TracRice

iniav Instituto Nacional de Investigação Agrária e Veterinária, I.P.

INSECT GENETICS IMProvement IMPlementation IMPact

EAAP European Federation of Animal Science

Optimizing COI Barcoding for molecular detection of hidden insect infestation in rice grains: evaluating primer efficiency and detection limits

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[4]GREEN-IT Bioresources for Sustainability, ITQB NOVA, Oeiras, Portugal

cost
EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

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RICE: a staple food

Portugal: 17kg/person/year

Source: FAOStat via HelgiLibrary
Created with MapChart

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WHAT'S BUGGING YOU?

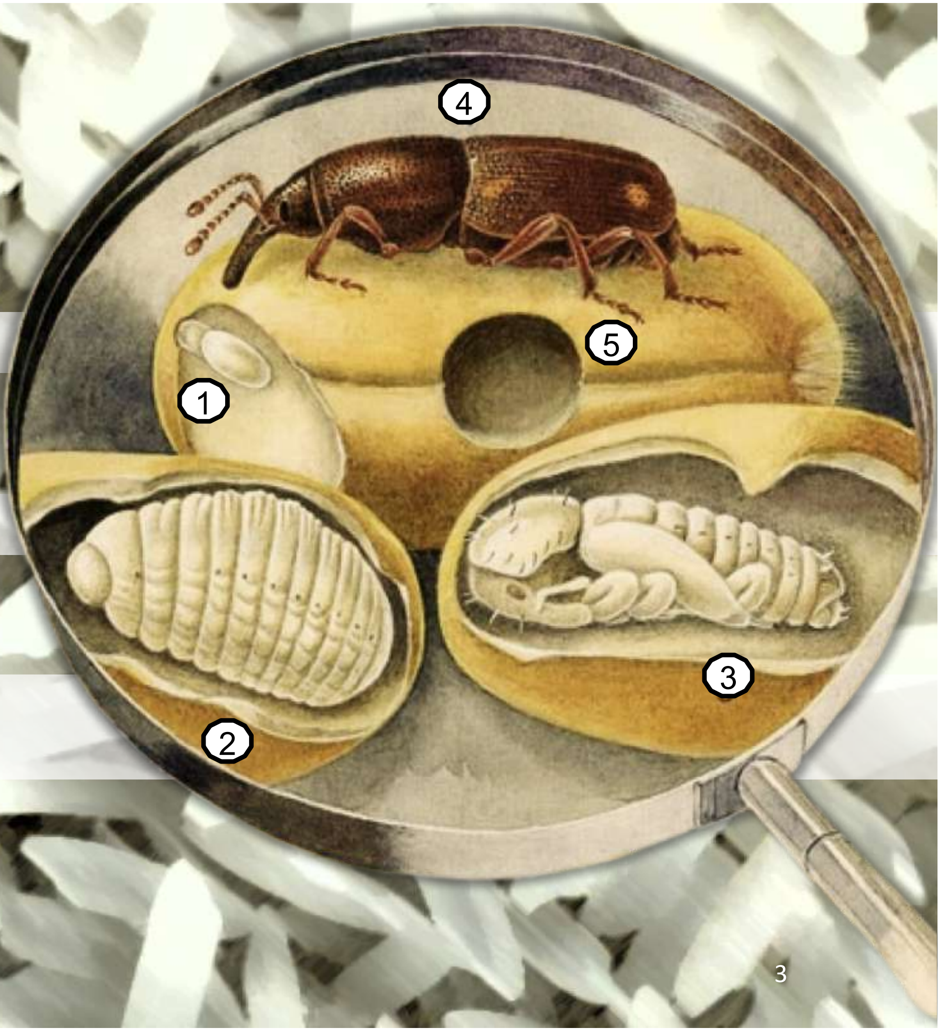
Sitophilus spp. cereal life cycle ± 28 DAYS

Favourable temperatures:
25 to 30 °C
High relative humidity:
70 to 80%

① Deposition of eggs inside the rice grain

② ③ The pupation process

④ ⑤ Exit hole is made, from which it then emerges the adult insect



WHAT'S BUGGING YOU?

ISO 6339-4:1987 - Cereals and Pulses—Determination of Hidden Insect Infestation

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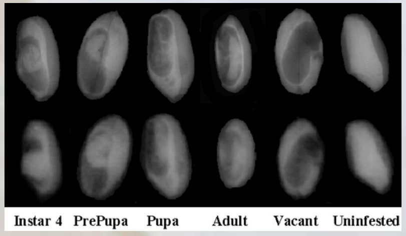
- DETERMINATION OF CO2 PRODUCTION METHOD
- NINHYDRIN METHOD

NEGATIVE TEST
Free α-amino group not present

POSITIVE TEST
Free α-amino group present

- X-RAY METHOD



- ACOUSTIC METHOD
- FLOTATION METHOD

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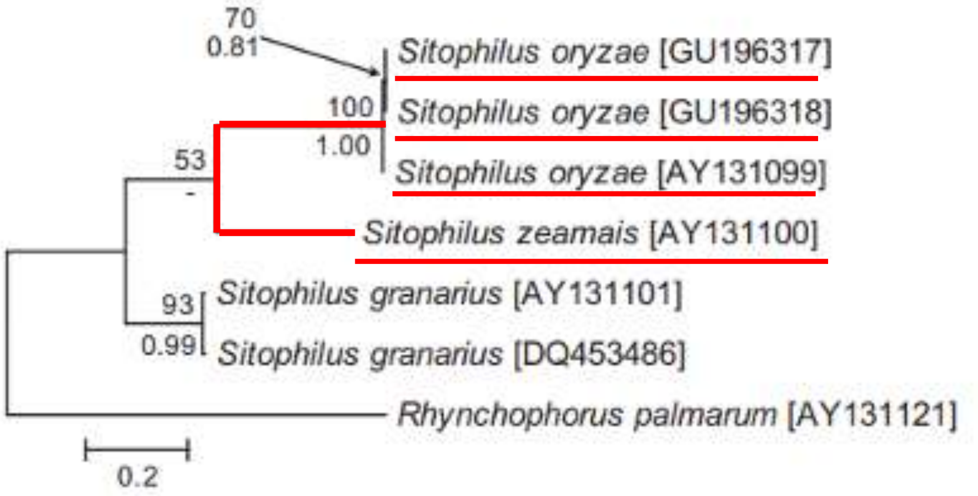
WHICH INSECTS
ARE MORE IMPORTANT TO DETECT ?

