



# AAP & WAAP & Interbull Congress 2023



Session 64

## Authentication of insect derived products: Methods and Findings from the FARMYNG project

*Presentation by Frédéric Debode, CRA-W*



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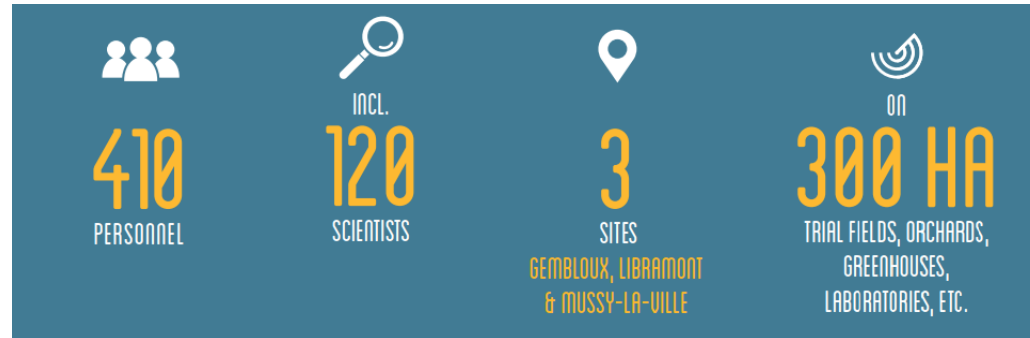
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TAKE-HOME MESSAGE

# CRA-W presentation



CRA-W's mission is to conduct basic and applied research for the benefit of the agricultural and agri-food sectors



# Role in the FARMYNG project

- Active in WP6 Quality, Safety, Purity assessment of insects based-products
  - Task 6.1 Quality and Nutritional Composition of the insect meal
  - Task 6.2 Microbial Safety Analysis
  - Task 6.4 Purity and authenticity**
  - Task 6.5 Benchmark with commercialized insect-based products

- Partners:



# Authorized insect species in feed



In Europe, the processed animal proteins obtained from seven insect species have been authorised for aquaculture by Commission Regulation (EU) 2017/893 since 1 July 2017.

- Yellow Mealworm (*Tenebrio molitor*)
- Black Soldier Fly (*Hermetia illucens*)
- Common Housefly (*Musca domestica*)
- Lesser Mealworm (*Alphitobius diaperinus*)
- House Cricket (*Acheta domesticus*)
- Banded Cricket (*Gryllodes sigillatus*)
- Field Cricket (*Gryllus assimilis*)
- **Silk worm (*Bombyx mori*)**



In 2021, this authorization has been extended to pig and poultry by regulation (EU) 2021/1372.



