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BeeConSel - Joint Effort for Honey Bee Conservation and Selection

# DELIVERABLE 6

# Mating control: evaluation of method

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# EXECUTIVE SUMMARY

Figure 1. Carniolan honeybees in Slovenia

Locally adapted honey bees are essential in maintaining biological diversity. The constant genetic improvement of the locally adapted honey bee populations was confirmed as a sustainable protection method. Genetic improvement is organised through breeding programs to obtain the next generation of individuals from animals with the highest genetic value. The crucial point for improving the population of honey bees (*Apis mellifera* L.) is controlled mating due to very specific reproductive biology. So far, controlled mating has not been fully implemented in SE Europe.

Within the BeeConSel project, beneficiary countries applied many approaches *in situ*, testing the success of the mating control, as reported in the previous deliverables. Additional validation of the approaches for controlled mating can be done by confirmation of the patrilines with molecular markers, which were part of WP4. Partners tested different mating approaches, the DNA was isolated, and polymorphism markers were identified. Patrilines were verified with bioinformatics tools, and finally, the patrilines were confirmed.

Within three seasons, three concepts for mating control were evaluated: geographical isolation, where four approaches were tested (deep forest, highland microsites, island, and alpine high-altitude valleys), time isolation with two approaches (labyrinth and cooling), and biological saturation.

The concept of geographical isolation was the most explored, searching for promising locations suitable for controlled mating of honey bees. The validations were applied for the locations identified as the most promising with the aim to be cost-effective.



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The deep forest location can be considered as partly isolated with verified presence of feral honey bee colonies. However, obtaining a known patriline origin of 65 % in mating honey bees could be an alternative for the breeding program where genetic diversity is desired.

Two of the eight highland microsites were the most promising, where 60-65 % of the bees in the colony had known origin. The reliability of the sites can be improved by providing a higher number of drones. On the island, mating control is challenged by unfavourable weather conditions, which reduce the colonies' survival rate and negatively impact the flying of drones and virgin queens. Nevertheless, 85 % of the known drones contributed to the mating of the queens. Alpine high-altitude valleys are encouraging and can offer high confidence in their use as mating stations, where reliability on average was 89 %

Time isolation or the delayed time mating flight model is an innovative alternative to classically isolated mating stations used in honey bees. Two alternatives, i.e. labyrinth and cooling, were tested for restricting free flights of queens and drones. The labyrinth method in the first iteration was promising, where 62 % of drones with known origin were verified in mated queens. The cooling method was even more auspicious in the first trial (75 % verified patrilines). However, in the second trial, neither method was verified due to observed unsatisfied nuptial flying patterns. The time isolation concept needs further testing before it can be routinely used in breeding programs.

The concept of biological saturation is based on the overflow of the area with many drones with known origins. The concept was validated in two seasons where 76-96 % of worker bees in colonies had validated patrilines. This approach is advantageous in mating several (thousand) queens and could be useful for commercial breeders or groups of breeders.

Croatia's preferred controlled mating systems were deep forest and biological saturation, where both approaches should be combined to perform controlled mating for breeding purposes. In N. Macedonia, two highland microsites and one island were validated as potential mating stations, while in Slovenia, which has very high apiary density, two Alpine high-altitude valleys were the most promising.



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# BACKGROUND

Locally adapted honey bees are essential in maintaining biological diversity. One of the approaches to protect local honey bees is by their constant genetic improvement (FAO, 2004). With this, superior characteristics can be maintained compared to introduced subspecies. Managing genetic improvement in the population of honey bees (*Apis mellifera* L.) is more complex than in other economically important species.

In breeding programs, it is crucial that the next generation originates from the most desirable animals, e.g., reproduce individuals with the highest genetic value. The genetic value of queens and drones can be for production (honey yield), for behaviour (hygienic, defensive, swarming), vitality (colony strength, brood development, resilience to parasites and diseases), and for maintaining diversity (breed against inbreeding, a sufficient number of colonies, keeping CSD (Complementary sex determiner) alleles diversity sufficient within a population). One of the deciding points in breeding is mating control (Du et al., 2021, 2023; Uzunov et al., 2022) due to the peculiarity of the honey bee reproductive biology.

