|China.Sichuan|Tephritidae|
Bactrocera minax|[63]|China.Sichuan|Tephritidae|
Bactrocera minax|[64]|China.Sichuan|Tephritidae|
Bactrocera minax|[65]|China.Sichuan|Tephritidae|
Bactrocera minax|[66]|China.Sichuan|Tephritidae|
Bactrocera minax|[67]|China.Sichuan|Tephritidae|
Bactrocera minax|[68]|China.Sichuan|Tephritidae|
Bactrocera minax|[69]|China.Hubei|Tephritidae|
Bactrocera minax|[70]|India|Tephritidae|
Bactrocera minax|[71]|India|Tephritidae|
Bactrocera tsuneonis|[72]|Tephritidae|
Bactrocera tsuneonis|[73]|China.Guangdong|Tephritidae|
Bactrocera tsuneonis|[74]|China.Guangdong|Tephritidae|
Bactrocera tsuneonis|[75]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[76]|China.Guizhou|Tephritidae|
Bactrocera tsuneonis|[77]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[78]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[79]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[80]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[81]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[82]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[83]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[84]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[85]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[86]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[87]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[88]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[89]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[90]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[91]|China|Tephritidae|
Bactrocera tsuneonis|[92]|China|Tephritidae|
Bactrocera tsuneonis|[93]|China|Tephritidae|
Bactrocera tsuneonis|[94]|Tephritidae|
Bactrocera tsuneonis|[95]|Tephritidae|
Bactrocera tsuneonis|[96]|China.Sichuan|Tephritidae|
Bactrocera tsuneonis|[97]|China.Guizhou|Tephritidae|
Bactrocera tsuneonis|[98]|China.Guizhou|Tephritidae|
Bactrocera tsuneonis|[99]|China.Guizhou|Tephritidae|
Unknown Specimen||

3. Principle and operational guidance of specificity- primers technique

分类号：
密　级：

单位代码：10019
学　　号：B1201009

中国农业大学

博士学位论文

我国检疫性实蝇分子鉴定技术体系的研究

Technique System for Molecular Identification of Quarantine Fruit Flies in China

本研究获国家科技支撑计划课题（2012BAK11B01）和农业部"948"项目（2009-Z41）资助

研　　究　　生：姜　帆
指　导　教　师：李志红　教授
合 作 指 导 教 师：

申请学位门类级别：农学博士
专　业　名　称：植物检疫与农业生态健康
研　究　方　向：检疫鉴定与处理
所　在　学　院：农学与生物技术学院

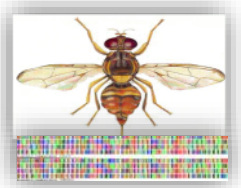
2015 年 6 月

How to design Specific primers

Sample collection and morphological identification

DNA extraction, PCR amplification and sequencing

Lco1490/Hco2198



DNA Barcodes database construction

CAUPQL+BOLD

Specific primers design, specificity and sensitivity test

DNA Barcodes database construction

	Number of species （BOLD/CAUPQL）	**Number of sequences （BOLD/CAUPQL）**
Anastrepha	7（7/2）	98（84/14）
Rhagoletis	12（12/2）	87（81/6）
Total	**181**	**2252**