**In 2021, this authorization has been extended to eight species by regulation (EU) 2021/1925.**

# Methods for authentication of insect-derived products

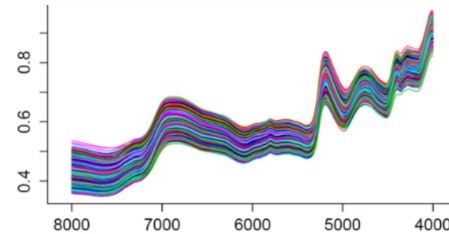
## Light microscopy



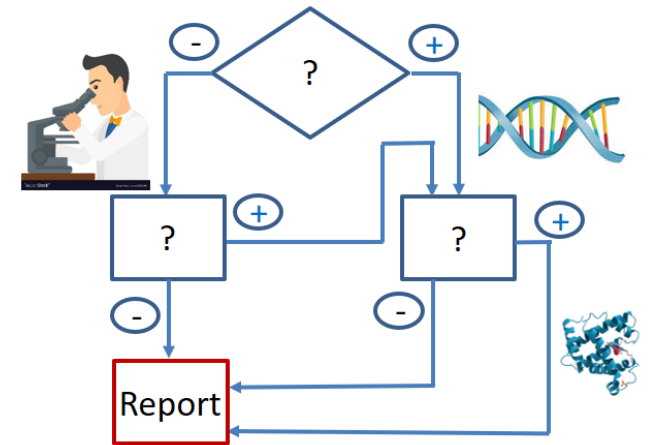
## Visual identification



## Near Infrared spectroscopy



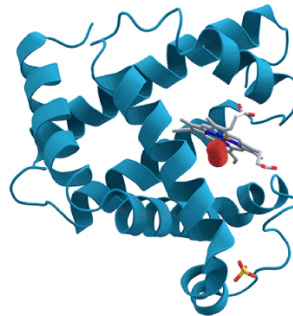
## Combination of methods



## Genomics



## Proteomics

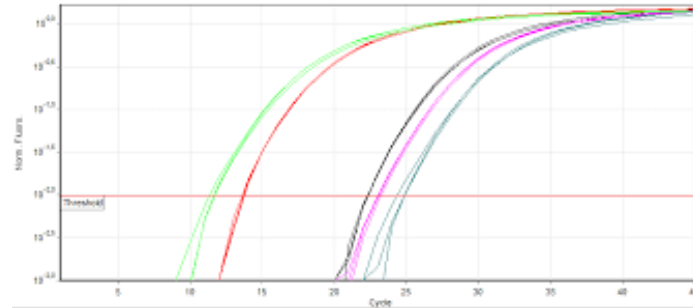


# GENOMIC METHODS



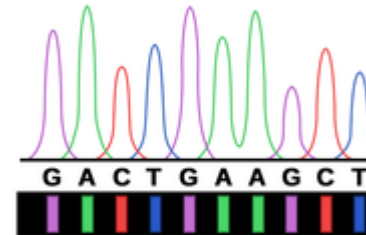
## Real-time PCR

- Targeted approach
- Can be quantitative
- Existing guidelines / performance criteria
- Recognized method for animal species detection



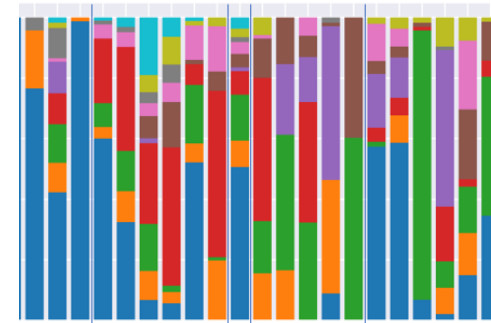
## Sequencing

- Sequencing of barcodes to identify species
- Only on pure products



## Metabarcoding combined to High Throughput Sequencing (and bioinformatics)

- Untargeted approach
- Detection of all authorized and unauthorized species,
- Detection of contaminant species
- Semi-quantitative?



# GENOMIC METHODS – Real time PCR

## PROGRESS SO FAR...

| Insect  | References/Ongoing                  | Other references                                    |
|---|-------------------------------------|---|
| Yellow Mealworm ( <i>Tenebrio molitor</i> )       | Debode et al., 2017                 | Koppel et al., 2019 (multiplex)                     |
| Black Soldier Fly ( <i>Hermetia illucens</i> )    | <a href="#">Marien et al., 2018</a> | Zagon et al., 2018                                  |
| Lesser Mealworm ( <i>Alphitobius diaperinus</i> ) | <a href="#">Marien et al., 2022</a> | Garino et al., 2022                                 |
| House Cricket ( <i>Acheta domesticus</i> )        | Close to be submitted               | Koppel et al., 2019 (multiplex) Garino et al., 2021 |
| Field Cricket ( <i>Gryllus assimilis</i> )        | Testing close to be completed       |   |
| Silk worm ( <i>Bombyx mori</i> )                  | Close to be submitted               | Kim et al., 2018<br>Zarske et al., 2021             |
| Common Housefly ( <i>Musca domestica</i> )        | Testing ongoing                     |   |
| Banded Cricket ( <i>Gryllodes sigillatus</i> )    | Testing ongoing                     |   |

Some methods are based on low copy targets and others on high copy targets : both are interesting