Figure 2. Instrumental insemination

In instrumental insemination the paternity can be assigned without doubt; however, the main constraints are in the number of queens served per time unit resources and in that it can be costly. Besides instrumental insemination, the use of mating stations and the delayed time mating flight models are feasible approaches to obtain mating control (Musin et al., 2021; Uzunov et al., 2022).

The idea behind the mating stations and delayed time mating flight models is to enable known mating partners (i.e. drones) for the virgin queen in the air. Mating stations are isolated locations where it is assumed that no drones other than those with known origin are present at the mating site. So far, even in countries where the use of mating stations is well established, the efficacy of mating stations was never measured.

The beneficiary countries (HR, MK, and SI) have not formally established mating stations so far (except Slovenia, where two mating stations operated



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in the past but were shown not to be isolated). Potential locations were identified through WP2 (reported in D2 - Knowledge transferred - Trainers trained), and *in situ* mating was observed and tested (WP3, reported in D5 - Pilot mating control *in situ*), the potential locations were identified, on site mating was observed and tested, and capacity was developed for further use of mating control. Newly formed colonies verified the mating success with the successful egg-laying and brood establishment. The aim of the BeeConSel project in WP4 was to evaluate the mating control via patrilines present in test honey bee colonies. For that purpose, several activities were performed:

a. Developing sampling protocol of biological material (D3).
b. Developing procedures for DNA extractions (D3).
c. Identifying the most cost-effective method for patrilines confirmation.
d. Designing bioinformatics tool for verification of the patrilines.
e. Evaluating the obtained mating control at tested mating stations (D5) through verification of patrilines.

# DEVELOPING SAMPLING PROTOCOL OF BIOLOGICAL MATERIAL

A sampling protocol suitable for field conditions was developed through WP2/WP4 (see D3 - Knowledge transferred - Test protocol developed). Simplicity and ease of use in the field while assuring adequate sample preservation were of our primary concerns. Pupae samples were preferred, however, if a sufficient number could not be sampled, larvae or young workers/drones just before or during emergence from their cells were also collected. In some cases, queen wing clippings were collected instead of whole queens, although whole queens were preferred.

Table 1. The planned number of collected samples depended on colony type/role

| | Drone brood | Queen | Worker brood |
|---|---|---|---|
| Mating nuc | / | 1 | 30 - 50 |
| DPC | 20 - 50 | 0-1* | / |

* In some cases, queens from DPCs were sampled by wing clip in 2023



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# DEVELOPING PROCEDURES FOR DNA EXTRACTIONS

Figure 3. Samples stored at -22° C

DNA was extracted from a single leg (worker brood and queen samples), antennae (drone brood), wing clippings (queens), or spermatheca content (queens). The process, including extraction success verification, consisted of the following six steps:

1. Sample dissection
2. Sample homogenisation
3. DNA extraction
4. PCR amplification of target microsatellite loci
5. Agarose gel electrophoresis of PCR products
6. Preparation of PCR products for capillary electrophoresis (microsatellite analysis)

The DNA extraction procedure varied between sample types. DNA extractions from brood and queen legs were performed using NucleoMag Tissue Kit as described in Moškrič et al. (2023) (ANNEX 1) and DNA extractions from queen wing clippings were performed using the QiaAmp DNA Mini Kit (Qiagen) or QIAamp DNA Investigator Kit following the protocol described in Bubnič et al. (2020).

