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Whole-genome sequencing data confirms the uniqueness of Ireland's native honey bee

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Recent evidence suggests a rise in the importation of non-native honey bee subspecies into Ireland with an associated potential rise in hybridisation followed by introgression. The purity of the *Apis mellifera mellifera (A. m. mellifera)* population in Ireland, given its decline elsewhere in Europe, makes the Irish population a very important biodiversity, and food security, resource that will also play an important role in conserving the genetic diversity of *A. m. mellifera*. To investigate if the Irish *A. m. mellifera* population can be distinguished from other European populations and to assess the introgression levels within Irish honey bees, we sampled 30 worker bees from each of 153 honey bee colonies for a pool-sequencing analysis. The assembled genomic data, also including pool sequencing data from other European studies, allowed us to confirm that pure Irish *A. m. mellifera* colonies are distinct from other *A. m. mellifera* populations and that introgression has become a serious issue.

Keywords *Apis mellifera mellifera*, Pool-sequencing, Introgressive hybridisation, High-resolution population networkA, Conservation

The *Apis mellifera* L. subspecies native to Ireland is *A. m. mellifera,* one of three subspecies currently assigned to the M lineage, and known for its ability to survive harsh winters and forage during wet and windy weather conditions[1]. While the native range of *A. m. mellifera* once included most of Northern Europe, the current range is greatly reduced, with pure (or almost pure) populations existing only on select islands such as Colonsay island in Scotland[2], Ouessant island in France[3] or in conservation areas, such as the Glarus conservatory in Switzerland[4]. The decline of native subspecies and the impact of hybridization due to the human-mediated movement of honey bees for apiculture has led to concerns about the loss of local biodiversity and potential extinction of native subspecies from parts of their original range[5–7]. Extensive introgression has been described for the United Kingdom (UK)[2], Norway[8], France[4], and Poland[9,10] and islands including the Canary islands,[11,12] and Malta[13]. Ireland has a widespread population of *A. m. mellifera*, however imports of non-native subspecies by beekeepers is beginning to impact this population also [e.g.[14,15]]. While hybridization, and subsequent introgression due to backcrossing, can provide additional alleles, boosting adaptive potential when populations are weak or small[16,17], introduced alleles can have a negative impact on a population that is diverse and well adapted[18–20]. However, the long-term effects of honey bee subspecies hybrids is not known[21]. The widespread use of a few commercially-oriented strains could cause homogenization and reduce genetic diversity of well adapted forms. Therefore, we consider protection of the biodiversity associated with local populations of native subspecies, such as the Irish population of *A. m. mellifera*, to be important.

Recent research has shown that *A. m mellifera* colonies from Ireland have a distinct phenotype compared to other *A. m. mellifera* populations on mainland Europe[14] and that most Irish beekeepers place a high level of importance on conservation of this subspecies[22]. This creates a significant opportunity to turn Ireland into a conservation area for *A. m. mellifera*. However, to achieve such a goal, it is important that the population would have retained a sufficient amount of non-introgressed *A. m. mellifera*. Even though most beekeepers in Ireland aim to manage *A. m. mellifera* bees[22], C-lineage honey bees, (primarily *A. m. ligustica* and *A. m. carnica*) and the commercial Buckfast strain, appear to be continually imported into the island[14,15]. This is likely due to the perception that they produce greater honey yields and are gentler compared to other subspecies[23,24], despite evidence that hybrids are more aggressive[1,25], and that introgression can cause the loss of locally adapted traits[20,26]. Queens of non-native subspecies, as well as the commercial Buckfast strain, are often purchased online by novice beekeepers, who are not aware of the difference between native or non-native subspecies, nor of the benefits of

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keeping locally adapted honey bees. This human-facilitated dispersal is behind the major threats facing *A. m. mellifera* and other threatened honey bee populations, including introgression caused by the backcrossing of hybridised colonies with native pure colonies[27]. Given the global trade of Italian (*A. m. ligustica*) and Carnolian (*A. m. carnica*) subspecies, introgressive hybridisation, and at times total replacement of one subspecies with another, has become a global problem, impacting native subspecies across Europe and Africa [e.g.[28–32]].

Previous studies carried out in Ireland have shown that besides a pure *A. m. mellifer*a population, introgressed colonies also exist. A very low introgression level (2.2%) was identified in 412 honey bees from 80 colonies (1:3 free-living:managed) sampled across Ireland via microsatellite and mtDNA data by Hassett et al.[33]. Browne et al.[34] obtained a similar result for a free-living honey bee cohort (76 colonies sampled primarily in 2016) using the same methods and single nucleotide polymorphism (SNP) genotyping. Within 10 years the pattern had changed with McCann and McCormack[15] and Valentine et al.,[14] showing higher introgression levels. Only 36 free-living colonies were included by McCann and McCormack[15] but 26.5% of mitochondrial sequences generated were of C lineage and 12.5% of the colonies examined showed introgression by microsatellites with only one bee sampled per colony. Valentine et al.[14] reported 25% of > 500 managed colonies as being introgressed with approximately 8% significantly introgressed. The percentage of queens imported into Ireland has also risen, from approximately 150 between 2012 and 2017 to 249 in 2019 followed by another further sharp rise of > 327% to 1063 in 2020 (*nihbs.org*) perhaps accounting for the increase reported from molecular data.