CAUPQL：6 genera 50 species 700 items

BOLD：5 genera 154 species 1552 items

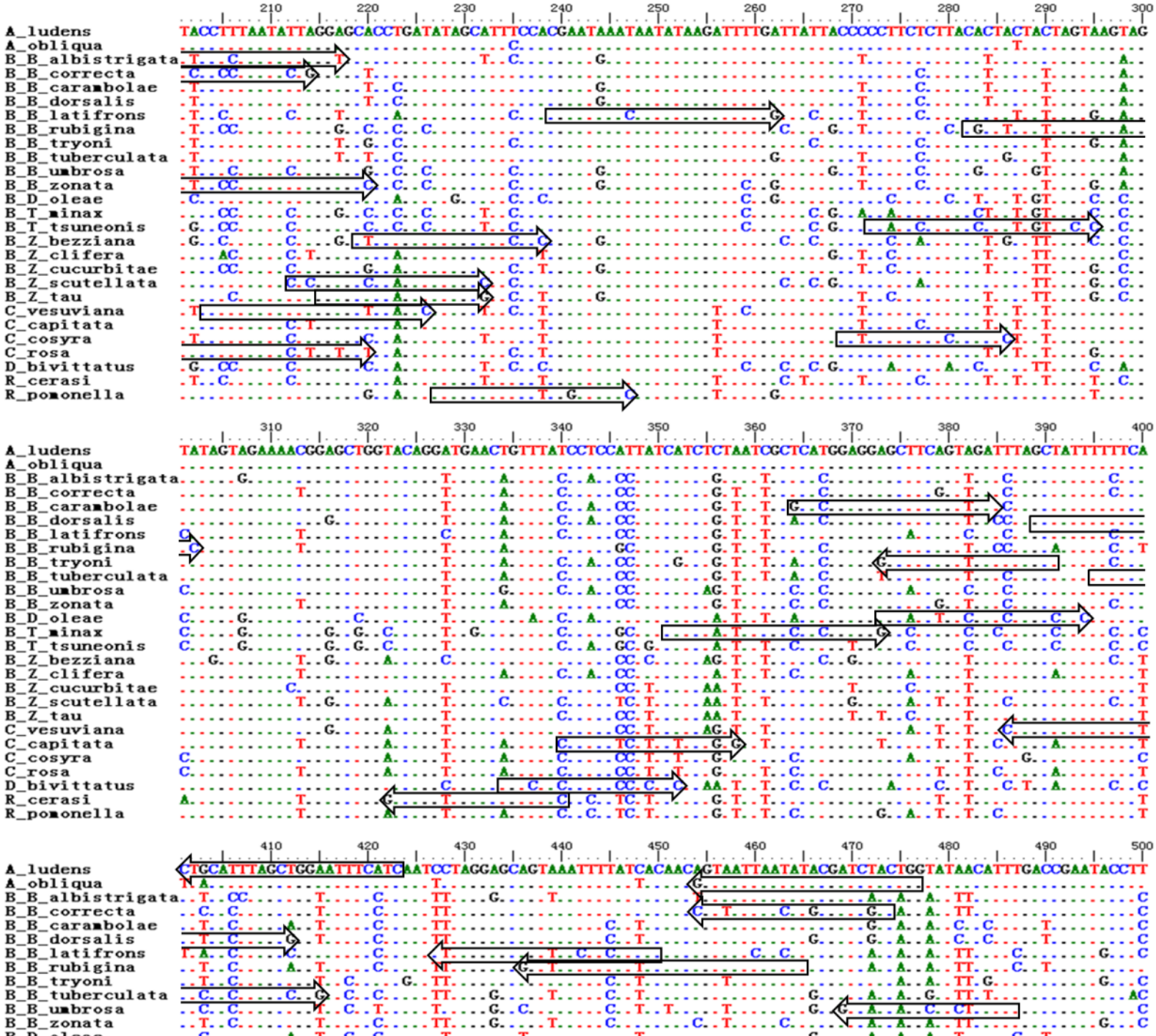
Specific Primer Design

1. Sequence alignment by using MEGA 7.0 software

2. C or G sites with intraspecific crosstalk and interspecific variation were selected by BioEdit software.

3. Design primers by Oligo 7.0 software.

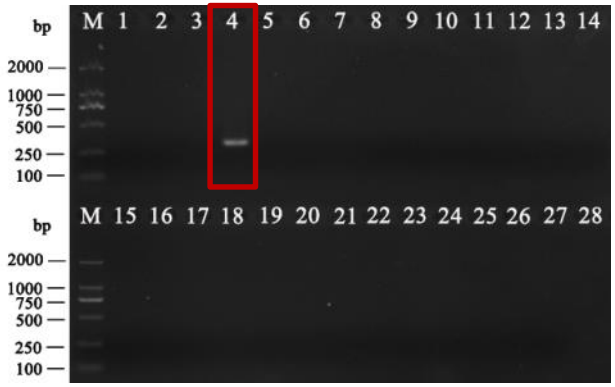
- The length of the primers was set as 25 bp.
- Species-specific sites were placed at the 3-ends of the forward and reverse primers
- The length between forward and reverse primers was not less than 200 bp.