Sitophilus zeamais

Sitophilus oryzae



Toju et al., Nature, 2013

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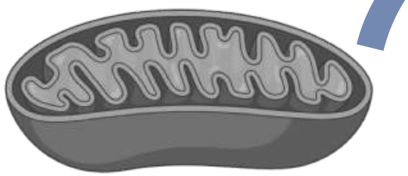
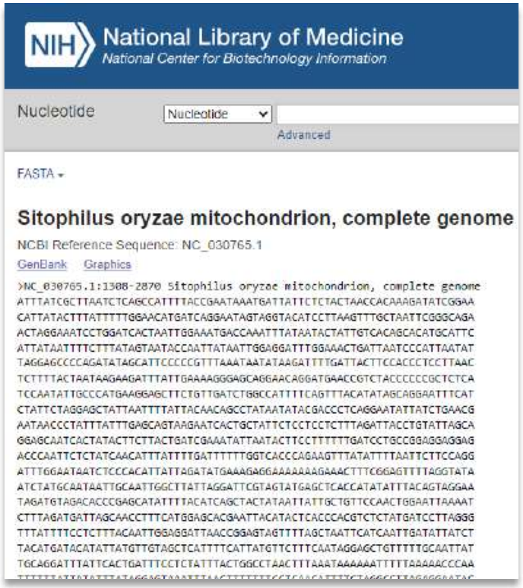
COI BARCODING

PRIMERS AND PROBES DESIGN



S. oryzae and *S. zeamais* mtDNA

Cytochrome oxidase subunit 1 COI



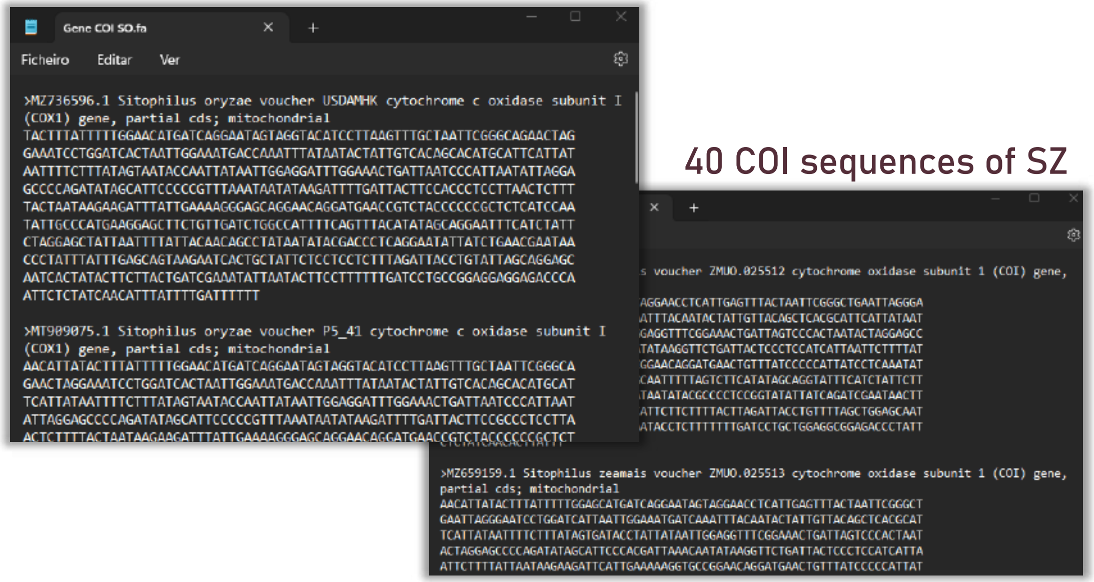
Sitophilus oryzae mitochondrion, complete genome

NCBI Reference Sequence: NC_030765.1

```
>NC_030765.1:1308-2870 Sitophilus oryzae mitochondrion, complete genome
ATTTATCGCTTAATCTCAGCCATTTTACCGAATAAATGATTATTCTCTACTAACCACAAAGATATCGGAA
CATTATACTTTATTTTTGGAACATGATCAGGAATAGTAGGTACATCCTTAAGTTTGCTAATTCGGGCAGA
ACTAGGAAATCCTGGATCACTAATTGGAAATGACCAAATTTATAATACTATTGTCACAGCACATGCATTC
ATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAACTGATTAATCCCATTAATAT
TAGGAGCCCCAGATATAGCATTCCCCCGTTTAAATAATATAAGATTTTGATTACTTCCACCCTCCTTAAC
TCTTTTACTAATAAGAAGATTTATTGAAAAGGGAGCAGGAACAGGATGAACCGTCTACCCCCCGCTCTCA
TCCAATATTGCCCATGAAGGAGCTTCTGTTGATCTGGCCATTTTCAGTTTACATATAGCAGGAATTTCAT
CTATTCTAGGAGCTATTAATTTTATTACAACAGCCTATAATATACGACCCTCAGGAATATTATCTGAACG
AATAACCCTATTTATTTGAGCAGTAAGAATCACTGCTATTCTCCTCCTCTTTAGATTACCTGTATTAGCA
GGAGCAATCACTATACTTCTTACTGATCGAAATATTAATACTTCCTTTTTTGATCCTGCCGGAGGAGGAG
ACCCAATTCTCTATCAACATTTATTTTGATTTTTTGGTCACCCAGAAGTTTATATTTTAATTCTTCCAGG
ATTTGGAATAATCTCCCACATTATTAGATATGAAAGAGGAAAAAAAGAAACTTTCGGAGTTTTAGGTATA
ATCTATGCAATAATTGCAATTGGCTTATTAGGATTCGTAGTATGAGCTCACCATATATTTACAGTAGGAA
TAGATGTAGACACCCGAGCATATTTTACATCAGCTACTATAATTATTGCTGTTCCAACTGGAATTAAAAT
CTTTAGATGATTAGCAACCTTTCATGGAGCACGAATTACATACTCACCCACGTCTCTATGATCCTTAGGG
TTTATTTTCCTCTTTACAATTGGAGGATTAACCGGAGTAGTTTTAGCTAATTCATCAATTGATATTATCT
TACATGATACATATTATGTTGTAGCTCATTTTCATTATGTTCTTTCAATAGGAGCTGTTTTTGCAATTAT
TGCAGGATTTATTCACTGATTTCCTCTATTTACTGGCCTAACTTTAAATAAAAAATTTTTAAAAACCCAA
```