The first molecular studies on the Irish population described above were limited by the coverage of the honey bee genome, the numbers of collection sites and numbers of individuals[14,15,33,34]. Chen et al.[35] suggested the inclusion of 10 worker bees to accurately assess colony genetic diversity given the number of patrilines present[36,37]. Valentine et al.[14] carried out an improved approach by including the antennae from each of 30 worker bees for each colony to generate an estimated introgression level based on the allele frequencies of 62 SNPs. Here we apply state of the art next generation pool-sequencing approach, on the DNA from 30 worker bees per colony, in a specific approach to increase the likelihood of each included bee contributing to the data. Three bioinformatics approaches were compared for their ability to detect introgression before being applied to investigate the possible impact of imports on colony genetic integrity. The generated whole-genome sequencing data will be used not only to test the hypothesis that the Irish *A. m. mellifera* is now under serious threat due to introgression, but also to test if the data is consistent with the presence of a distinct ecotype on the Island of Ireland. If both hypotheses are true, immediate conservation action becomes more crucial.

Methods

Data sampling: The dataset includes pool-seq data from 153 *A. m. mellifera* colonies from throughout Ireland. These included 75 free-living colonies, reported by members of the public into the wild honey bee study reporting tool between 2020 and 2022 (https://biodiversityireland.ie/surveys/wild-honey-bee-study/) and 78 managed honey bee colonies located at multiple apiaries for comparison of fully managed and low intervention management practices. Given that there is no way to confirm if wild honey bees have descended from a colony that has always been wild, or has swarmed from a nearby apiary (feral), the term free-living was developed[34] to describe those colonies that have colonized a cavity other than a beehive and lives there with no human intervention. Some of the free-living colonies are part of a monitoring program and therefore we know that they are established in the wild habitat, while for others we cannot confirm the origin. Two managed colonies (DRX3 and TD2) that showed a mixture of colour type in the worker bees, indicative of introgression were included. For these colonies two samples were taken; one of only black bees and one that contained worker bees that had orange banding on the abdomen. Both samples were processed separately to explore if the black bees were unhybridised and the result of a cross between a pure *A. m. mellifera* queen with mixture of native and non-native drones. Previous studies from Ireland did not include any colonies located in Donegal (North West Ireland), therefore we included 46 colonies from this area (21 free-living and 25 managed). In each case, colonies were sampled from the nest entrance using an insect hoover to collect a minimum of 30 foragers. All sampled colonies and their location are summarized in Supplemental Table 1 and in total the number of worker bees processed comprised 4,736. Each colony sample of honey bees was placed in a ventilated box on ice immediately after capture and transported to the laboratory. Upon arrival at the laboratory the bees were transferred to a −20 °C freezer and then freeze-dried to remove all moisture and maximise DNA yield. Three colonies were included that had been sampled opportunistically from nests of free-living forest honey bees in Northamptonshire and Oxford (UK) and five *A. m. carnica* colonies were also included for reference that were received from the Kirchhain bee research Institute in Germany.

DNA extraction, pool sequencing and variant calling: The honey bees collected from each colony were dissected in six groups of five individuals (N = 30) for the extraction of thoracic muscle in a method similar to Buswell et al.,[2]. The freeze-dried tissue from each group of five bees was ground into a fine powder using a sterile pestle and mortar. We used 25 mg of the fine powder, from each of the six tissue subsamples, to extract DNA using a ZymoBIOMICS™ Miniprep Plus kit (Zymo Research Corp., Irvine, CA, USA). DNA concentration was measured using a Qubit 4 fluorometer to ensure successful DNA extraction, while DNA quality was checked by nanodrop. Finally, the DNA extracted from each group of five bees was pooled together in equimolar amounts to create a single DNA sample (30 mL) for each colony. Next-generation sequencing (NGS) was outsourced to Novogene and performed using a NovaSeq6000[38] with a target depth of 50X coverage. Initial quality control of the NGS data was performed using FastQC (version 0.11.9)[39]. Adapters, low quality reads, and reads with less than 50 bp were removed using Trimmomatic (version 0.39, http://www.usadellab.org/cms/?page=trimmomatic)[40]. Raw reads were aligned to the honey bee reference genome Amel_HAv3.1,[41] using BWA-MEM2 (version 2.2.1, https://github.com/bwa-mem2/bwa-mem2)[42] with default parameters selected. Duplicate reads were marked and read groups were added to the alignment files using PICARD tools (version 2.20.4, https://broadinstitute.github.io/picard/). Indels were marked and variant call format (VCF) files were generated using LoFreq (version

2.1.3.1, https://csb5.github.io/lofreq/)[43]. VCF files were then annotated using SNPEff (version 5.1, https://pcingola.github.io/SnpEff/)[44] after creating a custom *A. mellifera* database using the reference genome and Gene Transfer Format (GTF) files.