# IDENTIFYING THE MOST COST-EFFECTIVE METHOD FOR PATRILINES CONFIRMATION

Identifying the most cost-effective method for patrilines confirmation (microsatellites loci, SNPs): The markers are bi-parentally inherited, thus making them more useful tools in parental determination and genetic polymorphic analyses (Estoup et al., 1995). A polymorphic microsatellite has more than one potential allele at a given locus. Given the fact that they are codominant, Mendelian inherited, and neutral markers, microsatellites are easily typed. In addition, they have a high distinctive power among closely



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related individuals, ultimately making them suitable candidates for determining the population structure. So far, 500 microsatellites in honey bees have been studied. Some of them are highly polymorphic, and some are less.

Microsatellites have previously been used to characterise honey bee populations of European origin, and Estoup et al. (1995) scored alleles for seven microsatellite loci among various subspecies and reported a high degree of genetic variation within the honey bee samples. The variation ranged between seven (7) to thirty (30) alleles per locus. Also, super sisters, i.e. honey bees from a similar patriline, clustered together as opposed to half-sisters.

All microsatellites used in the determination of patrilines in the BeeConSel project are unlinked and have an independent inheritance. In the determination of patrilines, five microsatellites were used (A7, A113, Ap43, Ap55 and B124). For all used microsatellites, various reports about their allele forms exist, depending on application in different sub-species. The number of alleles in the populations of beneficiaries' countries is generally high, where HR and SI accounted for about 30 %, while the population in MK is diverse, accounting for 40 % of all possible allelic forms (Table 2). Such a high number of alleles per microsatellite provides high resolution for adequately identifying the patrilines.

Table 2. The number of allelic forms in beneficiaries' population per microsatellite.

| Microsatellite | Possible alleles | Alleles found (%) | Alleles in HR (%) | Alleles in MK (%) | Alleles in SI (%) | Common alleles HR, MK, SI (%) | Common alleles HR, MK (%) | Common alleles HR, SI (%) | Common alleles MK, SI (%) |
|---|---|---|---|---|---|---|---|---|---|
| A7 | 27 | 55.56 | 25.93 | 29.63 | 22.22 | 11.11 | 11.11 | 14.81 | 14.81 |
| A113 | 16 | 50.00 | 25.00 | 37.50 | 25.00 | 12.50 | 18.75 | 18.75 | 18.75 |
| Ap043 | 16 | 68.75 | 37.50 | 43.75 | 18.75 | 18.75 | 25.00 | 18.75 | 18.75 |
| Ap055 | 15 | 66.67 | 26.67 | 53.33 | 46.67 | 20.00 | 26.67 | 26.67 | 26.67 |
| B124 | 15 | 80.00 | 46.67 | 53.33 | 33.33 | 20.00 | 33.33 | 26.67 | 26.67 |
| Total alleles | 89 | 62.92 | 31.46 | 41.57 | 28.09 | 15.73 | 21.35 | 20.22 | 20.22 |



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# DESIGNING BIOINFORMATICS TOOL FOR VERIFICATION OF THE PATRILINES

Designing bioinformatics tool for confronting genomic reads and verification of the patrilines:

All genomic forms of microsatellites were documented in a spreadsheet and stored in the project cloud. This spreadsheet contains information for unique sample codes corresponding to the origin of the sample (country, location, type of colony (DPC, mating nuc), type of the sample (worker bee, mated queen, DPQ – drone producing queen, drone) and two genomic forms identified for each of 5 microsatellites.

A special pipeline was designed for analyzing 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 validation confirmed that the data is following 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 of the genome of the mated queen (MQ) and the genomes of all possible known mates (drones) that were present at the tested location (D). Then, all possible combinations were mathematically calculated to build a reference genome for worker bees (WB) in the colony. To avoid any miss-labelled 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 the 5 microsatellites.
- Verification that all worker bees sampled from a particular colony originated from the queen of the same colony.



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- Reference drones' genome for each of the 5 microsatellites.
- The colony reference genome consisting of a list of all possible combinations of drones and the colony's queen.

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

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

- Total analysed samples per colony.
- Number of unique drones that had mated the queen of the colony.
- The total number of worker bees originating 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.