Compilation of DNA sequences from GenBank®

211 COI sequences of SO



```
>MZ736596.1 Sitophilus oryzae voucher USDAMHK cytochrome c oxidase subunit I
(COX1) gene, partial cds; mitochondrial
TACTTTATTTTTGGAACATGATCAGGAATAGTAGGTACATCCTTAAGTTTGCTAATTCGGGCAGAACTAG
GAAATCCTGGATCACTAATTGGAAATGACCAAATTTATAATACTATTGTCACAGCACATGCATTCATTAT
AATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAACTGATTAATCCCATTAATATTAGGA
GCCCCAGATATAGCATTCCCCCGTTTAAATAATATAAGATTTTGATTACTTCCACCCTCCTTAACTCTTT
TACTAATAAGAAGATTTATTGAAAAGGGAGCAGGAACAGGATGAACCGTCTACCCCCCGCTCTCATCCAA
TATTGCCCATGAAGGAGCTTCTGTTGATCTGGCCATTTTCAGTTTACATATAGCAGGAATTTCATCTATT
CTAGGAGCTATTAATTTTATTACAACAGCCTATAATATACGACCCTCAGGAATATTATCTGAACGAATAA
CCCTATTTATTTGAGCAGTAAGAATCACTGCTATTCTCCTCCTCTTTAGATTACCTGTATTAGCAGGAGC
AATCACTATACTTCTTACTGATCGAAATATTAATACTTCCTTTTTTGATCCTGCCGGAGGAGGAGACCCA
ATTCTCTATCAACATTTATTTTGATTTTTT

>MT989075.1 Sitophilus oryzae voucher P5_41 cytochrome c oxidase subunit I
(COX1) gene, partial cds; mitochondrial
AACATTATACTTTATTTTTGGAACATGATCAGGAATAGTAGGTACATCCTTAAGTTTGCTAATTCGGGCA
GAACTAGGAAATCCTGGATCACTAATTGGAAATGACCAAATTTATAATACTATTGTCACAGCACATGCAT
TCATTATAATTTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGAAACTGATTAATCCCATTAAT
ATTAGGAGCCCCAGATATAGCATTCCCCCGTTTAAATAATATAAGATTTTGATTACTTCCGCCCTCCTTA
ACTCTTTTACTAATAAGAAGATTTATTGAAAAGGGAGCAGGAACAGGATGAACCGTCTACCCCCCGCTCT
```