Merging pool-seq datasets: To create a Europe-wide population network of honey bees, we combined our Irish dataset with two previously described pool-seq datasets [[45], corrected dataset (SRA PRJNA666033)] and [32]. Each sample from the SMARTBEES dataset[45] was created by pooling organic material from 100 single honey bee workers gathered from 100 different colonies throughout the country of origin, while the pooled data from Switzerland used 500 individual workers from a single colony and included among others *A. m. mellifera* colonies from a conservation area and a selection program[46]. To combine the three different datasets, the Popoolation sync file, that contained the SmartBEES and Irish data, was transformed into an allele frequency file using a custom python script that allowed for multi allelic information. This was then merged with the allele frequency file of the Swiss data, leaving 540,491 genome-wide overlapping SNPs, which were used for subsequent analyses. A single *A. cerana* worker from SRA project accession PRJNA869845 sample SAMN30328048 was added to the dataset to act as an outgroup.

Estimating Introgression: To assess C-lineage introgression levels in the sampled *A. m. mellifera* colonies, we applied and compared three commonly used hybrid detection methods; F_{ST}, f-statistics, and NGSadmix[47–49]. Concordance between the three methods was assessed by ranking colonies according to C-lineage introgression levels estimated by each method, followed by calculating the Spearman's rank correlation between the methods using the top 35 most hybridized colonies. A Friedman's rank sum test was used to detect significant differences between the rankings of the three methods using the PMCMR package in R (version 4.4, https://cran.r-project.org/web/packages/PMCMR/index.html)[50]. For this analysis the SNP data set including 540,941 genome-wide SNPs was selected, which were in common with previously described SNPs to delineate honeybee subspecies[3]. To detect hybrids using F_{ST}, an mpileup file was generated with Samtools (version 1.1, https://www.htslib.org/), using the quality controlled bam files and a bed file containing the positions of the 540,941 SNPs[51]. This output was then processed with Popoolation 2 (version 1.201, https://sourceforge.net/p/popoolation2/wiki/Main/)[52] to produce a sync file that contained allele frequencies for each SNP across all sampled colonies. This sync file was then converted into a pooldata object using the PoolFStat package in R (version 3.1, https://cran.r-project.org/web/packages/poolfstat/index.html)[53] with the following thresholds: a minimum coverage of four reads per pool, a maximum of 500 reads per pool, and a minimum allele frequency (MAF) of 1%. The resulting pooldata object contained 359,499 genome-wide SNPs, enabling calculation of pairwise F_{ST} values between populations. For each *A. m. mellifera* colony, the final F_{ST} value was computed as the average pairwise F_{ST} between that colony and the reference C-lineage colonies, using both our dataset (N = 5) and C-lineage samples (N = 5) from the SmartBEES data set[45].

Using f3 statistics with PoolFStat[53], hybrids were detected based on known M-lineage and C-lineage populations selected as the hypothetical parent populations. A colony was considered hybridised if the f3-statistics yielded a Z-score less than -3 (p value < 0.0015)[53]. To further quantify the degree of introgression, an f4 ratio test[53] was performed for each colony using *A. cerana* as outgroup. Finally, model-based clustering NGSadmix (https://github.com/aalbrechtsen/NGSadmix)[49] was used to estimate C-lineage introgression levels in the sampled Irish *A. m. mellifera* colonies. This method relies on genotype likelihoods, which were derived from allele frequencies at each SNP, assuming Hardy–Weinberg equilibrium. For the comparative analysis, NGSadmix was run unsupervised with the number of ancestral population (K) set to 2 and a common applied MAF threshold of 5%. After MAF filtering, the analysis included 109,480 genome-wide SNPs from 145 of the 153 Irish *A. m. mellifera* colonies and five *A. m. carnica* reference samples.

Population network analyses: To generate a fine-scale population network of European honeybees, a Euclidean distance matrix was created using the custom allele frequency file that was then transformed into a Spearman's correlation matrix using R. The correlation matrix was used as input for NetView[54,55]. NetView utilises an unsupervised k-nearest neighbours algorithm to identify fine-scale population structures. Here, we applied the algorithm in its default setting (number of k nearest neighbours k-NN = 10) and used the program Cytoscape (version 3.91, https://cytoscape.org/index.html)[56] to visualize a fine-scale population network. Node shapes were then manually styled according to the original geographical location and data origin. Hybrids were detected using f3 and f4 statistics as described above and f4 ratio results for each honey bee colony in the Irish dataset were overlaid onto the network graph using the enhanced Graphics package within CytoScape[57].

To explore the differences between Irish and Swiss *A. m. mellifera* colonies, we applied NGSadmix[49] on a subset including 332 *A. m. mellifera* and 33 *A. m. carnica* colonies as C-lineage references (Galway dataset N = 5, Swiss dataset N = 28). NGSadmix was run unsupervised with a default MAF threshold of 5%, increasing the number of K from 2 to 5. After MAF filtering 178,396 genome-wide SNPs were included in the analysis. Cluster membership proportions of the colonies for the respective K clustering result were visualized using ggplot2 in R.