For each tested location, a summary of results, identifying its possible use of as a potential mating station, is also added.

The tool was communicated to the scientific community by presenting on EAAP 2023 (Andonov et al., 2023) The abstract and poster are given in ANNEX 2.

# EVALUATING MATING CONTROL VIA PATRILINES

The samples of drone-producing queens, drone brood, worker brood and test queens used in various test locations (see D5) were subject to validation. The validation was done using genetic markers, where the origin of desired patrilines was verified. It was confirmed that the geographical and temporal isolation sites and biological saturation approaches were all suitable methods for successful mating control.



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# Geographic isolation

Four approaches were tested on geographic isolation:

## Deep forest

Figure 4. Deep forest concept testing at Gorski Kotar

The concept of deep forest isolation was based on the fact that the area is not interesting for beekeepers outside the honeydew season (late July). It was tested in the Gorski Kotar (HR) location for two seasons. Without DPCs at the location in season 1, only queens and worker brood were sampled. The genetic composition of brood in the colony was challenged against the genetic composition found in queens' spermatheca. The comparison indicated that only 66 % of the genetic diversity in brood was also found in spermatheca.

The validation method was improved by fully exploiting five loci and all allele frequencies. On the site of Gorski Kotar, virgin queens were mated with, on average, 65 % of drones from the deployed drone-producing colonies (5 DPCs) in season 2. The lowest percentage of known drones' contribution to queen mating was 31 %, but there were cases where 100 % of the contribution to queen mating was with drones from DPCs.

It is evident that other - likely feral – honey bee colonies are present even at this deep forest location, which can be considered partly isolated at best. Acquiring a known patriline origin of 65 % in mating honey bees, with the contribution of many drones (10-25), could be used in the breeding program where diversity is desired for increasing genetic variation.

## Highland microsites

The highlands' micro-locations in MK seemed to be suitable as possible locations for mating stations in honey bees due to the configuration in the landscape. Therefore, many sites were explored either with DPCs or without



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DPCs. The potential sites were tested in season 1 without DPCs to identify the presence of other honey bee colonies. Virgin queens in mating boxes were deployed, and mated queens and their brood were thereafter sampled and explored. For the different locations, it was found that many of the queens were mated with 9 to 25 genetically different drones. The results of season 1 helped substantially in selecting the sites for season 2. On the two sites, queens were mated, with 60-64 % (in Toranica and Nikiforovo) of the drones from DPCs. However, high variations were observed (from 10 % to 95 % on one site), which was additionally tested in season 3. In the other locations, the variation ranged from 40 to 95 %.

The isolation of the sites seems long-lasting or permanent, and the sites have the potential to be used for mating stations in N. Macedonia. Their reliability can be improved by providing a higher number of drones, with special drone management or by increasing the number of DPCs.

## Island

The only natural island in MK is available in Lake Prespa. The island is relatively small and isolated by 2 km of water from the nearby mainland. The island has harsh environmental conditions that challenge colony survival throughout the year. The idea is to use the site only during the mating season. Due to high logistic costs, the island was tested in season 3 only by 24 mating nucs and 12 DPCs. However, only 9 nuc colonies survived and were tested. The preliminary results (5 colonies) showed that 85 % of DPCs patrilines contributed to mated queens, although these results could change once all colonies will be analysed.

Figure 5. Snake island in Prespa Lake, MK



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Even though we assumed that the island could provide about 95 % contribution of the known patrilines in mated queens, the results could be influenced by unfavourable weather conditions (i.e. extremely dry and hot in July). The weather events could affect the drone's ability to fly for an extended period, and queens in their nuptial flights could be prolonged.