40 COI sequences of SZ

```
s voucher ZMU0.025512 cytochrome oxidase subunit 1 (COI) gene,

AGGAACCTCATTGAGTTTACTAATTCGGGCTGAATTAGGGA
ATTTACAATACTATTGTTACAGCTCACGCATTCATTATAAT
GAGGTTTCGGAAACTGATTAGTCCCACTAATACTAGGAGCC
TATAAGGTTCTGATTACTCCCTCCATCATTAATTCTTTTAT
GGAACAGGATGAACTGTTTATCCCCCATTATCCTCAAATAT
CAATTTTTAGTCTTCATATAGCAGGTATTTCATCTATTCTT
TAATATACGCCCCTCCGGTATATTATCAGATCGAATAACTT
ATTCTTCTTTTACTTAGATTACCTGTTTTAGCTGGAGCAAT
ATACCTCTTTTTTTGATCCTGCTGGAGGCGGAGACCCTATT

>MZ659159.1 Sitophilus zeamais voucher ZMU0.025513 cytochrome oxidase subunit 1 (COI) gene,
partial cds; mitochondrial
AACATTATACTTTATTTTTGGAGCATGATCAGGAATAGTAGGAACCTCATTGAGTTTACTAATTCGGGCT
GAATTAGGGAATCCTGGATCATTAATTGGAAATGATCAAATTTACAATACTATTGTTACAGCTCACGCAT
TCATTATAATTTTCTTTATAGTGATACCTATTATAATTGGAGGTTTCGGAAACTGATTAGTCCCACTAAT
ACTAGGAGCCCCAGATATAGCATTCCCACGATTAAACAATATAAGGTTCTGATTACTCCCTCCATCATTA
ATTCTTTTATTAATAAGAAGATTCATTGAAAAAGGTGCCGGAACAGGATGAACTGTTTATCCCCCATTAT
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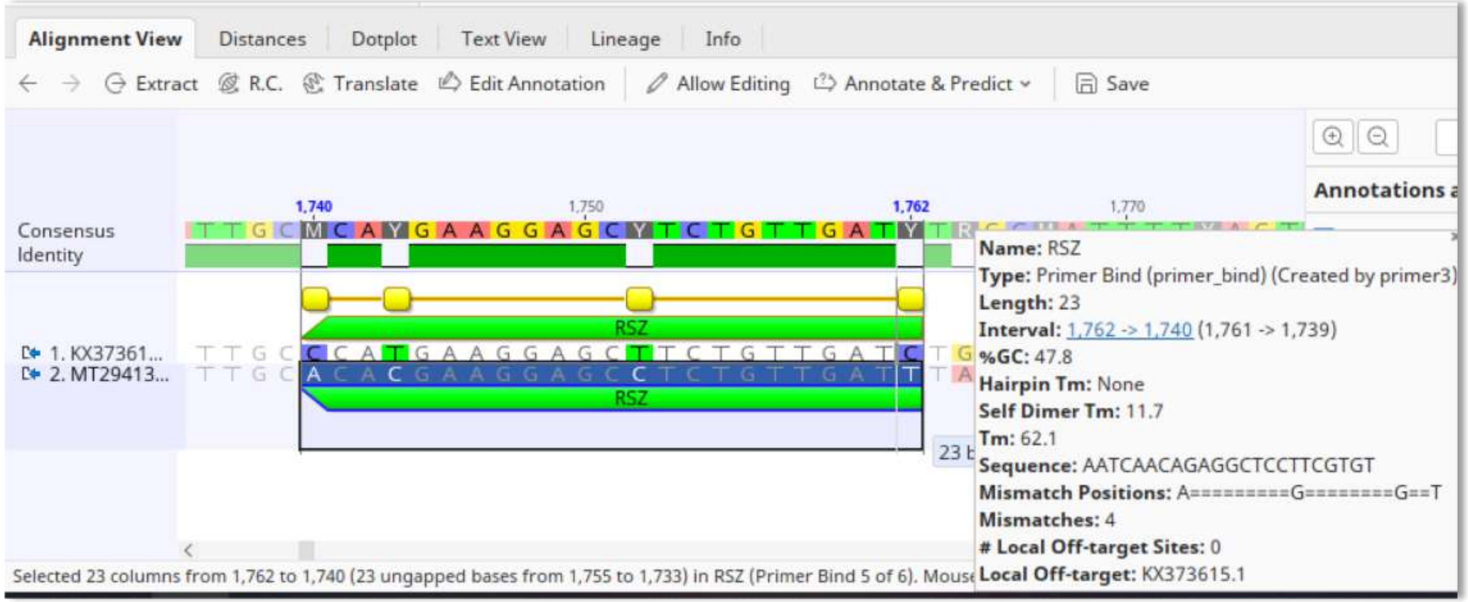
PRIMERS AND PROBES DESIGN

geneious prime

Sequence alignment and primer&probes design



Name: RSZ
Type: Primer Bind (primer_bind) (Created by primer3)
Length: 23
Interval: 1,762 -> 1,740 (1,761 -> 1,739)
%GC: 47.8
Hairpin Tm: None
Self Dimer Tm: 11.7
Tm: 62.1
Sequence: AATCAACAGAGGCTCCTTCGTGT
Mismatch Positions: A==========G=========G==T
Mismatches: 4
 # Local Off-target Sites: 0
Local Off-target: KX373615.1

Selected 23 columns from 1,762 to 1,740 (23 ungapped bases from 1,755 to 1,733) in RSZ (Primer Bind 5 of 6). Mouse

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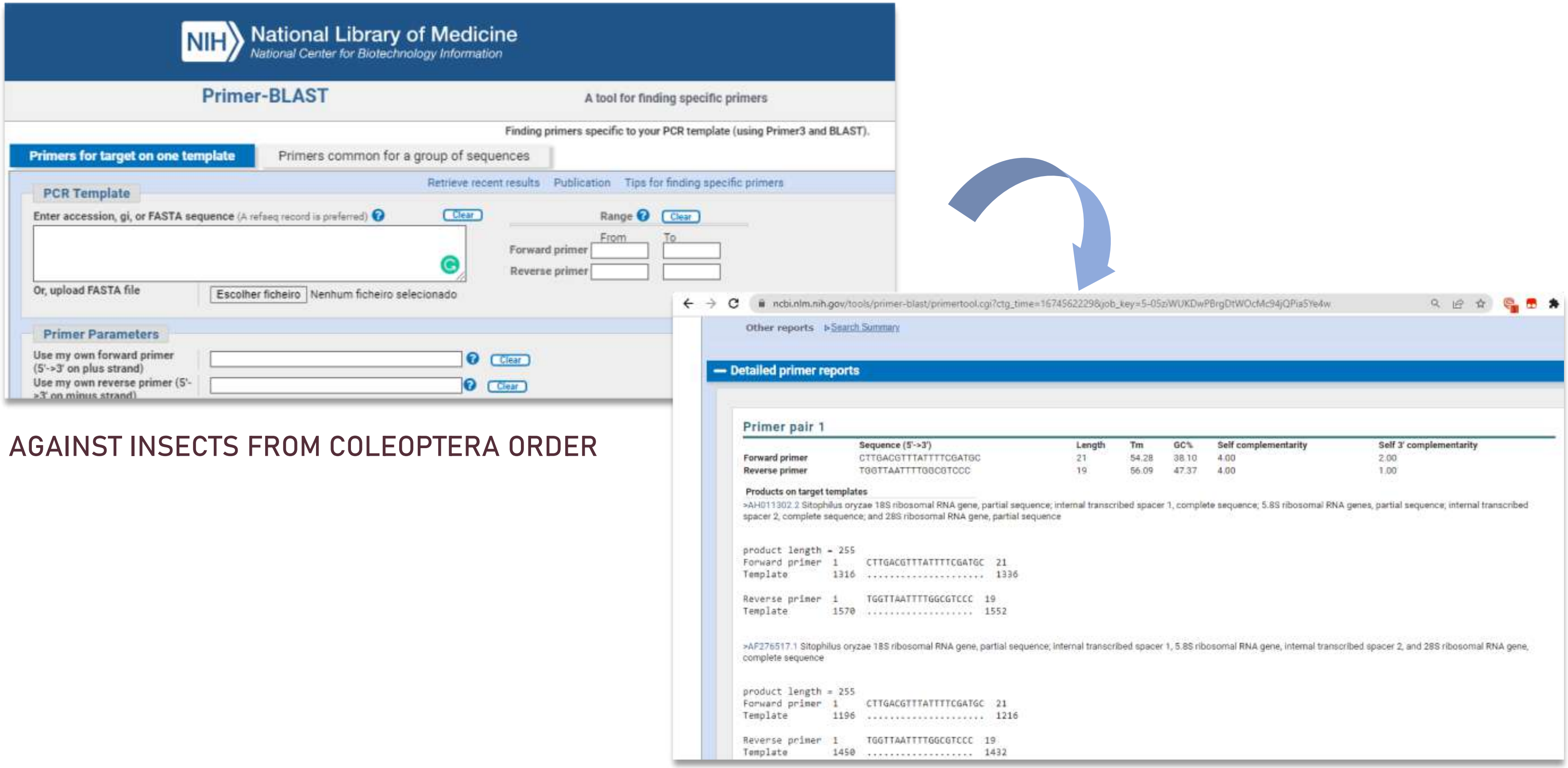
IN SILICO SPECIFICITY

PRIMER BLAST



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTTGACGTTTATTTTCGATGC	21	54.28	38.10	4.00	2.00
Reverse primer	TGGTTAATTTTGGCGTCCC	19	56.09	47.37	4.00	1.00

Products on target templates

>AH011302.2 Sitophilus oryzae 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; 5.8S ribosomal RNA genes, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
product length = 255
Forward primer  1     CTTGACGTTTATTTTCGATGC  21
Template        1316  .....................  1336

Reverse primer  1     TGGTTAATTTTGGCGTCCC  19
Template        1570  ...................  1552
```

