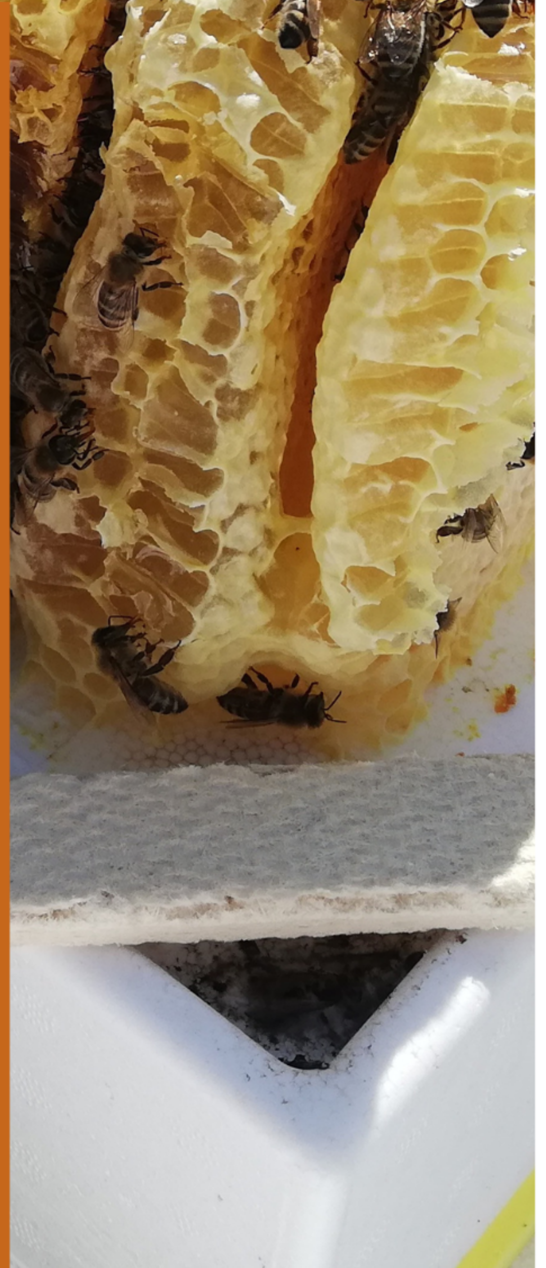

Iceland Liechtenstein Norway grants

Norway grants

BeeConSel - Joint Effort for Honey Bee Conservation and Selection

DELIVERABLE 3

Knowledge transferred

- Test protocols developed -

BeeConSel is funded by Iceland, Liechtenstein and Norway through the EEA and Norway Grants Fund for Regional Cooperation.

TABLE OF CONTENTS

EXECUTIVE SUMMARY 3
PROTOCOL FOR SYSTEMATIC OBSERVATION OF MATING FLIGHTS................ 4
Condensed protocol 5
SAMPLING PROTOCOLS FOR MOLECULAR ANALYSES 8
The Field Sampling Protocol.. 9
Protocol for molecular analyses 11

Deliverable driver: B Dahle

Authors: B Dahle, S Andonov, J Prešern, M Kovačić, A Uzunov, A Moškrič

Photos: J Prešern, A Uzunov, M Kovačić, B Kozinc, A. Moškrič, A. Marinč, K. Mole

Implemented by:

Kmetijski inštitut Slovenije Agricultural Institute of Slovenia · calis · ДИПОЗ CARPEA · NORGES BIRØKTERLAG · SLU

BeeConSel is funded by Iceland, Liechtenstein and Norway through the EEA and Norway Grants Fund for Regional Cooperation.

EXECUTIVE SUMMARY

To improve mating control in the beneficiary countries, potential locations for mating stations must be tested for their suitability. Due to the complex genetic and mating system of honey bees we developed a comprehensive set of protocols to be used in WP3-Assessment and implementation of best-suited mating control models.