# GENOMIC METHODS – Real time PCR

## Evaluation of the performances of the PCR tests

| Insect                        | Efficiency | Specificity              | Applicability | Sensitivity              | Robustness |
|-------------------------------|------------|--------------------------|---------------|--------------------------|------------|
| All insects                   | V          | V (due to plant species) | V             | /                        | /          |
| <i>Tenebrio molitor</i>       | V          | V*                       | V             | LOD6 = 20                | /          |
| <i>Hermetia illucens</i>      | V          | V*                       | V             | LOD6 = 5<br>LOD95% = 5   |            |
| <i>Musca domestica</i>        | V          | / (?)                    | /             | /                        | /          |
| <i>Alphitobius diaperinus</i> | V          | V*                       | V             | LOD6 = 5<br>LOD95% = 10  | V          |
| <i>Acheta domesticus</i>      | V          | V*                       | V             | LOD10 = 5<br>LOD95% = 10 | V          |
| <i>Gryllus assimilis</i>      | V          | V*                       | V             | LOD6 = 10<br>LOD95% = 20 | V          |
| <i>Bombyx Mori</i>            | V          | V*                       | V             | LOD6 = 10<br>LOD95% = 10 | V          |

# GENOMIC METHODS- Sequencing

Real-time PCR is not able to distinguish some close species

- Case of *Alphitobius diaperinus* and *Alphitobius laevigatus*, two commercialized species

Sequencing of COI sequences to distinguish the two species

(Marien *et al.*, 2022)

Correlation between  
sequences and  
visual inspection



Microscopic observation of the eye's shape allows distinguishing *A. diaperinus* from *A. laevigatus*



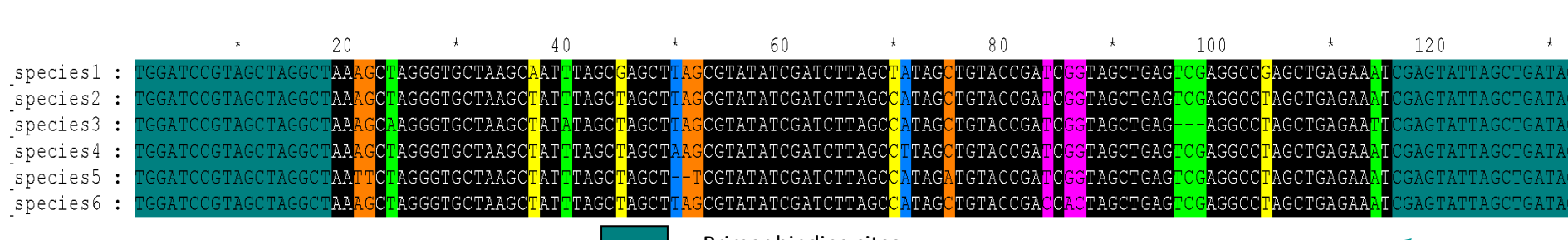
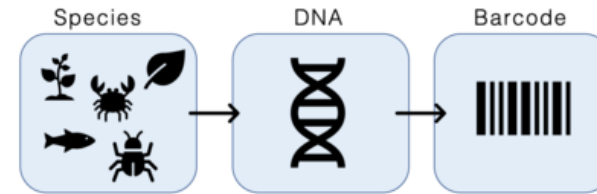
Picture : Gilles San Martin, CRA-W

- Similar job done for other insect species

Sequencing is also useful ▪ for the implementation of databases used for assignation of the reads (fill the gaps)  
▪ to check the sequences used for the design of real time PCR tests

# GENOMIC METHODS – Metabarcoding combined to HTS

## Assessing the composition of complex insect samples



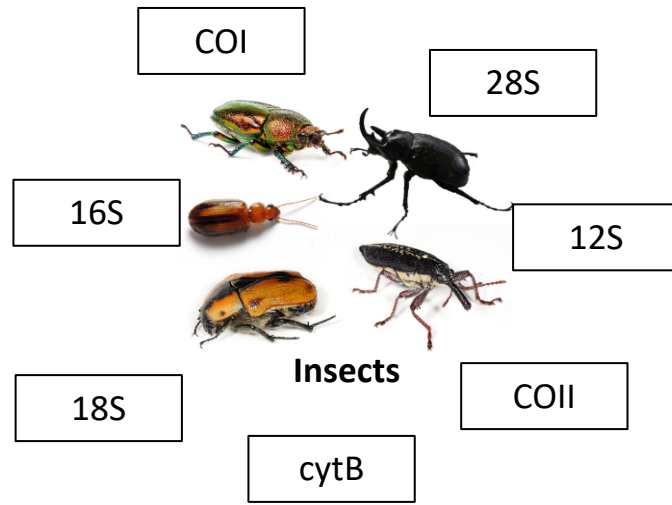
: Primer binding sites

Variable region bordered by conserved regions

→ Take advantage of inter-specific sequence variations to identify insect species