>AF276517.1 Sitophilus oryzae 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence

```
product length = 255
Forward primer  1     CTTGACGTTTATTTTCGATGC  21
Template        1196  .....................  1216

Reverse primer  1     TGGTTAATTTTGGCGTCCC  19
Template        1450  ...................  1432
```

AGAINST INSECTS FROM COLEOPTERA ORDER

INSECT PRE-IDENTIFICATION

GENITALIA MORPHOLOGY – SELECTING POSITIVE CONTROLS

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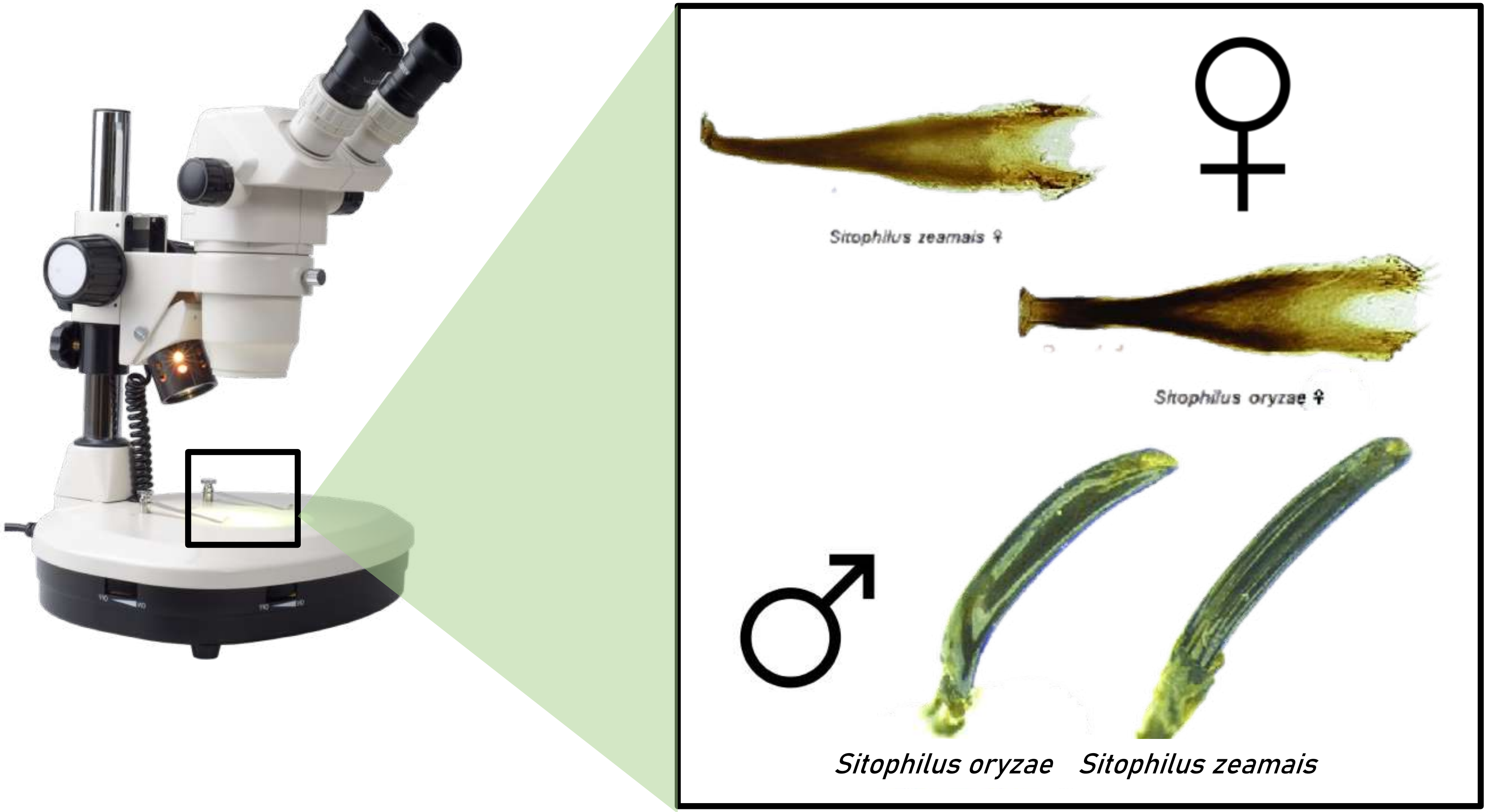

Hong et al., Journal of Asia-Pacific Biodiversity, 2018

INSECT DNA EXTRACTION

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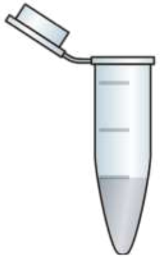
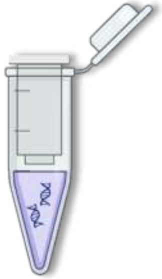
Grinding whole insects in a mortar

nzytech tissue gdna isolation kit



4h lysis at 56 °C



Purified DNA extract

qPCR

PROTOCOL OPTIMIZATION

TEMPERATURE GRADIENT

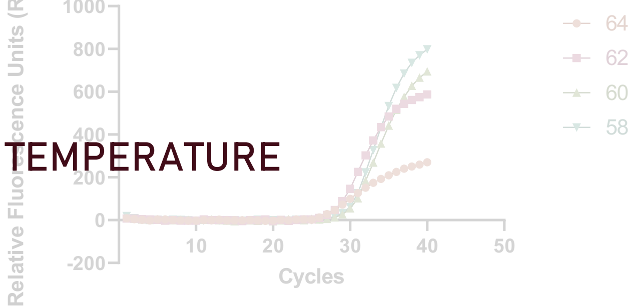
SO

Annealing Temperature (°C)	Mean Cq	Stand. Desv.	Maximum RFU
64	29,99	1,71	LOW
62	[illegible]	[illegible]	MEDIUM
60	29,76	0,38	HIGH
58	29,59	0,14	HIGH