1) Protocol for systematic observations of mating flights of honey bee queens and drones with the use of Tailor-made equipment which is an initial step to assess an area's suitability as mating station. Used in all beneficiary countries.

2) Sampling protocols for molecular analyses to ensure collection of suitable samples for genotyping the progeny (worker, drone larvae or pupae), their mothers (the mated queens) and their possible fathers (the drone producing queens or their brood). Used in all beneficiary countries and donor country.

3) Protocol for molecular analyses of the samples taken from the field including DNA extraction, DNA quantification, PCR amplification of microsatellite loci, agarose gel electrophoresis of amplified products and preparations of PCR products for capillary electrophoresis and peak calling. Protocol is extended with analysis of molecular markers for parentage confirmation with data verification per location, building of the reference genome for each colony and identifying paternity percentage.

BeeConSel is funded by Iceland, Liechtenstein and Norway through the EEA and Norway Grants Fund for Regional Cooperation.

PROTOCOL FOR SYSTEMATIC OBSERVATION OF MATING FLIGHTS

Honey bees (*Apis mellifera*) are characterised by a complex genetic and reproductive (mating) system. Queen bees are polyandrous and mate with several drones in air during mating (nuptial) flight in a place where drones from the area gather (so-called “drone congregation area”). Mating is lethal for drones; however, the sperm that is passed to the queen is alive within queens' specialised organ called spermatheca and used for laying fertilised eggs until the end of queen’s life. The mating of the queen with a drone takes up to five seconds, and the queen is usually mated with 10-20 drones. After the successful mating flight, queen returns to the colony and after a few days it starts laying eggs. The mating process of the queen is one of the riskiest parts of the life of a honey bee colony. If the queen is lost and there is no young brood in the colony from which a new queen could be reared, the colony will collapse within few weeks or months.

When planning and organizing the mating station, it is necessary to reduce as much as possible the risk of losing queens during mating. The initial step for assessing the area's suitability for a mating station is the collection of data on queens’ nuptial flights, such as flight frequency, duration of flights, mating success, survivability, spermatheca filling etc. Observing the behaviour of the virgin queens on the hive entrance also serves to estimate the distance from the potential drone congregation areas (DCAs) as well as for the detection of airborne drones in the area. Collected data may provide important information like distance of the mating station from the potential drone congregation areas. Several methods are available to collect data on flight behaviour of virgin queens. The flight behaviour of queens and drones can be monitored by direct observations of the hive entrance by an observer or by usage of technologies like Radio Frequency Identification technology (RFID) or video surveillance. However, these technologies have some limitations, and the method of direct observations was chosen as the most rational and convenient for this project. In this Deliverable we summarized the proposed and used methodology.

For the purpose of implementing this task, we developed a recordkeeping card for observation of queen and drone mating flight (submitted scientific review article "Standard methods for direct observation of honey bee (*Apis mellifera* L.) nuptial flights") and trained all partners who participated in this activity. The protocol was developed and standardized to collect all relevant information on the flight behaviour of virgin queens to successfully monitor, interpret, and compare data across locations.

Condensed protocol

The observation of virgin queens should start when they become sexually mature, usually 5-7 days post emergence. To document the nuptial flights, virgin queens together with several hundred worker bees are installed in mating boxes. An observation tunnel with a queen excluder is mounted in front of the mating box. The excluder, preferably transparent, allows the passing of the worker bees out and to the tunnel but at the same time prevents the queen from exiting or entering. The queens in the mating boxes should be marked, ideally by numbered and coloured opalite plates on the dorsal side of the thorax to enhance visibility and to verify that queens re-enter their box of origin upon arrival after a mating flight.

Figure 1. A mating box with an observation tunnel (©A. Uzunov).