A_ludens
A_obliqua
B_B_albistrigata
B_B_correcta
B_B_carambolae
B_B_dorsalis
B_B_latifrons
B_B_rubigina
B_B_tryoni
B_B_tuberculata
B_B_umbrosa
B_B_zonata
B_D_oleae
B_T_minax
B_T_tsuneonis
B_Z_bezziana
B_Z_clifera
B_Z_cucurbitae
B_Z_scutellata
B_Z_tau
C_vesuviana
C_capitata
C_cosyra
C_rosa
D_bivittatus
R_cerasi
R_pomonella
210
220
230
240
250
260
270
280
290
300
310
320
330
340
350
360
370
380
390
400
410
420
430
440
450
460
470
480
490
500

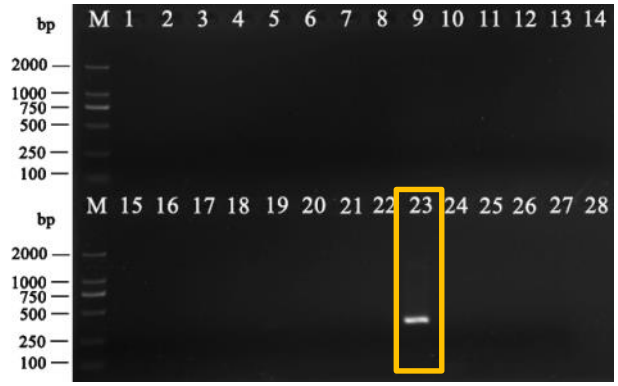
27 quarantine speices specific primers designed by CAUPQL

Species	Sequences（5’-3’）
A. lundens	CAATGTAATTGTAACAGCTCACACG
	GATGAAATTCCAGCTAAATGCAG
A. obliqua	ATAGTAATACCTATTATAATTGGG
	CCAGTAGATCGTATATTAATTACC
B. albistrigata	GACTTGTTCCTCTAATATTAGGT
	GCTCCTGCTAAAACTGGTAAG
B.correcta	TGACTTGTCCCCCTAATACTG
	GTCGATCGCATGTTAATAACG
B.carambolae	GCACGGAGGAGCTTCAGTTGAT
	GATAATAAAAGTAATAAAGCTGTTAATACT
B. dorsalis	GCTATTTTTTCACTTCACTTAACG
	AGTATTTAAGTTTCGGTCTGTTAG
B. latifrons	CGAATAAACAATATAAGATTTTGG
	GTGATGAAGTTAACTGCTCCTAAG
B. rubigina	CGCTTCTATTAGTAAGAAGTC
	ATATTAATTACTGTTGTAATAAAATTAACC
B. tryoni	ATTAATCGGAGACGATCAG
	AGCTAAATCAACTGAAACC
B. tuberculata	TTTTCACTCCACTTAGCCAGG
	GGGGTCAAAAAATGAAGTATTTAAGTTC
B. umbrosa	GCCCATTATAATCGGGTGC
	AAATGAGATGCCTGTTGAC
B. zonata	ACTTGTTCCCCTAATATTAGGAACC
	TGTTAATACAACTGCTCAGACGAAG
B. oleae	AGCATCTGTCGATCTAGCCATC
	TGGGTCGAAAAAGGAAGTATTC
B. minax	AATTTATAACGTAATCGTTACAGCC
	AAGTATTGTGATAGCTCCGGCTAGG

Species	Sequences（5’-3’）
B. tsuneonis	TAATGTAATCGTTACTGCTCTCACGCC
	CTGGGTCAAAGAAGGATGTATTTAG
Z. bezzianus	CTCCTGATATAGCATTCACC
	AAGTATAGTGATAGCTCCAACC
Z. cilifer	GGCTGTAAATTTTATCACTACAGTC
	CGGTCTGTCAAAAGTATAGTAATG
Z. cucurbitae	GGAGATGATCTAATCTATAATGTC
	GCTCAAACGAATAAAGGTAAC
Z.. scutellatus	CTCGGAGCCCCAGATATAACC
	GGGCTGTTAATACTACTGCTCAG
Z. tau	GGAGCACCAGATATAGCG
	GGTATTCGGTCAAATGTAATC
Ca. vesuviana	CCTTTAATATTAGGAGCTCCAGAC
	GCTAAGTGTAAAGAAAAAATAGCTAG
C. capitata	CCCTCCTCTTTCTTCTGTG
	TGGTAAAGATAATAATAGAAGTAGT
C. cosyra	CCTCCTTCTCTCACACTC
	GTTTAGATTTCGGTCAGTTAG
C. rosa	CTAGTACCTTTAATACTTGGTCCT
	ATAGAAGAAATTCCTGCTAAG
D. bivittatus	TGTCTACCCTCCCCTCTCC
	GACAACTGCTCAGACAAATAA
R. cerasi	GTAATTGTTACAGCCCATACC
	GTAAACAGTTCAACCTGTC
R. pomonella	ATAGCATTTCCTCGGATAAAC
	TCGATCAAATGAAATTCCAAC