Results

Comparison of methods to detect introgression from pool-seq data: Results of F_{ST}, f3/f4 and NGSadmix analyses results for the top 35 hybridised colonies are presented in Suppl. Table 2 along with the ranking of colonies according to introgression levels (Suppl. Table 3). A high correlation was found between the three different applied approaches for detecting introgression (Suppl. Figure 1). The top 10 most hybridised colonies were identified as such by both f4-ratio and F_{ST} with one ranked 12th instead by NGSadmix. The top 20 introgressed colonies were the same for all three methods, while looking at the top 30 yields one difference between f4 ratio and both other methods where one colony was ranked 31th in each case (instead of 28th or 29th). Overall, f4-ratio and F_{ST} had the highest correlation at 0.971, while F_{ST} and NGSadmix showed the lowest at 0.831. A p-value result of 0.5238 via a Friedman's rank sum test however, indicated no significant difference between the three methods.

Introgression in Irish Sampled Colonies: According to f3 statistics, 28 (18.42%) of the 153 Irish colonies showed signs of introgression with C-lineage (Z-score $\leq$ -3). There was a greater percentage of hybridised colonies in County Donegal compared to all other counties sampled while County Tipperary, (location of significant native Irish honey bee conservation), had only one of nine sampled colonies identified as hybridized (Fig. 1). The degree of introgression in both free-living and managed colonies was similar at 18.7% for the free-living colonies and 18.1% for the managed colonies Both samples from each of the two managed colonies that showed a mixture of worker colour-type showed introgression with the black workers showing an f4ratio of 0.81/0.77 and the banded workers with an f ratio of 0.85/0.75 for DRX3 and TD2 respectively (Supplemental Table 2).

Of the 28 detected introgressed colonies via F-statistics, nine had an f4 ratio between 0.9 and 0.94, suggesting a low level of introgression had occurred in these colonies. A further 10 colonies had an f4 ratio between 0.85 and 0.9. The remaining nine colonies were very introgressed with f4 ratios in the range of 0.73 to 0.89 (Suppl Table 1). On the other hand, more than half of all Irish colonies had a f4 ratio estimate greater than 0.96 (N = 97) with a subset of non-introgressed *A. m. mellifera* colonies that had an f4 ratio estimate of 1 (N = 38) (Suppl Table 1). The three honey bee colonies with the most C-lineage introgression as determined by f4 ratio testing were all sampled in the UK (Suppl Table 1). The three most introgressed Irish colonies were F74D19 (0.74), TD2NBD (0.75) and F152DL2021(0.76) from Counties Dublin, Wicklow and Donegal respectively.

Inishowen in County Donegal, the largest peninsula in the country, was the geographic location that returned the highest percentage of introgressed colonies (34.7%, Fig. 1). The largest urban area in Inishowen, the town of Buncrana with a population of slightly less than seven thousand people, had a lower introgression rate ($\sim$ 80%) than some of the more rural areas sampled (Fig. 1) such as the town of Moville where both colonies sampled were found to be introgressed. These findings are in contrast to that of the other highly sampled county, County Galway, where Galway City and its surrounds were found to be more introgressed ($\sim$ 55%) than the rural areas, specifically the North, West, and East of County Galway where no hybrid colonies were detected suggesting a large degree of variability at local scale.

Fine-scale population network of European A. mellifera: The European population network successfully grouped honey bee colonies into clusters based on the geographic origin of the bee samples regardless of the dataset they originated from (i.e. all *A. m. mellifera* from Ireland, all *A. m. mellifera* from Switzerland and all *A. m. carnica* from Germany). Most Irish colonies formed a distinct group, while other Irish colonies showed a more dispersed pattern connecting to other *A. m. mellifera* populations such as the Russian, Danish, and Swiss honey bee colony data (Fig. 2). Honey bees clustering in close neighborhood to Irish samples originated from SMARTBEES data, i.e. the Isle of Man and *A. m. iberiensis* from Spain and Portugal, respectively.

Overlaying f4 ratio statistics from Suppl. Table 1 onto the Irish colonies of the population network, suggests that the group of Irish samples forming a distinct cluster, was composed of *A. m. mellifera* colonies that showed little or no introgression, while the more introgressed colonies were those that build a hub between this group and other subpopulations. For the majority, a gradient of introgression can be observed, which highly correlates with the position within the network (e.g. colonies positioned at the edge of a core group also exhibited higher admixture levels). In this context, two samples from the UK, JIBWWC1 and JIBWCB1, (f4 ratios of 0.6888 and 0.7243 respectively) and the two most introgressed Irish samples, DRX3BLACK and F152DL2021, (f4 ratios of 0.7574 and 0.8454 respectively) are positioned next to the Swiss *A. m. mellifera* colonies. Of the three colonies sampled in the UK, two of them group with the introgressed M data while one (RPOT from Blenheim, Oxford) grouped with C lineage data. All five A. *m. carnica* samples from Germany clustered within the C lineage cluster. Two of the colonies deemed non-introgressed via f4-ratio (F149DL2022, F162DL2022 Fig. 2), clustered with the hybridised colonies. NGSadmix identified these colonies as hybridised with similar results to surrounding colonies in the network (Suppl Figure 2).