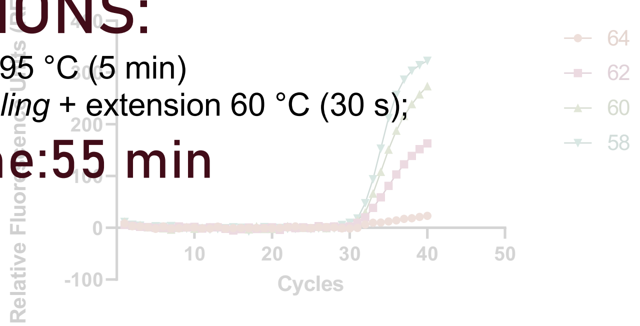
SZ

Annealing Temperature (°C)	Mean Cq	Stand. Desv.	Maximum RFU
64	31,46	1,20	LOW
62	30,68	0,45	MEDIUM
60	29,76	0,40	HIGH
58	29,72	0,67	HIGH

SO GRADIENT PLOT



SZ GRADIENT PLOT



BEST PRIMER ANNEALING TEMPERATURE

60 °C

PCR CONDITIONS:

INITIAL DENATURATION: 95 °C (5 min)

40 Cycles - denaturation 95 °C (5 s); *annealing* + extension 60 °C (30 s);

Total protocol time:55 min

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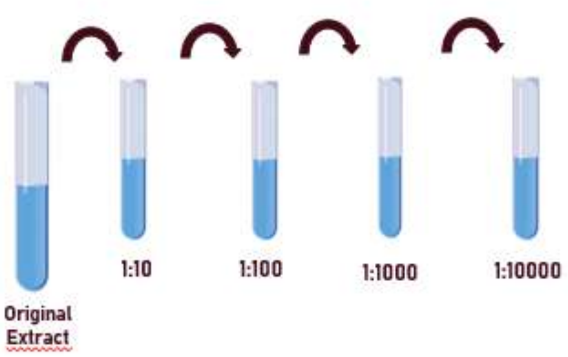
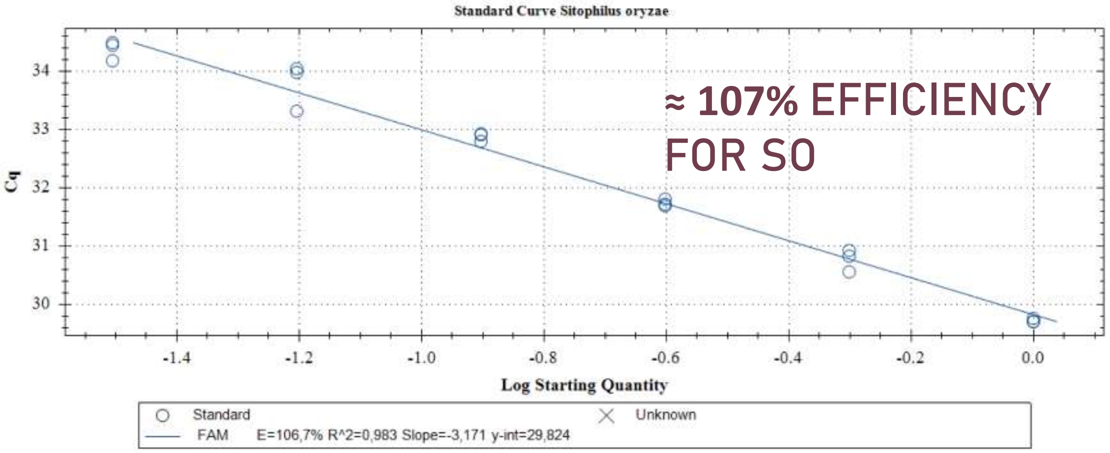
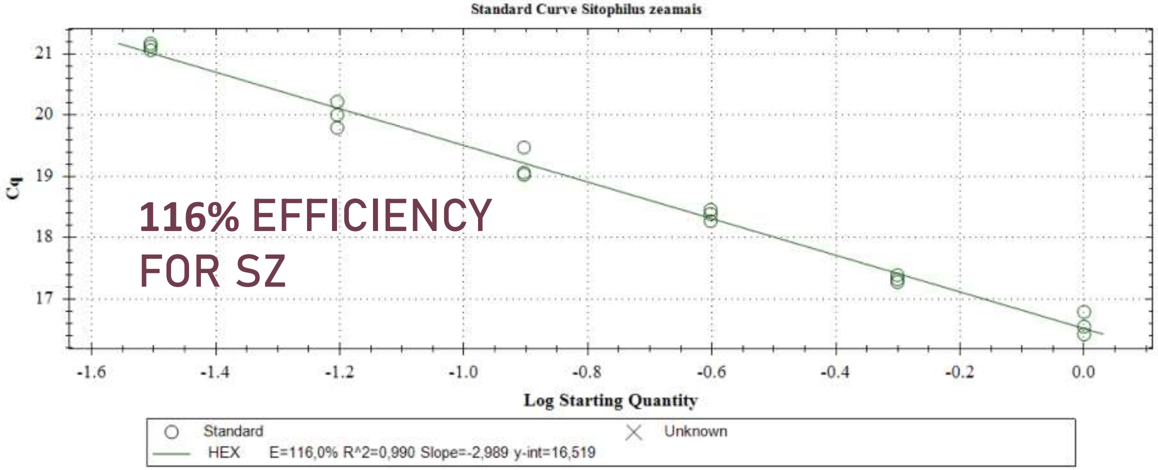
PRIMER EFFICIENCY

Serial dilution of the sample:





