BEEDET

IDENTIFICATION OF WILD AND DOMESTIC BEES USING NON-DESTRUCTIVE MOLECULAR METHODS

Métaprogramme BIOSEFAIR

Projet progress report: 2021 - 2024

July 2024

The decline of pollinating insects is now a proven fact, largely reported in mainstream media and scientific journals (Dicks et al, 2021). To halt this mass extinction, various actions have been taken in France, such as the Pollinators Plan (2021-2026), and in Europe with the drafting of Article 8 of the European Commission (June 2022), stating: “Member States shall reverse the decline of pollinator populations by 2030 and achieve thereafter an increasing trend of pollinator populations, measured every three years after 2030, until satisfactory levels are achieved. The Commission shall adopt implementing acts to establish a method for monitoring pollinator populations.”. However, at present, inventories and monitoring of pollinating insects face significant methodological barriers due to a lack of entomologists and reliable, standardized methods for species identification. In addition to methodological obstacles, there is also strong societal and institutional demand for monitoring pollinator populations in different environmental contexts using methods that preserve living specimens. In this context, we have proposed the development of a non-lethal protocol that can be easily replicated by institutes or associations to identify wild bees from the traces they leave on flowers when foraging, such as hairs, feces, or even saliva.

To develop this protocol, we set up an experimental system under controlled conditions. Four strawberry plants, commonly used as pollinometers, were placed in insect-proof enclosures or exposed to the open air for three days. In condition A, the strawberry plants were placed in contact with four bees belonging to three species that were captured by trapping in a net and photographed for identification (*Antophora plumipes*, *Andrena sp*., and *Lasioglossum sp*.). In condition B, the plants were exposed to the open air before the flowers were collected. In condition C, the strawberry plants were not exposed to pollinating insects. After three days, the flowers were harvested individually, frozen, and stored at -20°C before DNA was extracted from the traces.

Figure 1 : Expérimental setup

	A	B	C
Exposition conditions	**Exposure to the 4 bees captured**	**Open air exposition**	**No insecte contact**
Time exposition	3 days	3 days	3 days
Strawberry plants	4	4	4
flowers number harvested / plant	10	10	10

The sequencing of 250-bp 16S minibarcodes from insects (Clarke et al., 2014) was selected to develop this protocol. These minibarcodes are typically used to identify partially degraded DNA from environmental samples (eDNA). Prior to the eDNA metabarcoding work, we constructed a reference barcode database for wild bees in the Occitanie region (Marquisseau et al., in preparation 2024).

Principal results:

- Implementation of an optimised protocol that can be used routinely:

DNA extraction from insect traces
16S minibarcode amplification condition adapted to several genera.
MiSeq sequencing conditions (read depth, cost).
Bioinformatic analysis of the data.

- « Proof of concept »: under controlled conditions, preliminary analysis of results has shown that the passage of insects can be detected from pollinated flowers.

Detailed results

Optimising DNA extraction and amplification conditions

1/DNA extraction
Two extraction kits were tested: DNeasy Blood & Tissue (Quiagen) and Extract Me (Blirt). Only the DNeasy kit was selected, with a few modifications. Each flower was placed in a 2 ml tube and covered with lysis buffer containing proteinase K prior to 56°C incubation for 3 hours. The samples were then centrifuged to remove floral debris. The supernatant was placed on a NucleoSpin column for DNA purification, which was recovered in 120 µl of buffer and frozen at -20°C. DNA extractions were performed from 93 flowers: 33, 29 and 32 from condition A, B, and C respectively.

2/ 16S minibarcode PCR amplification
To optimise 16S minibarcode PCR amplification conditions from very small amounts of potentially degraded DNA, we tested different Taq polymerases and different DNA quantification methods (Qubit and agarose gel electrophoresis). PCR were performed in a final volume of 25µl with 3 µl of DNA, 0.25 µl of Taq polymerase (Eurobio), 1 µl of each primer (10 µM), 5 µl of MgCl2-containing buffer, and 14.5µl of water. The amplification parameters were 94°C for 5 min, followed by 45 cycles of 94°C for 30 s, 45°C for 45 s, 72°C for 45 s, and a final extension of 72°C for 20 min. After amplification, an aliquot was analysed on a 2% agarose gel (Figure 2). The positive control (T+) corresponds to DNA extracted from Antophora plumipes, one of the species that had been introduced into the pollination cage.

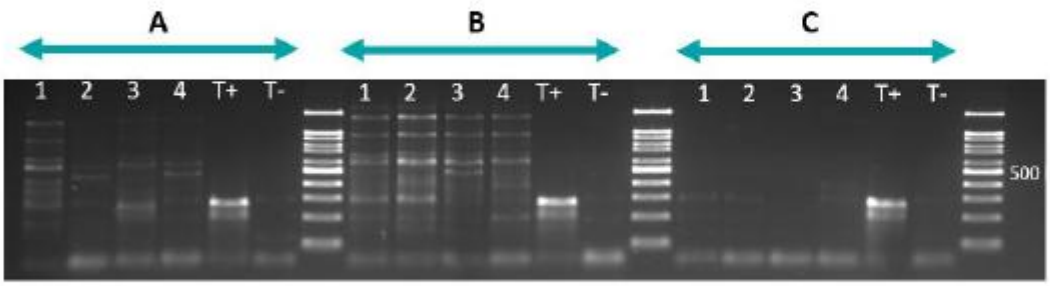


Figure 2: Agarose electrophoresis of 16S minibarcode amplification. A. flowers in contact with the three wild bee species; flowers freely exposed; C. flowers isolated from any contact with pollinating insects.