The population network has allocated the C-lineage honey bee colonies into a single cluster, however this cluster shows further substructures according to the geographical location, i.e. honey bees from Armenia were positioned at the top left corner followed by the 'Turkish group' thereafter Macedonia/Greece and Bulgaria, then Romania and Germany. Suppl. Figure 3 shows that the data generated here from five *A. m. carnica* samples obtained from the Kirchain Bee research Institute cluster with those *A. m. carnica* bees sampled from Germany/Austria/Slovakia within the SMARTBEES project. To the top right of the network in Suppl. Figure 3 are the Italian *A. m. ligustica*, the Swiss, US, Swedish, Norwegian C lineage bees with the Maltese bees remaining distinct.

At the first level of model-based clustering (K = 2) the Irish *A. m. mellifera* population exhibited significantly less introgression than the Swiss population (Fig. 3). In the Swiss population, only 15 out of 184 honey bee colonies showed a 100% membership proportion in the *A. m. mellifera* cluster, while all these colonies originated form the Swiss conservation program. In contrast, most colonies in the Irish population were assigned to the *A. m. mellifera* cluster with a 100% membership proportion. At the second clustering level (K = 3), the Irish population was distinguished from the Swiss population, and admixed Swiss colonies via K = 2 now showed, on average, a lower C-lineage introgression level. At the third (K = 4) and fourth (K = 5) clustering levels, Swiss colonies from the conservation program formed a distinct cluster, while colonies from the selection program showed further differentiation. In this context, it should be noted that at K = 2, 10 Swiss colonies from the conservation program exhibited low levels of C-lineage introgression. However, at higher clustering levels, there was no evidence of admixed colonies within the Swiss conservation program, although a few Irish colonies showed a low membership proportion to the Swiss conservation cluster.

Discussion

The food security of all countries is highly dependent on the health of their pollinator populations, of which honey bees arguably form the most important component[58]. The causes behind pollinator decline are very complex[59], encompassing invasive pathogens due to globalization[60], to trade of queens[30], pesticide use and lack of flowers[61]. In addition to these more recognizable elements, the more insidious and negative effects of