≈ 107% EFFICIENCY FOR SO



116% EFFICIENCY FOR SZ

IDEAL EFFICIENCY RANGE

SPECIFICITY ASSAY

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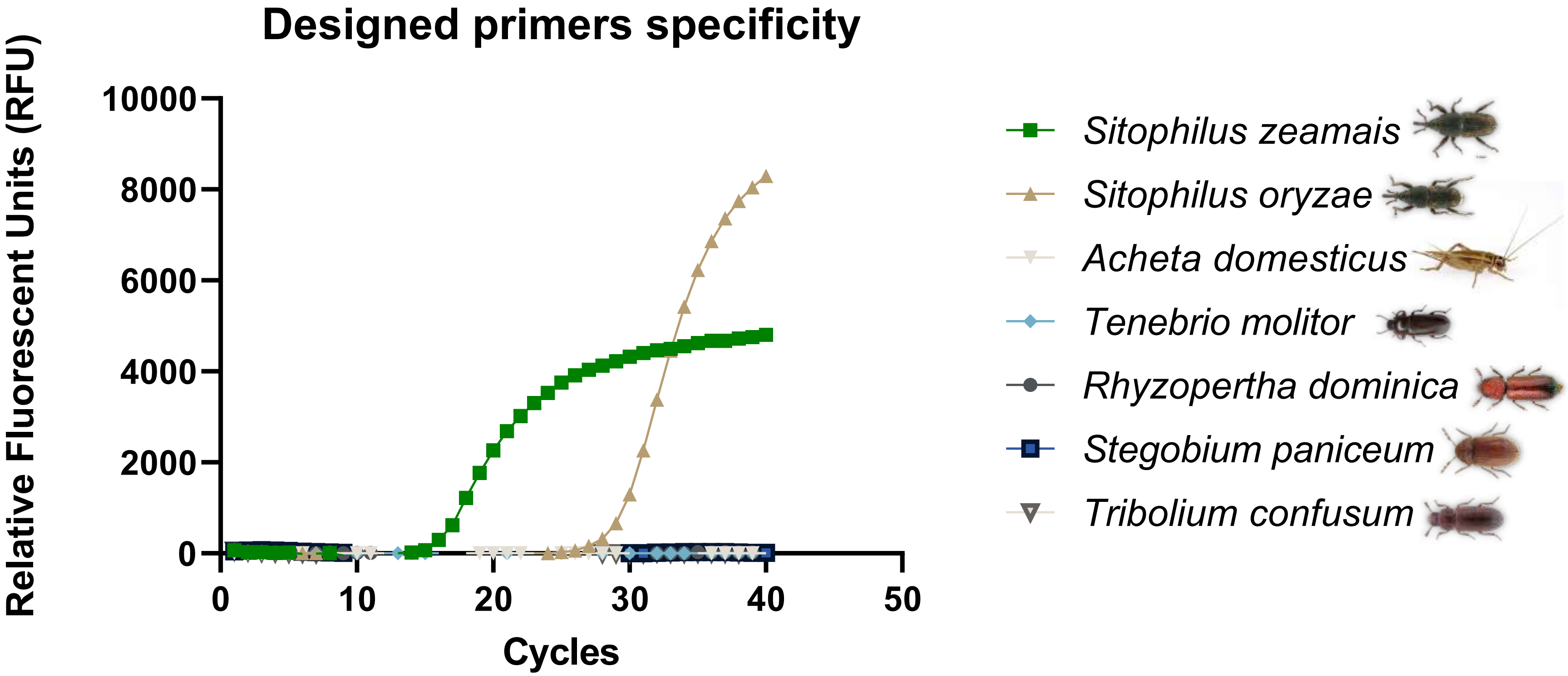

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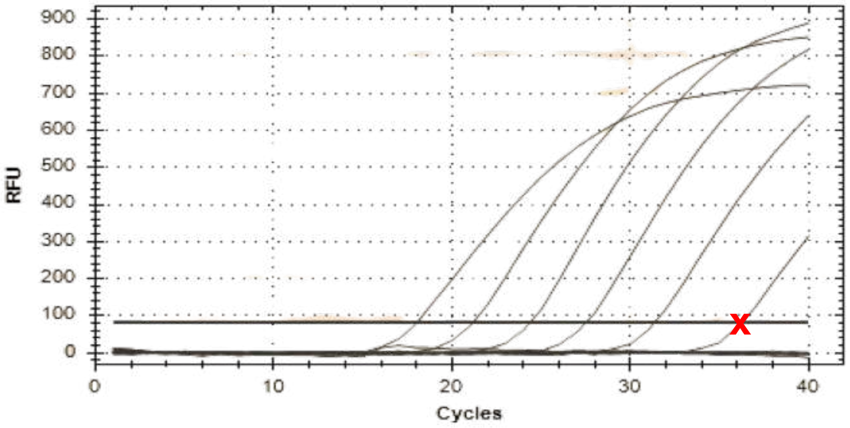
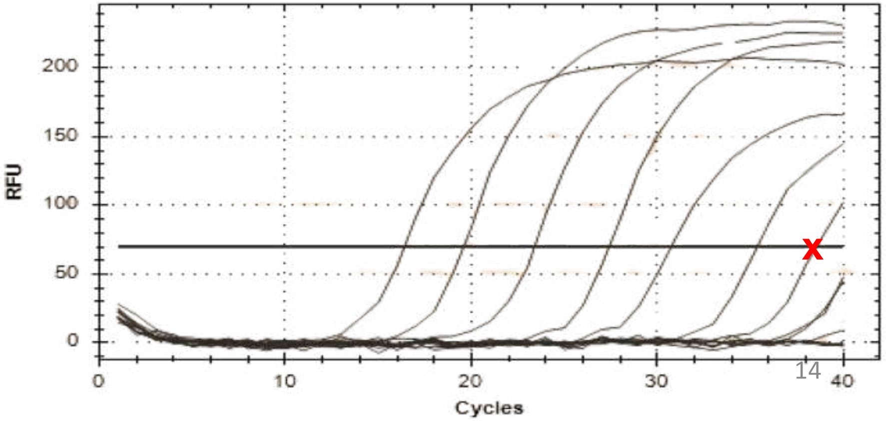
EXPERIMENTAL LIMIT OF DETECTION
OF THE qPCR