### Alpine high-altitude valleys

The beneficiary partner followed the guidance in D2 and chose to work on two valleys (Krma and Vrata) in the Alpine region due to the high colony density in the rest of SI. The location in Krma was tested in two seasons, both times with DPCs. Drones belonging to DPCs contributed to brood at an average of 89 % (94 % and 86 % in two cycles, respectively). These results suggest that the site can be used as a mating station successfully with sufficient DPCs. In the location Vrata, we have found evidence of feral colonies, as the queens were mated with 17-24 genetically different drones.

Figure 6. Geographical isolation in the alpine Valley Vrata

Combined with the observed behaviour (see D5), the results of the two Alpine high-altitude valleys are promising and can offer high confidence in their use as mating stations in a breeding program for honey bees in SI.

Additionally, we also engaged spermatheca test to improve the lab throughput, but only 62 % brood genotype was found also in spermatheca, suggesting the low value of the method for these purposes.

## Time isolation

The time isolation approach - also referred to as the delayed time mating flight model - is an innovative alternative to classically isolated mating stations used in honey bees. The idea behind this approach is to manipulate the virgin queens and drones of known origin to engage in mating flights



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outside of the time interval of other colonies, inclined to the same drone congregation area. The time interval in which the queens and drones engage in mating flights heavily depends on the length of the day (e.g., geographic latitude of the location) and weather conditions.

Two methods were tested to manipulate nuptial flights of the virgin queens during the mating season: labyrinth and cooling.

## Labyrinth

Adding a labyrinth in the front or in the mating box physically restrains the young queen from flying out until it is desired to do so but, at the same time, allows worker bees to pass through for forage. The biological function of the labyrinth is to prevent physical damage to the queen on the restraining mechanism due to light stimulation, which drives the queen for the nuptial flight. Namely, the labyrinth is constructed in such way as to effectively blocking all light. The system was tested with DPCs by partners in MK and NO for two seasons (seasons 2 and 3) and SI in one season (season 2).

Figure 7. Time isolation experiment in SI. Photo: T Vidmar.

The first iteration in MK was promising, where the virgin queens were mated with 62 % of drones originating from DPCs. The second season testing was repeated in MK, where some modification of the labyrinth and ventilation of the box was done. However, the results from observations were not so affirmative due to unidentified factors affecting flying drive, and molecular analysis was not performed.

In Slovenia, the experiment took place in the Ljubljana marsh region (i.e. Ljubljansko barje), just south of the capital, which is an area with high colony density. Two rounds of experiments were performed, both with two late-flying test groups and one control. As described in D5, there was no significant difference in success rate between the late control group and the two test groups, meaning that flying drones were available and likely



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dominated the drones from the three time-manipulated DPCs. Due to this, no paternity analysis was performed.

In Norway, the experiment was carried out to see whether this method could also work in a cold northern climate where it is already challenging to have the queens mated even without time restrictions. Under favorable weather conditions, high mating success was achieved. However, paternity analyses are needed to verify the success of this method.

## Cooling chamber

The alternative way to the time isolation approach is the use of a cooling device, where the nucs (virgin queens and worker bees) are kept in a dark and cold place (13-15˚ C) to manipulate the perception of bees about the daytime. The chamber was constructed and tested by the MK partner. Neither queens nor worker bees were observed to fly out before the selected time point (17:30, see D5). The contribution of the drones from the manipulated DPCs in the mating of the queens was 75 %. In the second year, as in the case of the labyrinth, the results from observations and successful mating flights were unfavorable.

Time isolation showed clear potential, particularly in the first testing season results. The method still needs a lot of tuning and modification until it can be considered a routine.

Figure 8. Cooling chamber in MK



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# Biological saturation

Biological saturation is a concept which compensates for the lack of geographical barriers (e.g., in flat land) by flooding the drone congregation areas where the mating takes place with an extremely high number of drones from DPCs of known origin (~100).

It was tested in one site, Batina, in HR for three seasons. In season 1 for verification, 4 loci were used due to the insufficient laboratory consumables supply affected by the COVID-19 pandemic. The method used was in lower resolution, concluding that 96 % of the matings of the virgin queens were performed by drones from the DPCs. In the following season (2), the method was improved to include an additional microsatellite locus, where the resolution in detecting different genotypes was more precise. However, the queens were mated with 76 % of drones from DPCs, while the remaining 24 % are from the neighboring honey bee population.