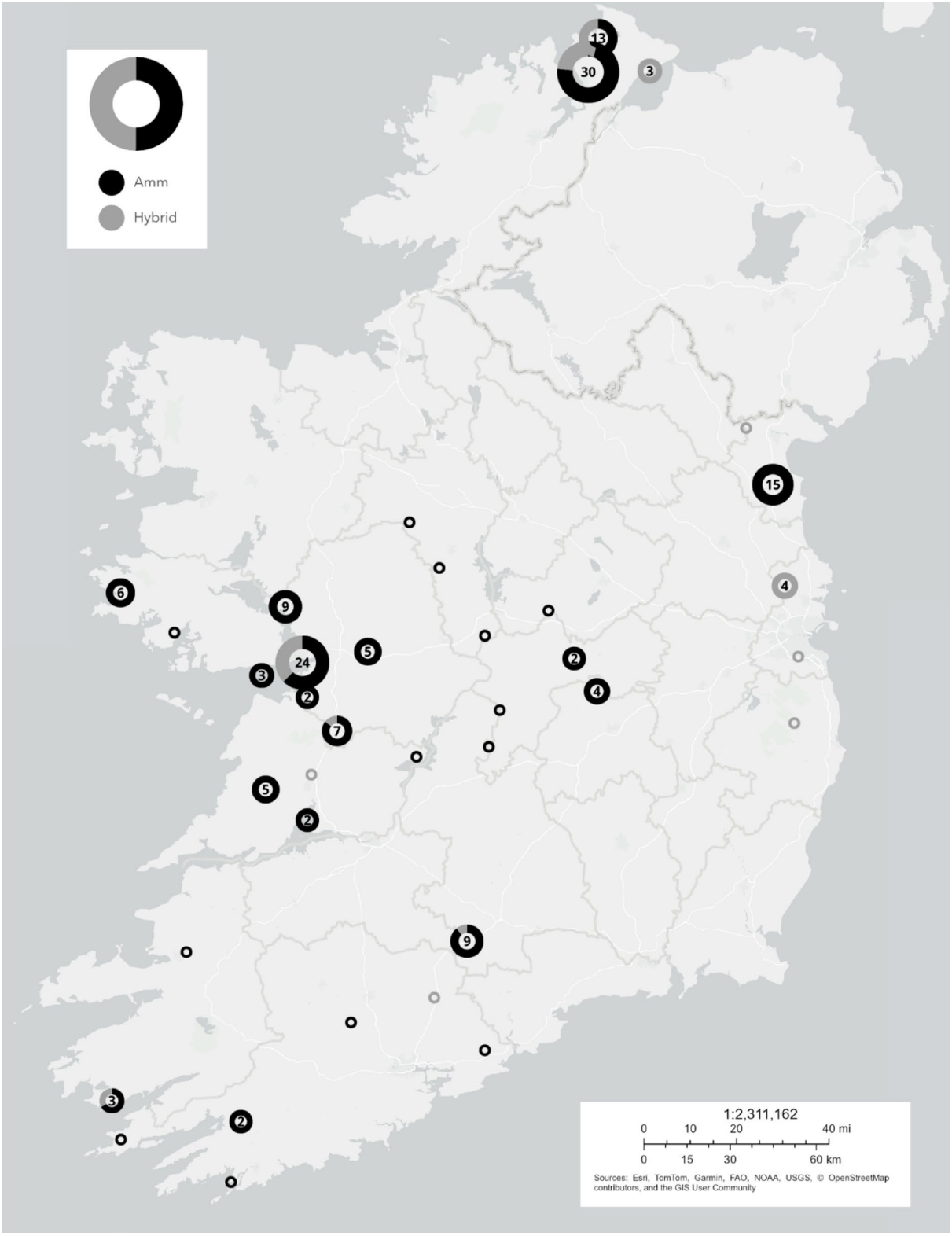


Fig. 1. Map of Ireland showing locations of sampled colonies. Each ring reflects the proportion of *A. m. mellifera* (black) and introgressed (grey) colonies at each location. The number of colonies represented by each ring is displayed in the centre, rings with no number refer to one colony having been sampled. The map was generated in ArcGIS (Esri "World Topographic Map https://www.arcgis.com/home/item.html?id=30e5fe3149c34df1ba922e6f5bbf 808f. May 25, 2017) under license.

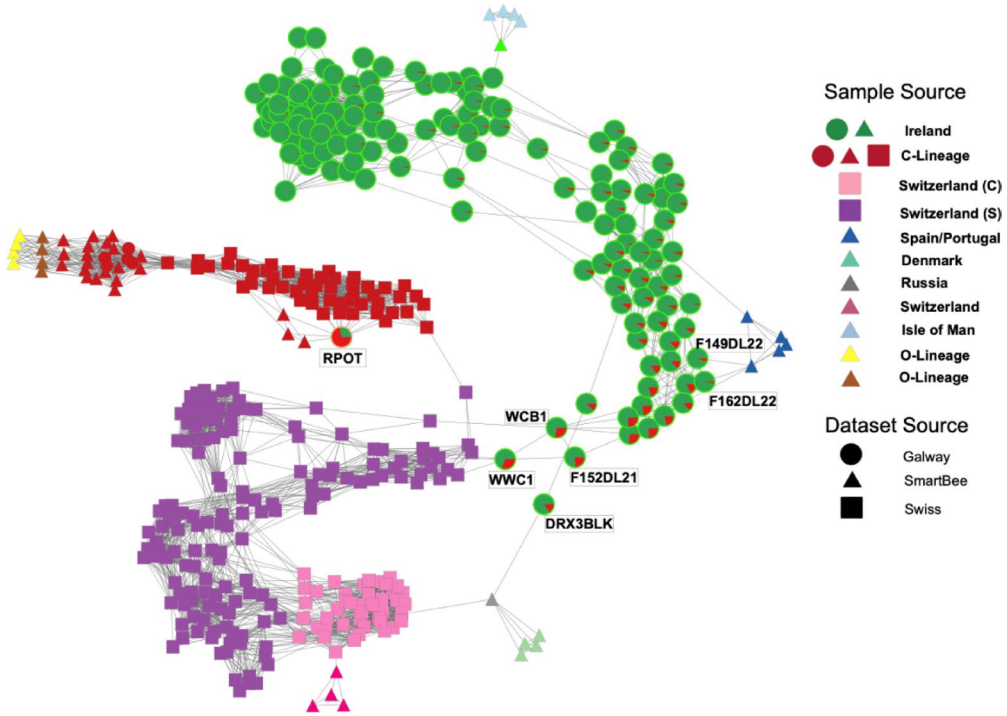


Fig. 2. European honey bee dataset population model created with NetView superimposed with introgression levels of colonies processed during this study via f4 ratios . Nodes are coloured and shaped by country and dataset of origin.