The mating boxes should be arranged in a half circle, differently coloured and about one meter apart. Each observer can handle 6-12 mating boxes, depending on experience, and training. Observations should cover the normal flight period, usually from solar noon until 6 h past solar noon. The queens' attempt to depart the mating box for orientational or mating flight is halted by the observation tunnel until the observer lets the queen out. Similarly, the observer will let the queen in when she returns to the mating box (Figure 1). This setup allows the observer to

Figure 2. Queen bee returned from the nuptial flight with a mating sign (©M. Kovačić).

document time and duration of queens' flight and observe whether the queen was successfully mated, judged from the presence of a mating sign or an open abdomen (Figure 2).

All relevant information during the observation period should be entered in the tailored recordkeeping card (Figure 4). Such information includes weather data as well as the queens' flight activity. Depending on the weather condition the observations should last from 6 days under ideal weather conditions, to up to 10-12 days if the weather conditions limit the queens' mating flights (cold and rainy conditions).

For comprehensive assessment and execution of the protocol additional information about flight and mating behaviour of the drones is needed. Two basic approaches are used to observe drones' behaviour:

Figure 3. Flight observations using queen pheromone bait. (©B. Kozinc).

- Direct observations on the DPC hive entrance, and
- flight observations using queen pheromone bait (9-ODA or alternatively so-called "queen compote") to attract airborne drones and to identify flight patterns and periods. This approach is also used for identification of DCA.

The flight period, range, pattern, and frequency of the drones is important to identifying the optimal time of queen release when temporal isolation (Jo Horner/Moonshine mating method) is used as a means of mating control. However, use of video surveillance and RFID is not excluded as a reliable method for drone observations.

Iceland Liechtenstein Norway grants Norway grants

Uzunov et al. 2021

ДИПОЗ CARPEA

Date:		Location:		Observer:	

		Flight 1	Flight 2	Flight 3	Flight 4	Flight 5	Flight 6	Flight 7
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							

		Flight 1	Flight 2	Flight 3	Flight 4	Flight 5	Flight 6	Flight 7
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							
Mating box No.	Departure (hh:mm)							
	Arrival (hh:mm)							
	Flight duration (minutes)							
	Mating sign (+/-)							
	Mating sign removal (minutes)							

Weather conditions

Hour:		T °C		Humidity %		Wind cat. *		Cloud coverage %	
Hour:		T °C		Humidity %		Wind cat. *		Cloud coverage %	
Hour:		T °C		Humidity %		Wind cat. *		Cloud coverage %	
Hour:		T °C		Humidity %		Wind cat. *		Cloud coverage %	
Hour:		T °C		Humidity %		Wind cat. *		Cloud coverage %	
Hour:		T °C		Humidity %		Wind cat. *		Cloud coverage %	

Abbreviations/signs	
Mating sign	(+ mated) (- non mated) (o open)
*Wind caegories	(1 = low), (2 = medium), (3 = strong)
Cloud coverage	Percentage of cloud coverage on horizon

Comment

Figure 4. Recordkeeping card

SAMPLING PROTOCOLS FOR MOLECULAR ANALYSES

The genotyping of the progeny (worker, drone larvae or pupae), their mothers (the mated queens) and their possible fathers (the drone producing queens or their brood) is assessed from samples of all the relevant partners using microsatellite markers (A0007, A0113, Ap043, Ap055, B0124).

Patrilines are assigned through molecular analysis and the proportion of known and unknown drones that had inseminated the queens is determined. For the purpose of the molecular analysis a clear protocol as well as a sampling kit containing the vials and stickers is provided by AIS and delivered to all the partners.

Figure 5. A sampling kit before shipping. (©A. Moškrič)

<u>BeeConSel Sampling Kit, provided by AIS, contains:</u>

- prelabelled 2.0 mL Eppendorf tubes, filled with absolute ethanol, for sampling of mated queens and queens from DPCs
- prelabelled 50 mL Falcon tubes, filled with absolute ethanol, for sampling of worker brood or drone brood. Tubes also contain a piece of tracing paper with the voucher inside each tube to prevent sample mislabelling if the codes on the tube get erased.
- steel forceps
- lighter
- tube with 70 % ethanol for cleaning of the forceps between sampling of different colonies
- nitrile laboratory gloves
- back-up Eppendorf and Falcon tubes filled with absolute ethanol, labels, tracing paper
- pencil
- voucher table to record the collected samples per colony per apiary

The voucher IDs on the tubes correspond to IDs in an Excel document at the BeeConSel SharePoint, where any relevant information regarding the samples is filled in. The quantity and types of tubes are adjusted to the partners' needs after the consultation before the experimental season.