Thailand wax apple



Tanzania mango

Guangxi, China bitter gourd

Sichuan, China citrus

Lanes 1-36：*A. ludens*；*A. obliqua*；*B. albistrigata*；*B. correcta*；*B. carambolae*；*B. dorsalis*；*B. latifrons*；*B. rubigina*；*B. tryoni*；*B. tuberculata*；*B. umbrosa*；*B. zonata*；*B. oleae*；*B. minax*；*B. tsuneonis*；*Z. bezzianus*；*Z. cilifera*；*Z. cucurbitae*；*Z. scutellatus*；*Z. tau*；*Ca. vesuviana*；*C. capitata*；*C. cosyra*；*C. rosa*；*D. bivittatus*；*R. cerasi*；*R. pomonella* and ddH2O；Lane M：D2000 Marker；

insects

MDPI

Article

New Species-Specific Primers for Molecular Diagnosis of *Bactrocera minax* and *Bactrocera tsuneonis* (Diptera: Tephritidae) in China Based on DNA Barcodes

Linyu Zheng [1,†], Yue Zhang [1,†], Wenzhao Yang [1], Yiying Zeng [1], Fan Jiang [2], Yujia Qin [2], Jiafeng Zhang [3], Zhaochun Jiang [4], Wenzhao Hu [5], Dijin Guo [6], Jia Wan [6], Zihua Zhao [1], Lijun Liu [1] and Zhihong Li [1,*]

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2 Institute of Plant Quarantine, Chinese Academy of Inspection and Quarantine, Beijing 100176, China; 13426369960@163.com (F.J.); qinyujia@cau.edu.cn (Y.Q.)
3 Hunan Plant Protection and Plant Quarantine Station, Changsha 410006, China; zhbao804@21cn.com
4 Guizhou Plant Protection and Plant Quarantine Station, Guiyang 550001, China; zbjzc@163.com
5 Chongqing Plant Protection and Plant Quarantine Station, Yubei 401123, China; yidao2003090012@163.com
6 Sichuan Plant Protection and Plant Quarantine Station, Chengdu 610041, China; guodijin2008@aliyun.com (D.G.); wan527jia@163.com (J.W.)
* Correspondence: lizh@cau.edu.cn; Tel.: +86-010-62733000
† These authors contributed equally to this work.