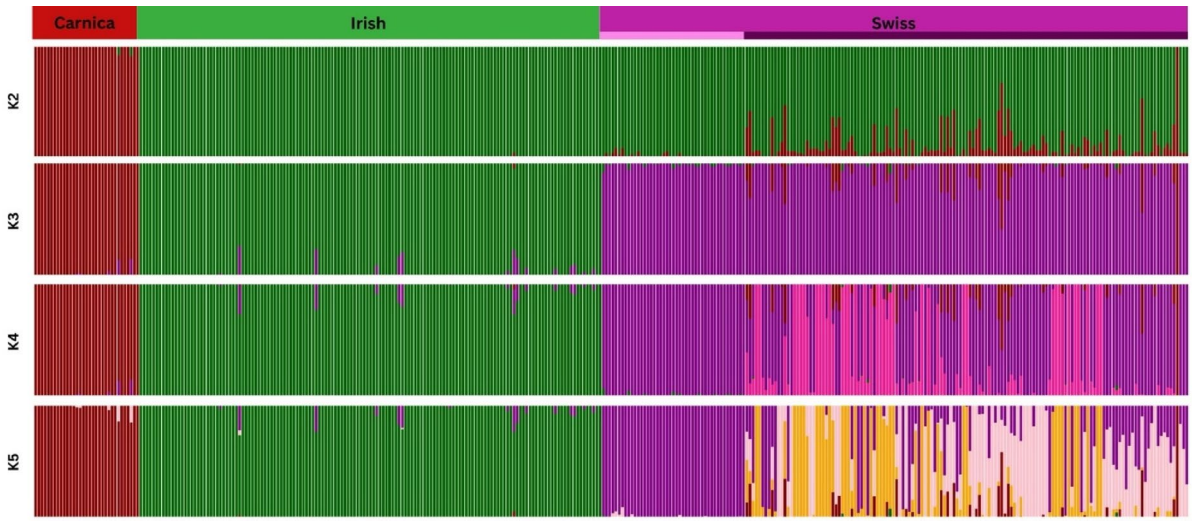


Fig. 3. NGSadmix results displaying the difference in purity levels found between the Irish and Swiss populations of *A. m. mellifera.* Each bar is a honey bee colony and within each bar the colour depicts the probability that the colony belongs to a certain population. Group membership is shown with the colour-bar at the top of the plot. Red for German and Swiss *A. m. carnica*, green for Irish, and purple for Swiss (dark purple for selection area, pink for conservation area.) Top plot (K2) displays the results obtained when setting the number of populations (K) to 2 during the analysis (M and C lineage model), bottom plot (K5) is the results with the number of populations set to 5.

losing alleles that allow for local adaptation of bees to climate or floral temporal cycles, due to introgression from introduced individuals, requires serious consideration. Island populations and free-living cohorts provide important genetic diversity and adaptations that are valuable to protect for their own right as well as to provide a source of alleles and traits for apiculture[6,62], the latter[62] calling for the establishment of a "European network of conservation islands" both for preservation and further study of honey bee biodiversity. Ireland could be an

excellent candidate for such an island network, however, introgression from traded managed stock continues to impact such important populations including Ireland.

Here, all three methods for detecting introgression that we compared performed similarly well in detecting and ranking hybridised or introgressed *A. m. mellifera* colonies. Therefore, the choice of method for hybrid detection using pool-seq data largely depends on other factors, such as selecting an optimal *A. m. mellifera* and *A. m. carnica* reference population and defining an appropriate threshold level. This can be seen in the results for colonies F149DL22 and F162DL22. These two colonies were deemed not to be introgressed via f-statistics but were hybridised via NGSadmix. In the network these two samples grouped close to the SMARTBEES *A. m. iberiensis* and not the non introgressed Irish group, indicating possible detection by NGSadmix of introgression from lineage A that could come from Buckfast colonies in the area, further indicating that NGSadmix was particularly effective in detecting admixture when the reference populations were not optimally matched. It has been recently demonstrated that admixture analyses are highly sensitive to the choice of reference populations[63]. Our results align with these findings, showing that the Irish *A. m. mellifera* colonies may not be an ideal reference for assessing introgression in Swiss *A. m. mellifera* colonies, as some colonies from the Swiss conservation program appeared admixed when compared to the Irish reference (Fig. 3, K = 2). However, these colonies showed no evidence of admixture once the Swiss population was differentiated from the Irish population (Fig. 3, K = 3). This could be due to some recent shared ancestry between Swiss *A. m. mellifera* and *A. m. carnica* due to geographical proximity and greater movement of honey bees that is not evident between the Irish and Swiss / *A. m. carnica* populations. Sharing of some alleles in this context could have been illuminated by comparing to a geographically distant population such as Ireland.

Overall, the percentage of introgressed colonies in the Irish dataset was 18.4%, much higher when compared to studies carried out, ten years ago[33,34] but consistent with more recent work[14]. The earlier studies did not sample from the most introgressed region identified in this analysis, County Donegal, an area that accounts for 53% (N = 15) of the introgressed samples from this study. To more accurately compare the results obtained here with these previous studies we re-analysed introgression levels with the Donegal data removed (N = 46) which led to a reduction in introgression levels to 12% (13/106) for the rest of the country. This result is in line with[15] where 12.5% of Irish colonies from non-Donegal sites were found to contain evidence of introgression via microsatellites. We did not test for introgression from the A lineage and given that Buckfast is also imported by some Irish beekeepers it is possible that additional introgression could be present that was not picked up here. Genetic data exists from few Buckfast samples from Ireland as yet and they were all M/C hybrids [[33];Hassett unpublished PhD thesis] however, some with an A lineage component could well have been imported.

The presence of non-native honey bees in Ireland is a clear risk to the integrity of the native and locally-adapted *A. m. mellifera* population. This is especially of high concern because we have identified that the majority of the sampled colonies have very little introgression, and within this group a subset (15%) where no detectable C-lineage introgression could be detected. The level of introgression detected here in the Irish population of *A. m. mellifera* is lower than recent studies carried out on this subspecies in other countries within its native range, e.g. 37% of all Norwegian colonies from 2020 were introgressed[8] while 87% and 84%, respectively of colonies from the UK were introgressed[2,64] and it is likely to be the most significant population of *A. m. mellifera* left in Europe. Moreover, given that Ireland has a widespread cohort of free-living colonies, that are freely mixing with and show similar levels of introgression to managed colonies, imports of non-native colonies puts this valuable element of biodiversity also at risk.