Considering the cost of managing the mating site and the reliability of the results, we suggest that in flat lands, where isolated locations are more challenging to obtain, saturation can be explored as an approach for mating control. However, occasional testing of the reliability of the biological saturation approach is necessary when it is used in continuous breeding programs.

Figure 9. Biological saturation in Batina, HR. Photo: B Kozinc.



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Table 3. The number of locations samples and verification methods by country and years.

| | Year | Mode of mating control | Location | Micro-locations | Mating boxes/ queens | DPC | Verification of patrilines purity | Use of spermathec aas the | Notes |
|---|---|---|---|---|---|---|---|---|---|
| **HR** | 2021 | G/S | Flatland | 1 | 120 | 96 | 96.3% | | 4 MS only; 15.4 patrilines |
| | | G | Deep Forest | 1 | 40 | 0 | | 63% | 12.6 patrilines |
| | 2022 | G/S | Flatland | 1 | 20 | 96 | 76.6% | | |
| | 2023 | G | Deep Forest | 1 | 30 | 6 | 64.9% | | |
| | | G | Flatland | 1 | 30 | 96 | | | |
| **MK** | 2021 | G+TC+T | Mrshevci | 1 | 40 | 0 | | | 9.5 patrilines |
| | | G | Krivolak | 3 | 30 | 0 | | | 17.7 patrilines |
| | | G | Krivolak | 2 | 16 | 0 | | | |
| | | G | Galicica | 1 | 16 | 0 | | | |
| | | G | Mavrovo | 3 | 36 | 0 | | | 15.0 patrilines |
| | | G | Mavrovo | 2 | 18 | 0 | | | 15.3 patrilines |
| | | TC+TL | Mrshevci | 1 | 42 | 0 | | | 14.5 patrilines |
| | 2022 | G | Ravna Reka | 1 | 23 | 0 | | | 24.2 patrilines |
| | | G | Nikiforovo | 1 | 31 | 35 | 64.5% | | |
| | | TC+TL | Radishani | 1 | 41 | 13 | 66.0%<br>TC: 75%<br>TL: 62% | | |
| | | G | Toranica | 1 | 29 | 20 | 59.7% | | |
| | 2023 | TC+TL | Radishani | 1 | 28 | 10 | | | |
| | | G | Toranica | 1 | 30 | 10 | | | |
| | | G | Snake Island | 1 | 24 | 14 | 85.1% | | |
| **SI** | 2021 | G | Krma | 5 | 30 | 5 | 94.2% | 62.4% | |
| | | G | Krma | 5 | 30 | 5 | 85.5% | | |
| | | G | Vrata | 5 | 30 | 0 | | | 24 patrilines |
| | | G | Vrata | 5 | 30 | 0 | | | 15 patrilines |
| | 2022 | G | Krma | 3 | 30 | 5 | | | |
| | | G | Krma | 3 | 30 | 5 | | | |
| | | G | Vrata | 2 | 30 | 4 | | | |
| | | G | Vrata | 2 | 30 | 4 | | | |
| | | TL | Ljubljana | 1 | 30 | 0 | | | |
| | | TL | Ljubljana | 1 | 30 | 3 | | | |
| | 2023 | G | Vrata | 2 | 30 | 8 | | | |
| | | G | Vrata | 2 | 30 | 0 | | | |
| **NO** | 2022 | TL | Ås | 1 | 30 | 6 | | | |
| | | TL | Ås | 1 | 30 | 6 | | | |
| | 2023 | TL | Aurskog | 1 | 30 | 6 | | | |
| | | TL | Aurskog | 1 | 30 | 6 | | | |

**Mode of mating control*: G: geographical isolation, S: biological isolation with many DPCs, TC: delayed mating flight with cooling method, TL, delayed mating flight with labyrinth method.