The Field Sampling Protocol

General instructions

1. The individuals should be sampled using DNA-free steel forceps.
2. During sampling it is important that the forceps are cleaned between samples by quickly dipping into 70 % ethanol and then through the flame to prevent cross-contamination. Since worker brood from each mating nuc is treated as one sample there is no need to clean the forceps in between sampling of individuals from the same mating nuc.
3. Use gloves and change them during sampling should they get dirty.
4. Add absolute ethanol to the tubes after sampling if it appears that the liquid level in the tube is too low.
5. It is best to keep the tubes with samples refrigerated before the transport.

Figure 6. Brood sampling. (©A. Moškrič)

The BeeConSel **sampling protocol** covers two different experimental setups, depending on the mating experiment properties in the field - with or without drone-producing colonies (DPCs).

Setup without DPCs

This method examines the isolation of mating yard and approximates the number of foreign colonies in the surrounding. In this setup we sample:

- Successfully mated queens from mating nucs, one queen into one 2.0 mL microcentrifuge tube filled with absolute ethanol. Each single queen represents one sample.
- Worker brood (30 to -50 individuals) from mating nucs, brood from each nuc should be pooled and stored together in a 50 mL centrifuge tube filled with absolute ethanol. Pupae are required, preferably older. Each pool represents one sample.

Figure 7. Brood sampling in the field (©A. Moškrič)

Setup with DPCs installed at the mating yard

This method addresses the source of drones that mated with the queens in the mating nucs. In the case of presence of foreign (from unknown sources) drones, the ratio of drones from foreign and known sources is assessed. In this setup the collection of samples includes:

- Drone-producing colonies (DPCs): Material from each DPC is sampled in one 50 mL tube filled with absolute ethanol. 20 to 50 drone pupae as mature as possible from one DPC, pooled together in one 50 mL tube represent a single sample.
- Drone-producing queens from DPCs, one queen into one 2.0 mL microcentrifuge tube filled with absolute ethanol. Each queen represents one sample. Alternatively, to keep the queen alive, a wing clipping from each drone-producing queen may be sampled. One wing clipped into one 2.0 mL microcentrifuge tube filled with absolute ethanol represents one sample.

Protocol for molecular analyses

Samples taken from the field are further analysed in the laboratory. Each unit is represented by one mated queen and up to 30 individuals of worker brood. Each drone producing colony is represented either by a drone producing queen or alternatively by 30 drone pupae in a pooled sample. The molecular analysis protocol consists of the following steps:

- DNA extraction
- DNA quantification
- PCR amplification of microsatellite loci
- Agarose gel electrophoresis of amplified products
- Preparations of PCR products for capillary electrophoresis and peak calling

1. DNA extraction

Steel tweezers should be washed by immersion into 96 % ethanol and flame heating before handling of each individual sample to prevent cross contamination.

DNA extraction procedure depends on the starting sample type. NucleoMag Tissue Kit (Macherey-Nagel) is used for DNA extraction from queens, workers, or drones. QiaAmp Blood and Tissue Mini Kit (Qiagen) is used for DNA extraction from wing clippings.