# GENOMIC METHODS – Metabarcoding combined to HTS

- Various taxonomically-informative regions of the genome are studied





**35 targets are considered in the FARMYNG project:**

- listed by Eurofins
- listed by CRA-W
- developed in the project

- Millions of reads (sequences) are generated by HTS  
→ Use of bioinformatic pipelines to handle/sort all this information

Development of bioinformatic pipelines for sequencing via

- Illumina 
- Nanopore 

# GENOMIC METHODS – Development of mock samples

## A. 8 species authorised in feed

- *Tenebrio molitor* (Coleoptera)
- *Alphitobius diaperinus* (Coleoptera)
- *Acheta domesticus* (Orthoptera)
- *Gryllus assimilis* (Orthoptera)
- *Gryllodes sigillatus* (Orthoptera)
- *Hermetia illucens* (Diptera)
- *Musca domestica* (Diptera)
- *Bombyx mori* (Lepidoptera)



*Tenebrio molitor*



*Alphitobius diaperinus*



*Acheta domesticus*



*Gryllus assimilis*



*Gryllodes sigillatus*



*Hermetia illucens*



*Musca domestica*



*Bombyx mori*

## B. 2 contaminating species

- *Ephestia kuehniella* (Lepidoptera)
- *Cadra cautella* (Lepidoptera)