Sitophilus oryzae LoD plot



LoD=0,0025 ng of DNA per µL

Sitophilus zeamais LoD plot



LoD=0,0005 ng of DNA per µL

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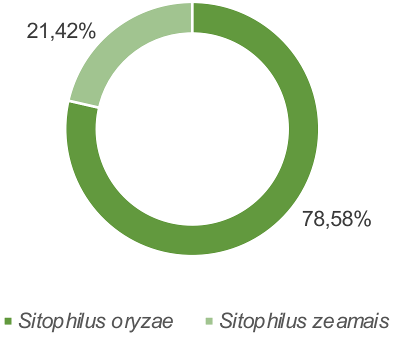
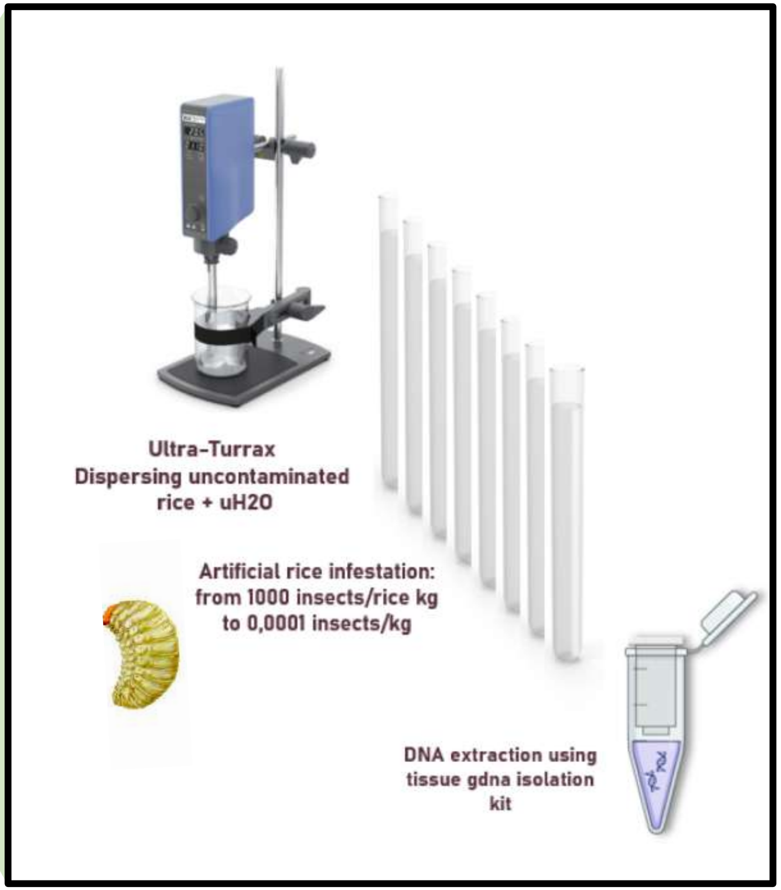
EXPERIMENTAL LIMIT OF DETECTION
OF THE TECHNIQUE – RICE MATRIX

75% OF RICE SAMPLES WERE POSITIVE AT LEAST FOR ONE OF THE STUDIED SPECIES

Sitophilus spp. **occurrence in supermarket rice samples**



NEGATIVE RICE SAMPLES


Ultra-Turrax
Dispersing uncontaminated rice + uH2O

Artificial rice infestation: from 1000 insects/rice kg to 0,0001 insects/kg

DNA extraction using tissue gdna isolation kit

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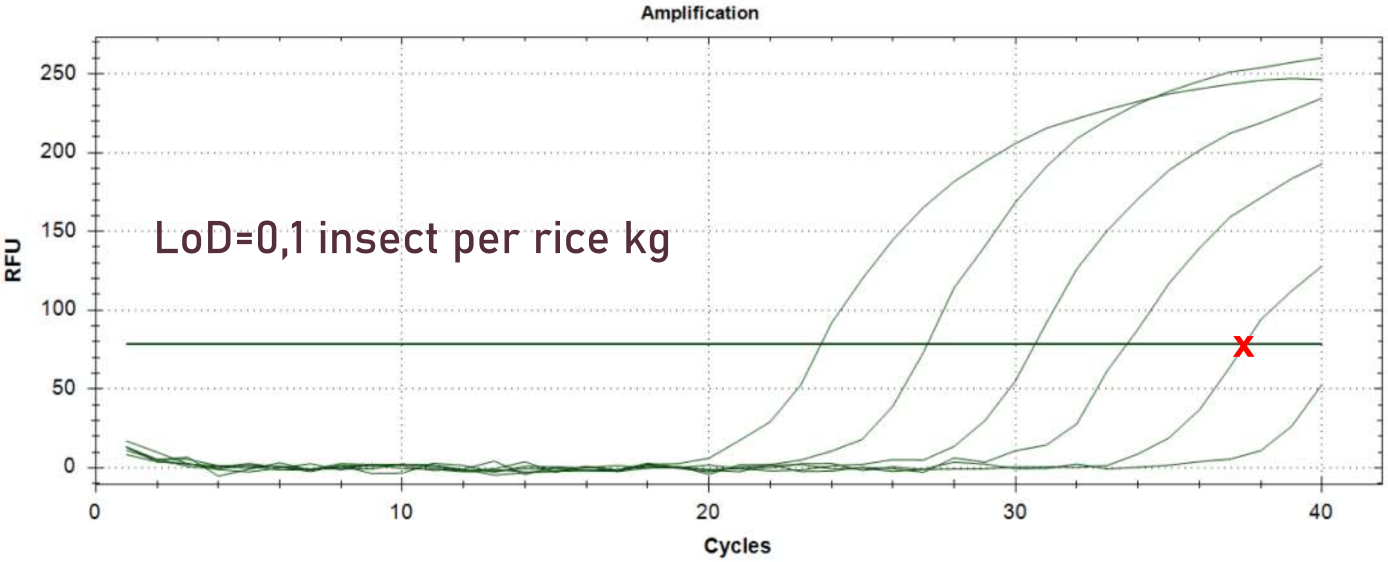
EXPERIMENTAL LIMIT OF DETECTION
OF THE TECHNIQUE – RICE MATRIX

Sitophilus zeamais LoD plot



LoD=0,1 insect per rice kg

Sitophilus oryze LoD – on going
some associated limitations

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WHAT'S NEXT?

- OPTIMIZATION OF METHODOLOGY DETECTION LIMIT

  STUDY OF OTHER GRIDING ALTERNATIVES (BEAD MILLS, CRIOTECHNIQUES)

- EXTEND THE SPECIES IDENTIFICATION PANEL

  ANOTHER PREVALENT SPECIES: *RHYZOPERTHA DOMINICA, TRIBOLIUM SPP.*

ACKNOWLEDGEMENTS

Instituto Nacional de Investigação Agrária e Veterinária, I.P.

PRIMA
PARTNERSHIP FOR RESEARCH AND INNOVATION IN THE MEDITERRANEAN AREA

TRACE RICE

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THANKS TO MY WONDERFUL TEAM AT INIAV!


Dr. Carina Almeida


Dr. Carla Brites


Dr. Ricardo Oliveira


Inês Sousa
(PhDstudent)

cost
EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

INSECT-IMP

EAAP
European Federation of Animal Science

We acknowledge the funding from the COST Action 22140 Insect-IMP: Improved Knowledge Transfer for Sustainable Insect Breeding.

And to the Scientific Committee for the approval and recognition of our work to be presented at such an esteemed conference.

THANK YOU FOR YOUR ATTENTION!

ANY QUESTION?