I. **Queen or worker:** all available or up to 30 worker pupae are transferred to a sterile Petri dish using sterile forceps. Using sterile tweezers, a leg is removed from each of the pupae for further molecular analysis while the remainder of the sample is transferred to an individual 2 mL microcentrifuge tube prefilled with 800 μL of absolute ethanol. The tweezers are flame heated between handling of the individual pupa. In the case of a queen, the imago is partially or fully lifted out of the

original sampling tube and held with one pair of sterile tweezers while the leg is removed with another. The queen imago is then returned to the original sampling tube and the leg allowed to dry in a 1.5 or 2.0 mL** microcentrifuge tube. 100 µL of Lysis Buffer T1 and 10 µL Proteinase K are added. The sample is ground into smaller pieces using a pestle.**

II. **Drones:** If neither the queen from DPC nor her wing clipping is available, the DNA is extracted from her drone brood instead as a pooled sample to recover the queen's genotype. Thirty drone pupae are placed into a sterile dish. An antenna from each is removed and all 30 antennae are placed into a 1.5 mL (or a 2.0 mL)** tube and let dry of ethanol. The drone brood from which antennae were collected is transferred into a new 50 mL tube filled with absolute ethanol. After the antennae have dried, 100 µL of Lysis Buffer T1 and 10 µL Proteinase K are added. Antennae are then ground into smaller pieces using a plastic pestle.**

III. **All samples**: The following steps apply for all sample types except for wing clippings.

a) Samples are then incubated at 56 °C overnight.

b) The next day the plates for the extraction using MagMax processor are prepared:

Well A: 100 µL of Binding Buffer MB2 and 10 µL of B-Beads
Well B: 150 µL of Wash Buffer MB3
Well C: 150 µL of Wash Buffer MB4
Well D: 200 µL of Wash Buffer MB5
Well E: 50 µL of Elution Buffer MB6

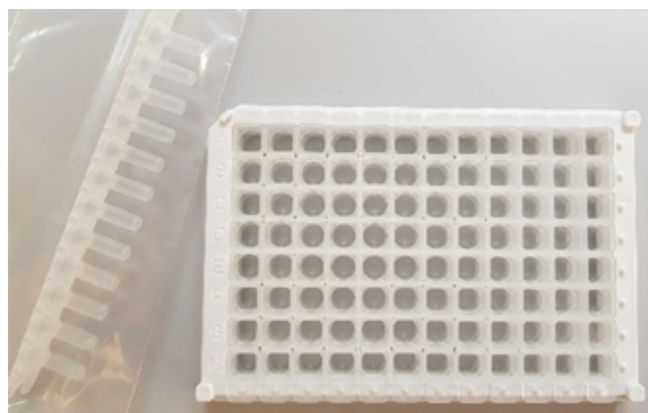

Figure 8. The plate and the comb for extraction of DNA using MagMax processor. (©K. Mole)

Figure 9. MagMax automatic processor for DNA extraction. (©A. Marinč)

90 µL of the sample lysate is transferred into well A. Two plates with 12 samples each are transferred into the MagMAX processor. The extraction steps follow the manufacturer's protocol. After the DNA extraction is complete, 50 µL of the extract (DNA dissolved in Elution Buffer MB6 in well E) is transferred into 250 µL PCR tubes in strips. DNA extracts are stored at 4 - °C for short term storage (days to a couple of weeks). During this time, they are further processed. Long term storage is at -20 °C.

*If the brood is younger and does not have legs yet, a small piece (approximately equal to the volume of a leg) of head or abdomen tissue is used instead.

**To save time and labour the Stainless Steel Beads (QIAGEN, 5 mm) and TissueLyser (1 min and 25 Hz, 2 times) may be used instead of manual grinding of the sample with pestle. Both methods yield DNA of sufficient quantity and purity for the subsequent fragment amplification (PCR).

IV. **<u>Wing clipping:</u>** Instead of sacrificing a drone producing queen an alternative non-lethal sampling method can be used. A wing clipping may be the source of extracted DNA for genotyping of each drone producing queen.