*Ephestia kuehniella*

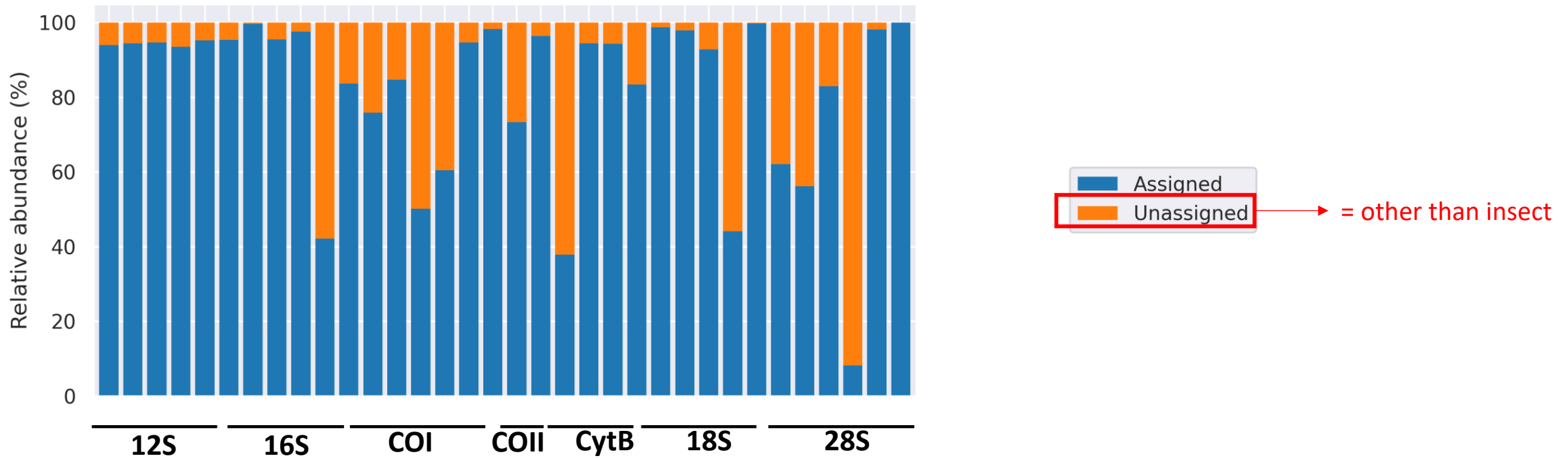


*Cadra cautella*

# GENOMIC METHODS – Development of mock samples

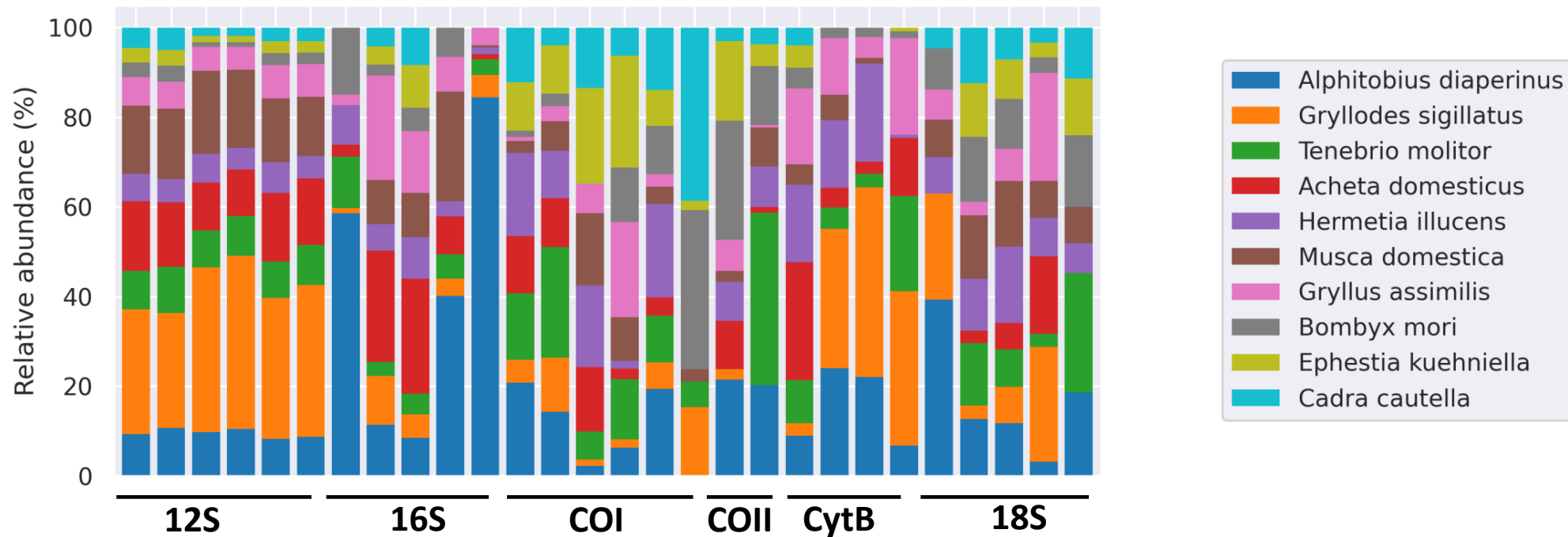
## Difficulty met:

- DNA extracted from insects can be contaminated (sources : microflora, nutrition)
- can (strongly) affect the results