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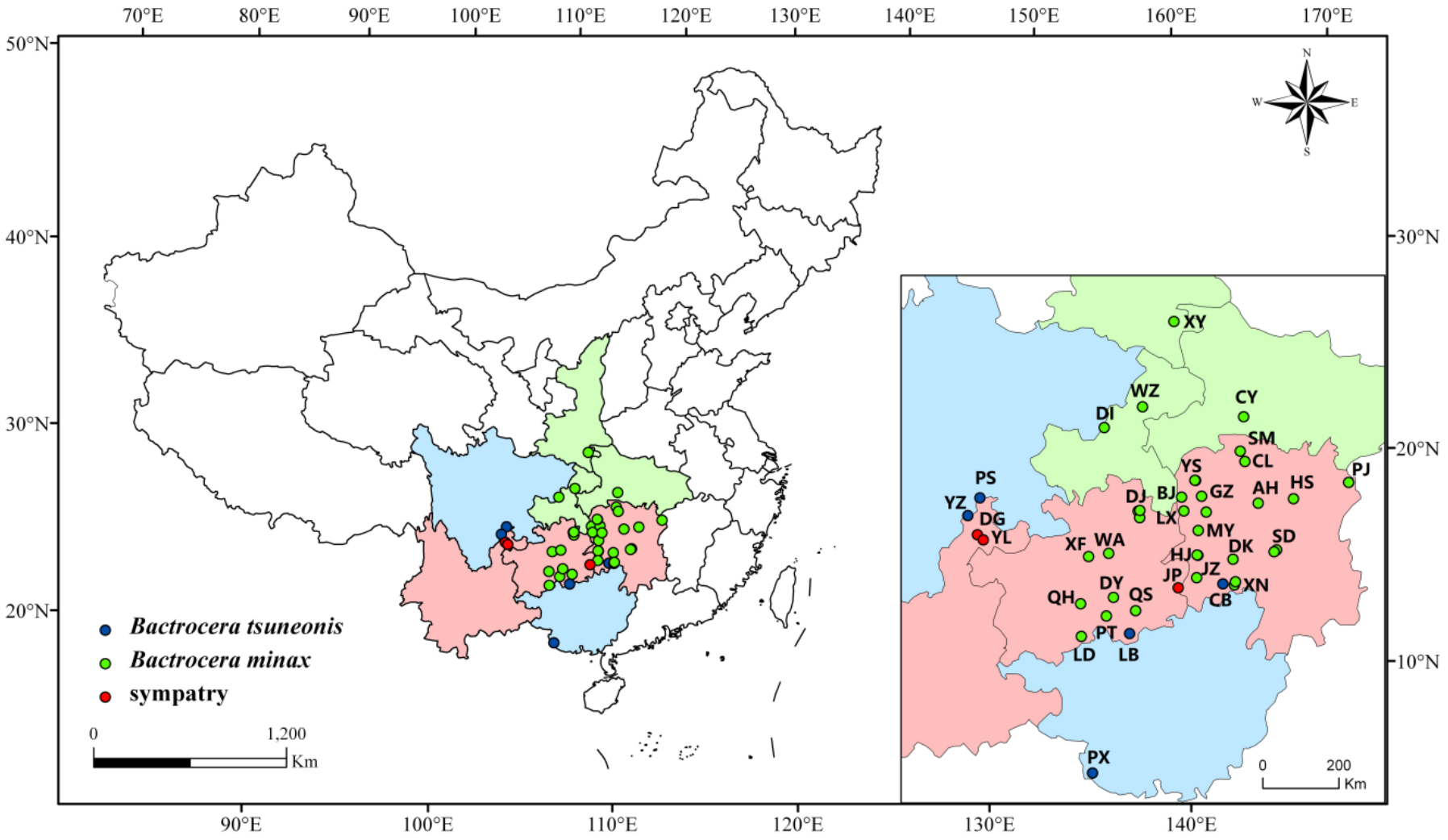
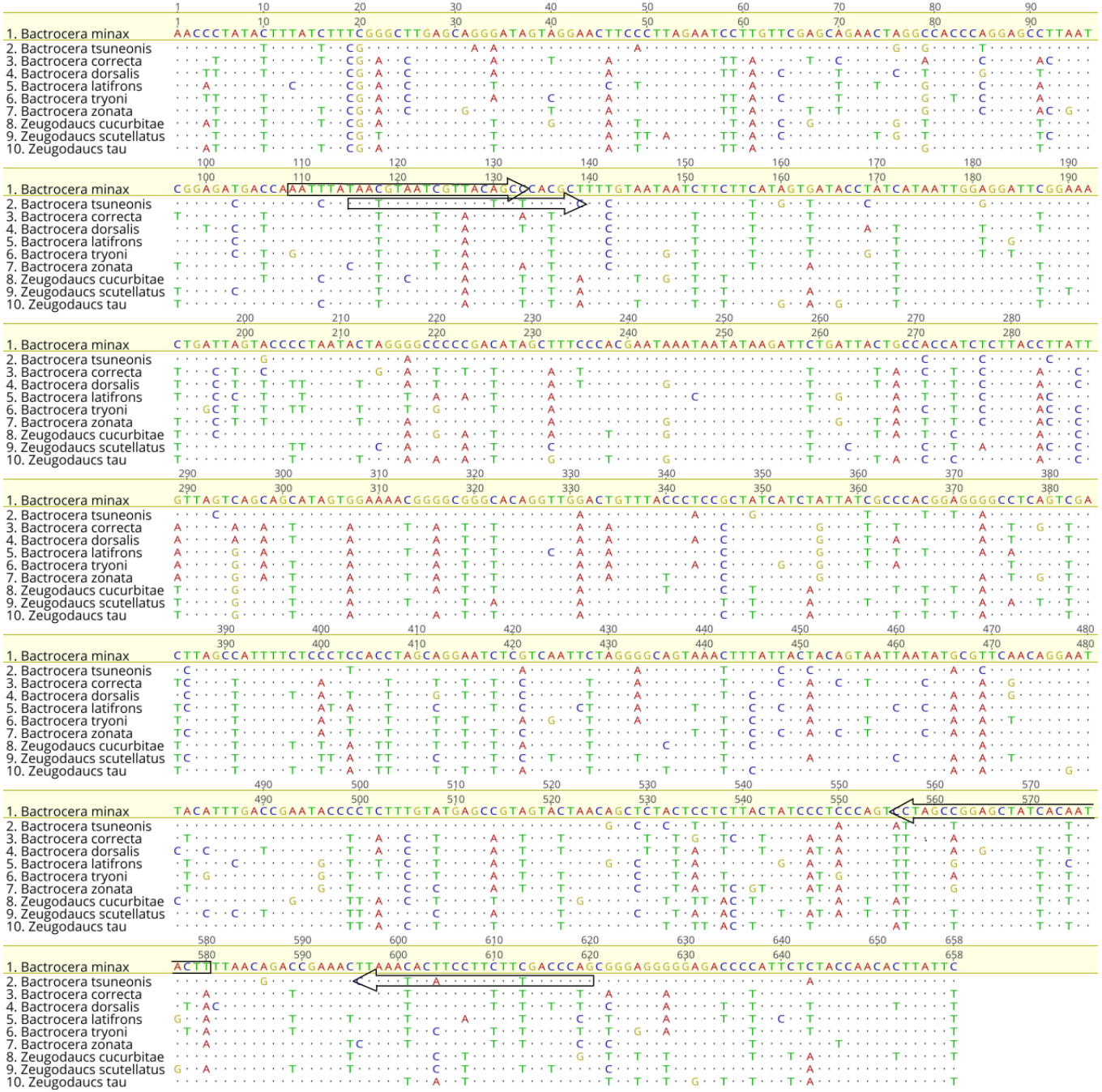