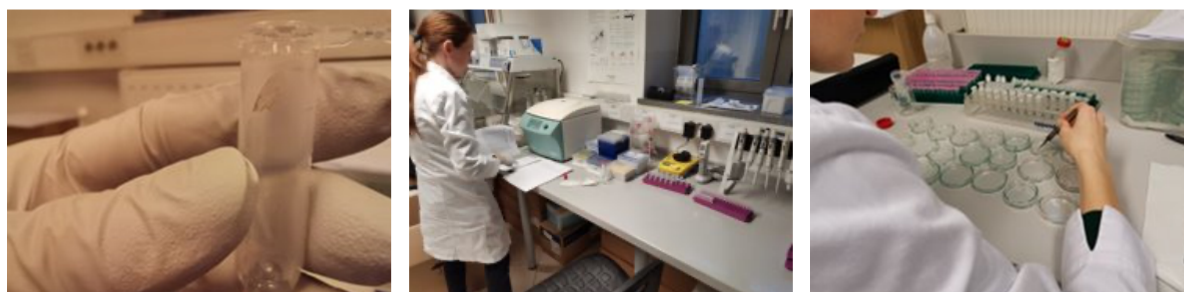

Figure 10. Extraction of DNA from queen wing clippings. (©A. Marinč)

All DNA extractions were performed using the QiaAmp DNA Mini Kit (Qiagen) following the protocol based on the paper: Bubnič J, Mole K, Prešern J, Moškrič A. Non-Destructive Genotyping of Honeybee Queens to Support

Selection and Breeding. Insects. 2020 Dec 21;11(12):896. doi: 10.3390/insects11120896

2. DNA quantification

DNA concentrations in DNA extracts are measured using Qubit fluorometer (Thermo Fisher Scientific) using the corresponding Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific) following the manufacturer's protocols.

3. PCR amplification of microsatellite loci

The protocol is based on the paper: Muñoz, I., De la Rúa, P. Wide genetic diversity in Old World honey bees threaten by introgression. Apidologie 52, 200–217 (2021). https://doi.org/10.1007/s13592-020-00810-0

The modification is the usage of Type-it Microsatellite PCR Kit (Qiagen). PCR for 5 loci - A07, A113, Ap43, Ap55 and B124 - performed in a multiplex reaction

(MP1 – multiplex reaction 1). Master Mix was prepared according to the table below, so the final volume is 8 µL. Working solutions of all primers are 20 µM, so the final concentration of each primer equals 0,2 µM.

Program: *Initial denaturation at 95 °C for 5 min, followed by 28 cycles of denaturation at 95 °C for 30 s, primer annealing at 57 °C for 90 s and extension at 72 °C for 30 s. Final extension is at 60 °C for 30 min.*

	1 x 8 µL
2x MasterMix Type-It	4
Q solution	0
A113_F (20 µM)	0,08
A113_R (20 µM)	0,08
A07_F (20 µM)	0,08
A07_R (20 µM)	0,08
Ap43_F (20 µM)	0,08
Ap43_R (20 µM)	0,08
Ap55_F (20 µM)	0,08
Ap55_R (20 µM)	0,08
B124_F (20 µM)	0,08
B124_R (20 µM)	0,08
DNA	0,5
dH20	2,7

4. Agarose gel electrophoresis of amplified products

Amplified PCR products are visualized using 1 % agarose gel electrophoresis in 0.5 x TBE buffer with 1.3 µL ethidium bromide. 1 µL 6x TriTrack DNA Loading Dye (Thermo Fisher Scientific) is added to 5 µL PCR products before loading. GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific) is used for standard. The electrophoresis is run at 100 V for 35 min.