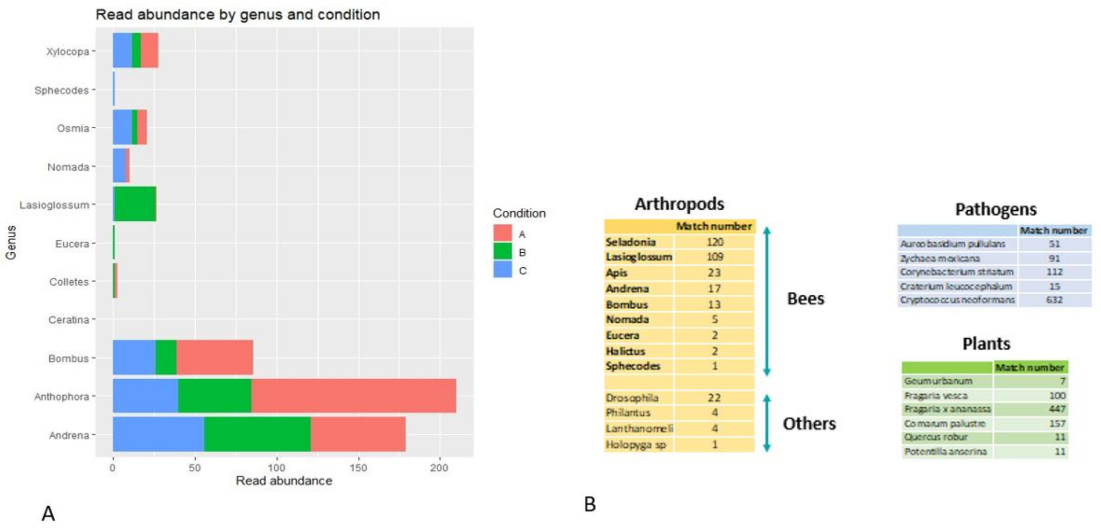
Multiband profiles are observed for all samples, with a higher tendency for flowers in contact with bees (A) or exposed to open air (B). Teste of enrichment of target sequences wre also performed (not shown).

3/ MiSEQ sequencing
A significant amount of time was spent testing for the conditions that would enable sequencing to a sufficiently high read depth for small amounts of target DNA detection, while remaining compatible with the financial resources allocated.

Bioinformatics data analysis

Preliminary analysis of the results (Figure 3) showed that it was possible to detect two of the species we had introduced manually: *Antophora* and *Andrena* (A). The detection of *Lasioglossum* requires further work, as we found that the 16S mini barcode is particularly rich in repeated AT sequences in this genus and requires the adaptation of blast parameters for searching global databases. The detection of sequences under condition C (no manual introduction of bees) is due to the fact that the strawberry plants used in this experiment came from external nurseries. A more detailed examination of a flower sample from condition A (manual introduction of bees) shows that several species of wild bees belonging to different genera are also detected, as well as sequences from other pollinating insects such as hoverflies (Philantus). We also found a lot of contamination from Homo sapiens (results not shown) and sequences corresponding to 16S DNA from strawberry plants.

Figure 3: A: List of wild bees classified per genera and detected by DNA metabarcoding on the 93 flowers studied. B: list of arthropod, pathogen and plant DNA identifiedon a flower from condition A (contact with three wild bee species).



Arthropods

	Match number
Seladonia	120
Lasioglossum	109
Apis	23
Andrena	17
Bombus	13
Nomada	5
Eucera	2
Halictus	2
Sphecodes	1
Drosophila	22
Philantus	4
Lanthanomeli	4
Holopyga sp	1

Pathogens

	Match number
Aureobasidium pullulans	51
Zychaea mexicana	91
Corynebacterium striatum	112
Craterium leucocephalum	15
Cryptococcus neoformans	632

Plants

	Match number
Geum urbanum	7
Fragaria vesca	100
Fragaria x ananassa	447
Comarum palustre	157
Quercus robur	11
Potentilla anserina	11

Feedback

It appears necessary to limit contamination from *Homo sapiens* and strawberry, having a much higher number of sequencing reads than bee species. This is a known issue for eDNA samples, and solutions exist, such as the use of blocking oligonucleotides, which aim to reduce the amplification of this contamination.

Valorisation of results

Presenting the project and preliminary results:

- EurBee 9 congress, Belgrade, Serbia, 20-22 September 2022.
- Workshop on automated methods for biodiversity inventorying, part of the Biosefair Metaprogram Lyon, the 7th and 8th of September.
- Journée scientifique ADN environnemental de la ZA PYGAR le 8 Novembre 2022,
- Barcoding/metabarcoding workin group, GDR Pollineco 5-7 October 2022.
- Apimondia congress, Santiago, Chili, 4-8 September 2023
- International Environmental and Agronomical Genomics symposium (Toulouse 2024)