963 samples were collected from 44 locations from eight provinces in China


1. Bactrocera minax
2. Bactrocera tsuneonis
3. Bactrocera correcta
4. Bactrocera dorsalis
5. Bactrocera latifrons
6. Bactrocera tryoni
7. Bactrocera zonata
8. Zeugodacus cucurbitae
9. Zeugodacus scutellatus
10. Zeugodacus tau

List of specific primer sequences for *B. minax* and *B. tsuneonis*.

Species	Primer	Primers Sequence (5′-3′)	Size (bp)	Tm (°C)
B. minax	Bm-F	AATTTATAACGTAATCGTTACAGCC	422	53.9
	Bm-R	AAGTATTGTGATAGCTCCGGCTAGG		60.2
B. tsuneonis	Bt-F	TAATGTAATCGTTACTGCTCACGCC	456	59.9
	Bt-R	CTGGGTCAAAGAAGGATGTATTTAG		56.1

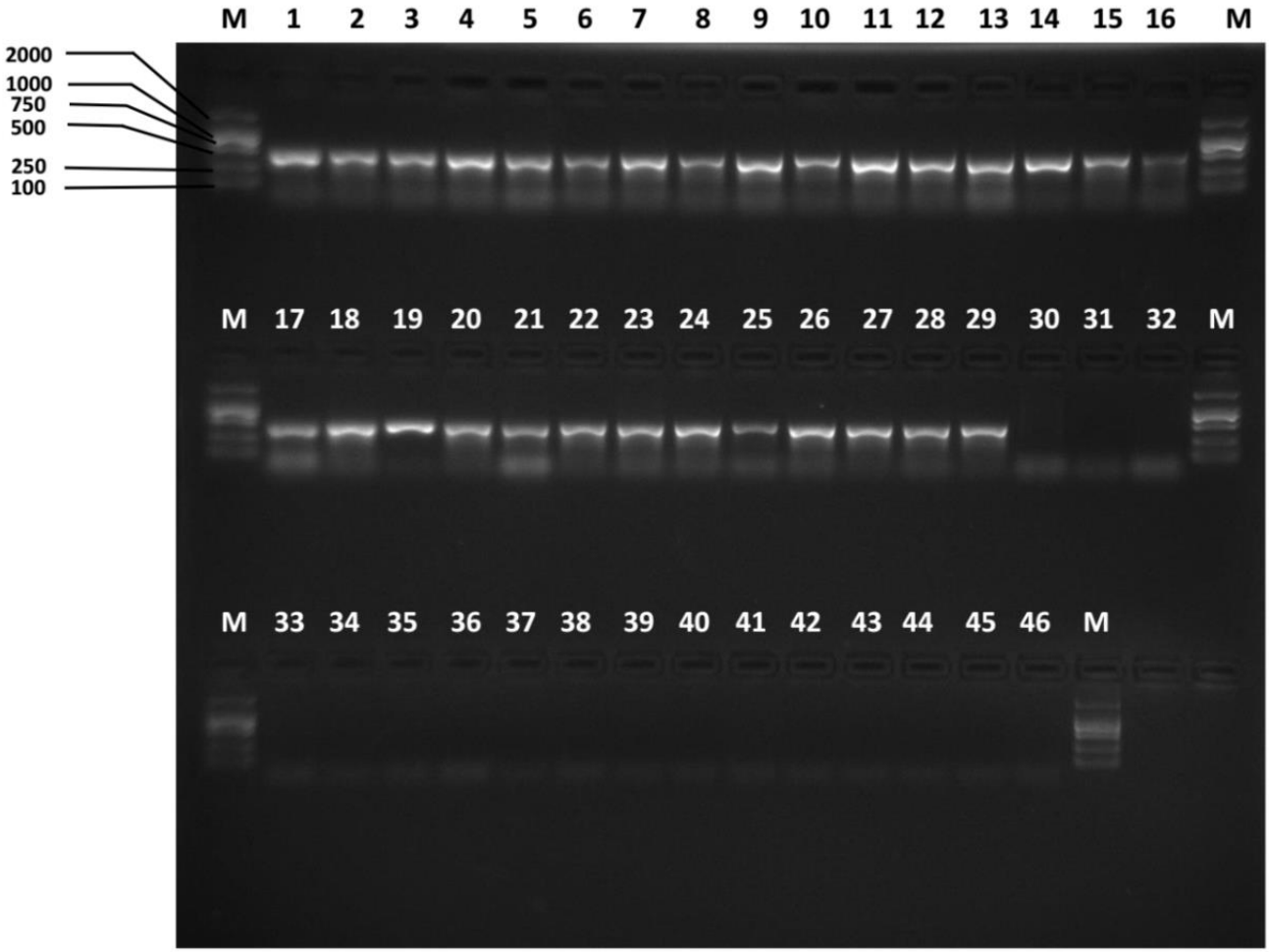


Figure 2. Specificity of the Bmina-F/Bmina-R *B. minax*-specific primer pair Lanes 1–29: *B. minax* from 29 geographical populations (Table S3); lanes 30–38: *B. tsuneonis* from nine geographical populations (Table S3); lane 39: *B. correcta*, lane 40: *B. dorsalis*; lane 41: *B. latifrons*; lane 42: *B. tryoni*; lane 43: *B. zonata*; lane 44: *Zeugodacus cucurbitae*; lane 45: *Z. scutellatus*; lane 46: *Z. tau*; lane M: D2000.