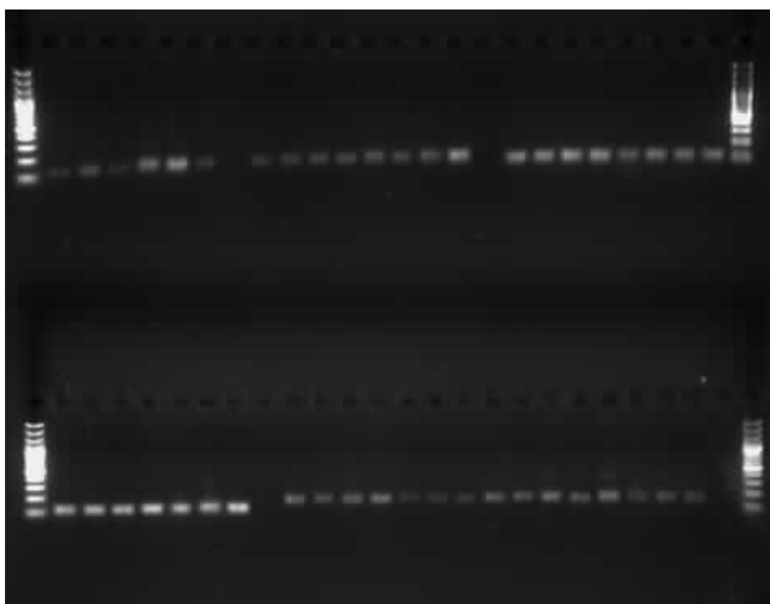

Figure 11. PCR products visualized on 1% agarose gel, EtBr.

5. Preparations of PCR products for capillary electrophoresis and peak calling

Successfully amplified samples are prepared for capillary electrophoresis. Each well of a 96-well plate is filled with 10 µL formamide, 0.35 µL size standard LIZ500 and 1.3 µL of amplified DNA. The samples are denatured at 95 °C for 2 min and then for 2 min incubated on ice. Microsatellite analysis is performed on 3500 Genetic Analyzer for Fragment Analysis (Applied Biosystems). The traces are visualised, and peak calling is performed using the microsatellite plugin in Geneious Prime (Biomatters, NZ; www.geneious.com).