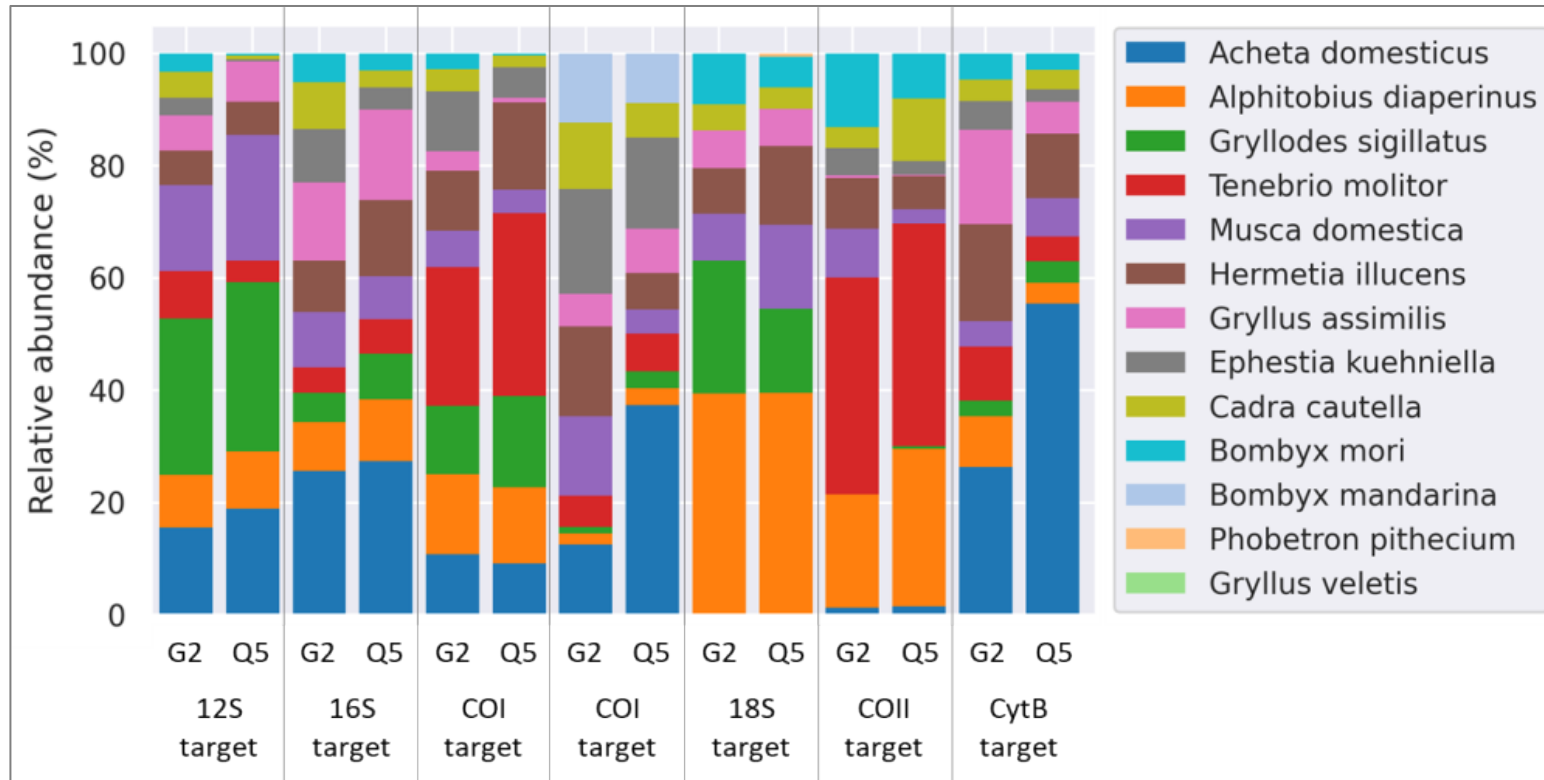
# GENOMIC METHODS – Metabarcoding combined to HTS

## Evaluation of targets



# GENOMIC METHODS – Metabarcoding combined to HTS

## Influence of reagents



- No major differences
- but some targets could be over- or under-represented

Taxonomic composition of a 10-species mock community inferred from different PCR targets using an amplicon high-throughput sequencing approach. The amplicons were generated with either a traditional (**G2**) or a high-fidelity (**Q5**) DNA polymerase.

# TAKE-HOME MESSAGE

- **Several real time PCR methods to detect authorized species are now available**

Based on single or multicopy regions – Tm can change following the considered target - Evaluation of performances is essential

- **We developed tests based on sequencing and allowing to distinguish close insect species thanks to a focus on more variable regions**

- **We have tested a large number of barcodes for metabarcoding approaches combined to HTS**

- Several problematic points that can be encountered with this approach were identified. They must be taken into account in the evaluation of the targets
- We developed bioinformatic pipelines for the analysis of MinION and Illumina results
- We constructed databases of sequences for the assignation of the results
- We exchanged material and reagents in order to ensure the transferability of the methods
- Testing on mock communities is essential
- An analysis can not be based on a single barcode



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# *Thank you for your attention*

## **AUTHENTICATION OF INSECT-DERIVED PRODUCT: METHODS AND FINDINGS FROM THE FARMYNG PROJECT**

**B. Dubois<sup>1</sup>, A. Marien<sup>1</sup>, S. Guillet<sup>2</sup>, J.F. Morin<sup>2</sup>, G. San Martin<sup>1</sup>, S. Covin<sup>3</sup>, B. Lorrette<sup>4</sup>, F. Debode<sup>1</sup>**

1 Walloon Agricultural Research Centre (CRA-W), 5030 Gembloux, Belgium

2 Eurofins Analytics France (EAF), 42301 Nantes, France

3 Eurofins Laboratoires de Microbiologie Ouest (ELMO), 44300 Nantes, France

4 Ynsect, 91000 Evry, France



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