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# CONCLUSIONS

Most promising approaches to mating control tested in the field were subject to verification by applying molecular methods. To the best of our knowledge, many of the existing mating stations used in honey bees all over the world assumed that geographical isolation was sufficient to improve genetic progress in breeding programs compared to the present system with very limited mating control. All partners adapted the approaches that had the most potential for each country, but the experiences and skills were largely shared over the project lifetime. However, specifics in the countries are still dominant and this will need to be taken into account in decision making.

## Croatia

HR tested two isolation approaches: isolation by deep forest and biological saturation with drones. When deep forest isolation is used for mating stations, one should expect the queens to be mated with about 2/3 drones originating from DPCs provided on the location. The purity of the mating satiation could provide a consolidated genetic background for further breeding. Still, if it is used as the only technique for many consecutive years, the genetic diversity may be maintained and blurred genetic progress should be expected.

The biological saturation of the mating sites with many drones proved to be a better solution regarding the contribution of 3/4 of the known origin drones to the mated queen. However, the system works if many virgin queens need to be mated in several cycles on the same spot. This approach is advantageous in producing many mated queens (several thousand) and can be used for commercial breeders or groups of breeders. On the other hand, introducing many queens mated with the same DPCs every year can narrow the population's genetic composition. Hence, regular checks of the genetic diversity should be performed in 3-5 generations when using the biological saturation approach.

Based on our results, both approaches should be combined in performing controlled mating for breeding purposes.



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## N. Macedonia

Driven by a pronounced interest in breeding programs and mating behaviour in honey bees, MK opted to test many approaches and locations. Two geographical isolations (highlands microsites and island) and temporal isolation with two modalities were tested. However, many highland microsites were identified, and two of the most promising were intensively tested. The results from the molecular analysis suggest that both have similar performance, providing almost 2/3 controlled mating for the queens. The variation among mated queens indicates room for improvement, reaching above 70 % of drones' contribution with known origin.

The most isolated site on the island can provide high reliability in controlled mating but at a very high cost (loss of the queens, expensive installation, and unpredictable weather conditions). In D7, the specific modelling should give a clear answer if the island as a controlled mating station can benefit future breeding programs.

The temporary delay mating methods are still being tested, even though they already showed promising results. Therefore, this approach should not be broadly used in controlled mating stations in MK until regular satisfactory results are reached.

## Slovenia

The partner in SI opted for three locations, where two locations have naturally isolated spots in two valleys. The sites are supposed to be isolated and free from surrounding apiaries. However, in season 1, evidence of the unmanaged or unknown honey bee colonies within mating range was confirmed. Hence, in the next season (2), the close availability of DPCs was considered in the two locations. Even though the test was done on a relatively small number of nucs, the contribution of the drones from DPCs to queen-mated was as high as 90 %. The results are promising, particularly because Slovenia has a high apiary density, and finding a spot without bees is always challenging. In addition, the prolonged mating time was tested on a third location, but the approach still needs further development to be implemented on a large-scale in the breeding program.



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# ANNEX 1

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## Cutting corners: The impact of storage and DNA extraction on quality and quantity of DNA in honeybee (*Apis mellifera*) spermatheca