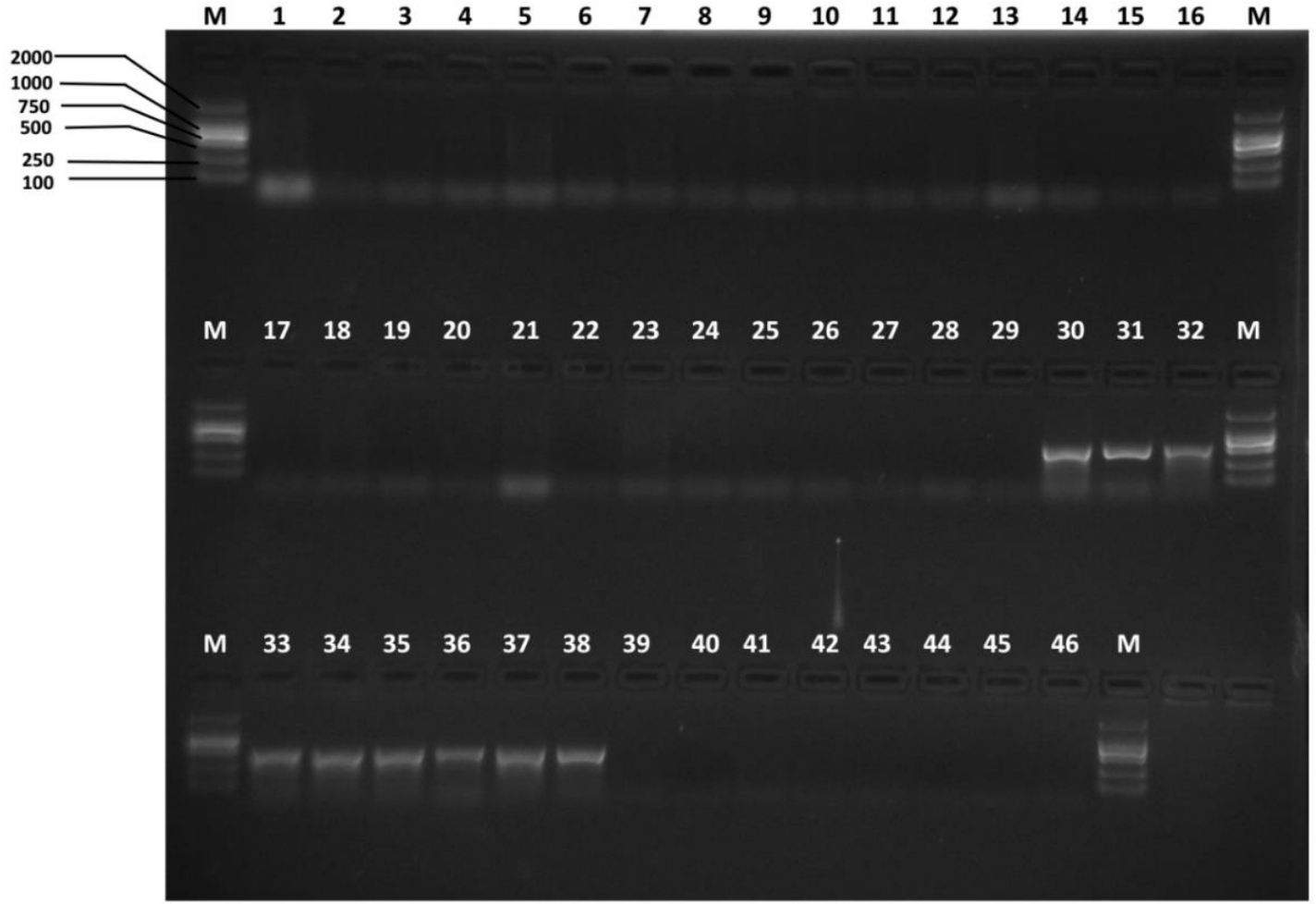


Figure 3. Specificity of the Btsun-F/Btsun-R *B. minax*-specific primer pair Lanes 1–29: *B. minax* from 29 geographical populations (Table S3); lanes 30–38: *B. tsuneonis* from nine geographical populations (Table S3); lane 39: *B. correcta*, lane 40: *B. dorsalis*; lane 41: *B. latifrons*; lane 42: *B. tryoni*; lane 43: *B. zonata*; lane 44: *Zeugodacus cucurbitae*; lane 45: *Z. scutellatus*; lane 46: *Z. tau*; lane M: D2000.

PCR to amplify the DNA Barcodes

- **PCR reaction system**

Template 2 ul

Forward primer (10uM) 2 ul

Reverse primer (10uM) 2 ul

2×Taq PCR Mastermix 25 ul (Taq polymerase, dNTP, reaction buffer)

ddH2O 19 ul

- **Reaction condition**

95℃ 3 min

95℃ 15 s

60℃ 1min

60℃ 1 min } 30×

List of species-specific primer pairs of 2 fruit flies species

Species	Primer	Primers Sequence (5′-3′)	Size (bp)	Tm (°C)
B. minax	Bm-F	AATTTATAACGTAATCGTTACAGCC	422	53.9
	Bm-R	AAGTATTGTGATAGCTCCGGCTAGG		60.2
B. tsuneonis	Bt-F	TAATGTAATCGTTACTGCTCACGCC	456	59.9
	Bt-R	CTGGGTCAAAGAAGGATGTATTTAG		56.1

Practices and Experiment

1. Molecular identification of fruit flies based on DNA barcodes
2. Molecular identification of fruit flies based on specific- primers

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