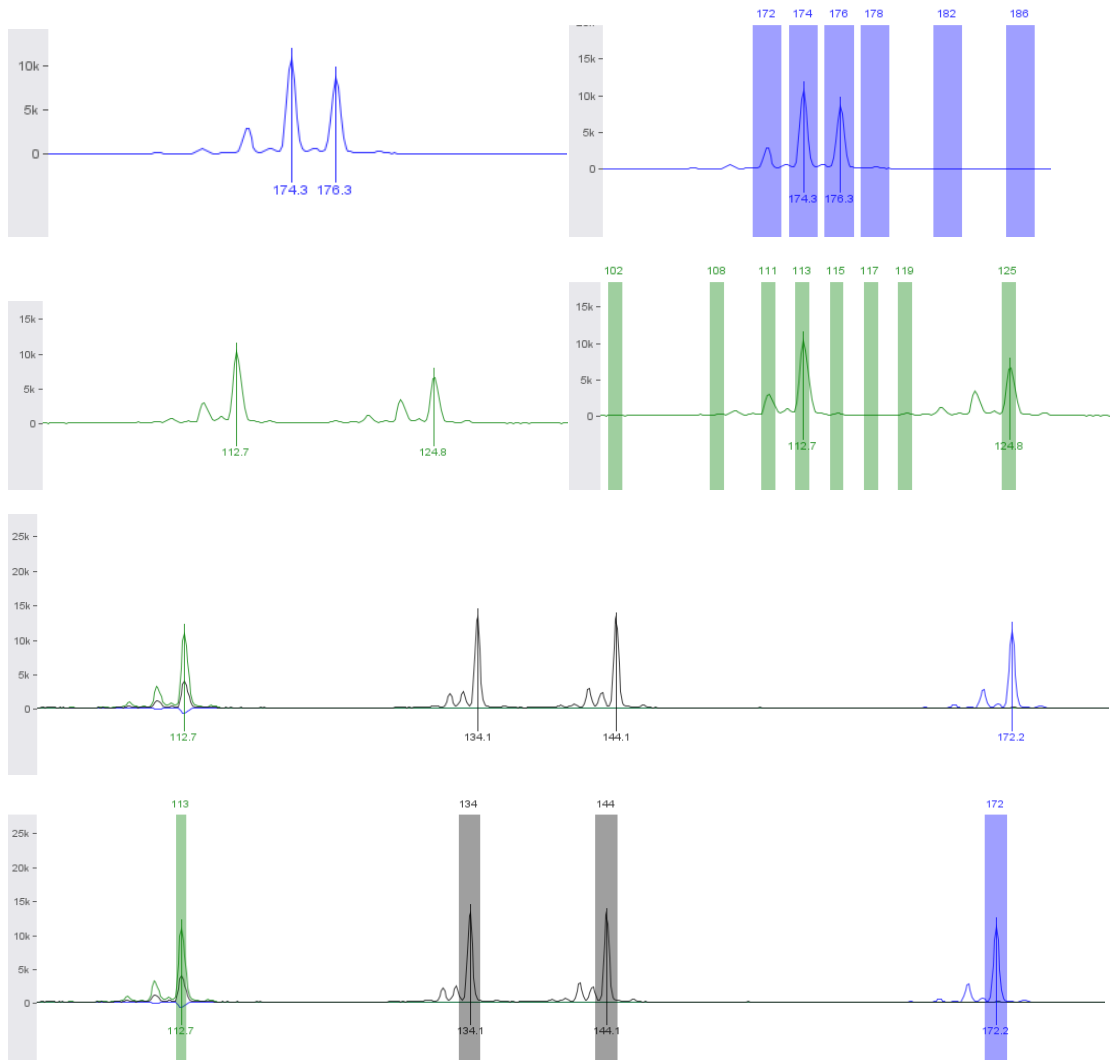

Figure 12. Geneious Prime UI for viewing microsatellite data.

6. Analysis of molecular markers for parentage confirmation

All genomic forms of microsatellites were documented in an excel document and stored on the project cloud. The excel sheet contains information for unique sample codes corresponding to the origin of the sample (country, location, type of colony (DPC, mating nuc) and type of the sample (worker bee, mated queen, DPQ – drone producing queen, drone)) and two genomic forms identified at each of 5 microsatellite loci.

A special pipeline was designed for analysing the microsatellites' genomic form matching combinations. The pipeline was written in the Linux script Bee Reference Genome Paternity (BRGP). BRGP aims to confirm paternity and verify data samples and the purity of the colonies' samples. BRGP runs in 3 steps.

Step 1: Data verification per location. The verification confirmed that the data is in accordance with the data in the excel file and there are no errors in the data reading. It is done by accounting for basic information:

- Number of colonies;
- Total number of worker bee samples;
- Total number of worker bee samples per colony;
- The total number of drone samples.

Step 2: Building the reference genome for each colony. The reference genome is composed from the genome of the mated queen (MQ) and the genomes of all possible known mates (drones) that were present at the particular location (D). Then all possible combinations are mathematically calculated to build a reference genome for worker bees (WB) in the colony. To avoid any miss-labelling or other possible mistakes, verification of WB is performed by confirming the presence of at least one MQ genomic at each of the five microsatellites. The outputs of step 2 are:

- Reference mated queen's genome for each of 5 microsatellites;
- Verification that all worker bees sampled from a particular colony originated from the queen of the same colony;
- Reference drones' genome for each of 5 microsatellites;
- The colony reference genome consists of a list of all possible combinations of drones and the colony's queen.

If all known genomic forms for each microsatellite locus are in the population, it results in over 23 trillion possible combinations.

Step 3: Identification of paternity percentage is done when the presence of each WB unique genomic form is matched in the colony reference genome. After matching, the results are summarized:

- Total analysed samples per colony;
- Number of unique drones that had successfully mated the queen of the colony;
- The total number of worker bees originated from known drones that had mated the queen of the colony;
- Number of unique drones that had mated the queen of the colony;
- % of worker bees with known paternity origin.