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The purpose of our study was to investigate methods of short-term storage that allow preservation, transport and retrieval of genetic information contained in honeybee queen's spermatheca. Genotyping of the honeybee colony requires well ahead planned sample collection, depending on the type of data to be acquired. Sampling and genotyping of spermatheca's content instead of individual offspring is timesaving, allowing answers to the questions related to patriline composition immediately after mating. Such procedure is also cheaper and less error prone. For preservation either Allprotect Tissue Reagent (Qiagen) or absolute ethanol were used. Conditions during transportation were simulated by keeping samples 6–8 days at room temperature. Six different storing conditions of spermathecas were tested, complemented with two DNA extraction methods. We have analysed the concentration of DNA, RNA, and proteins in DNA extracts. We also analysed how strongly the DNA is subjected to fragmentation (through amplification of genetic markers ANT2 and $tRNA^{leu}$-COX2) and whether the quality of the extracted DNA is suitable for microsatellite (MS) analysis. Then, we tested the usage of spermatheca as a source of patriline composition in an experiment with three instrumentally inseminated virgin queens and performed MS analysis of the extracted DNA from each spermatheca, as well as queens' and drones' tissue. Our results show that median DNA concentration from spermathecas excised prior the storage, regardless of the storing condition and DNA extraction method, were generally lower than median DNA concentration obtained from spermathecas dissected from the whole queens after the storage. Despite the differences in DNA yield from the samples subjected to different storing conditions there was no significant effect of storage method or the DNA extraction method on the amplification success, although fewer samples stored in EtOH amplified successfully in comparison to ATR storing reagent. However, we recommend EtOH as a storing reagent due to its availability, low price, simplicity in usage in the field and in the laboratory, and capability of good preservation of the samples for DNA analysis during transport at room temperature.





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# ANNEX 2

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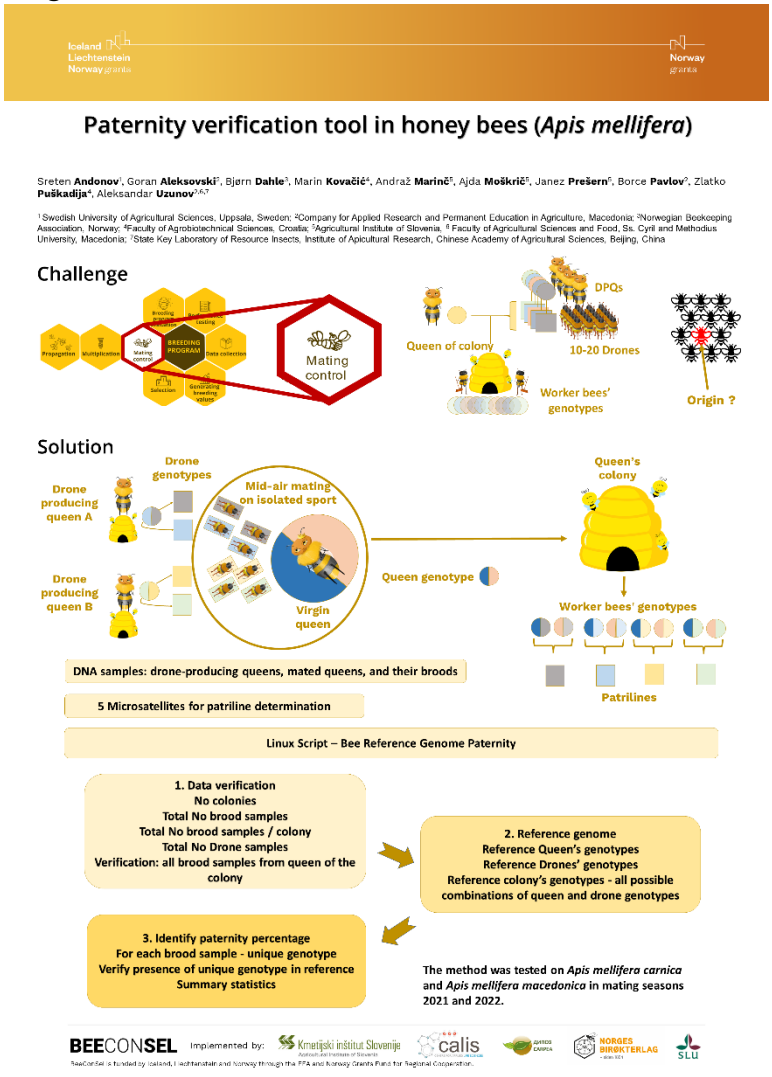




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