Population network analysis illuminated that the non-introgressed *A. m. mellifera* population present in Ireland is distinct from other *A. m. mellifera* populations included from across Europe. There has been no agreement on a definition for ecotypes[65], but broadly an ecotype could be regarded as a subset of a bigger population that has adapted sufficiently to a local environment that they possess morphological, genetic, and/or behavioural differences[66]. Under this view, given the morphological differences found by[15], the behaviours described for Irish and British honey bees by[25] and noted by beekeepers, and the genetic distinctiveness indicated here and in Huml et al. (in review) we propose that the Irish population should be considered an ecotype of *A. m. mellifera*.

The Irish population sampled within the SMARTBEES project and this dataset clustered next to each other in the population network, signifying that it is possible to combine pool-seq data from multiple studies, even though the organic material was sampled (different number of pooled workers) and processed differently. This was also noticed for the C-lineage cluster wherein C-lineage honey bee samples from all three datasets successfully grouped together. However, while the observed clustering pattern across the three different data does not suggest a batch effect, we cannot exclude the possibility of a batch effect and further interrogation of the data is required for additional population genomic analyses.

When associating the Irish nodes according to hybridisation status of the colony via f4 ratios it became apparent that the main Irish group is composed of largely non introgressed *A. m. mellifera* colonies, while the more diffuse clusters contained introgressed colonies. The main Irish cluster contained only those colonies with very high f4 ratio results. None of the honey bee colonies from Donegal grouped with this Irish cluster despite some of them having high population assignment values. This further suggests the importance of local diversity in determining patterns of introgression of honey bee populations. Patterns of imports in the early 1900s were different in the South and North of Ireland. In the South the focus was more on importing *A. m. mellifera* from the Netherlands with some imports of *A. m. carnica* and *A. m. ligustica,* as well as Buckfast bees[67], while in Donegal there were predominantly imports of *A. m. ligustica,* with Buckfast playing an important role in recent times (Verspuij, personal communication to GPM, 2025]. Also important is the proximity of the Northern Ireland border to Donegal and the movement of bees across the border. We detected higher introgression in Donegal in recent samples and, even in colonies with a higher proportion of *A. m. mellifera* identity, the allele frequencies were quite different from those further South (possibly indicating multiple generations of backcrossing with

A. m. mellifera drones). It is also of interest that data from these colonies were closer to *A. m. iberiensis* on the network than were other Irish *A. m. mellifera* colonies. Whether the Donegal honey bee population holds a larger remnant of a native honey bee lineage that arrived in Ireland via Iberia, in addition to different import history, or has experienced a higher influx of Buckfast and other non native colonies into the area throughout their history remains to be investigated. The addition of data from museum specimens from pre and post 1900 would shed light on this, as demonstrated by Parejo et al.[66].

The result of the NGSadmix analysis is also important to consider for conservation practice as it showcases the impacts of different conservation strategies on a honey bee population[46]. The Irish *A. m. mellifera* population has largely been isolated from other populations apart from Dutch imports in the 1920s and minor imports since. Furthermore, the majority of beekeepers favour the native bee and conservation of the black bee has been a strong focus of some groups since the 1980s culminating in the formation of the Native Irish Honey Bee Society (NIHBS) in 2012[68]. Strong natural selection and reduced transhumance compared to mainland European populations has maintained a high level of reproductive isolation between *A. m. mellifera* and other subspecies leading to the existence of a majority of non introgressed *A. m. mellifera* across the country, despite a lack of formal protection. By comparison the two Swiss cohorts included belong to areas that are governed by either a conservation strategy, where isolation and restrictions on movement are employed, or a selection strategy, where the local beekeepers selectively breed the honey bees in the area based on morphometric, resistance, or behavioral traits. The conservation area is tightly regulated with a 4 km buffer zone in place and a cantonal law that prohibits breeding any sub-lineage other than *A. m. mellifera*. It appears that this more regulated and enforced conservation strategy has resulted in a population that has a higher M-lineage assignment probability compared with the breeding population. This is an important finding in terms of how Ireland could approach the conservation of the Irish honey bee.

The Galtee Bee Breeding Group commenced the Voluntary Conservation Area (VCA) project in 1991 with the first area designated as a conservation area for the native Irish honey bee. The project, now led by NIHBS has approx 400 VCAs distributed across the island of Ireland ranging from carparks and gardens to large areas of land [[69]; nihbs.org]. The results from this study on the differences in introgression patterns between the Swiss conservation and selection areas suggests that the power of designating a conservation area for native bees is limited in that people can, and do, bring in non-native bees into the area which can undo years of breeding focus. Admixture interpretation is a solid first step in explaining a population structure or history and can identify groups of samples for future comparisons and studies[70]. In this case it shows us that the Irish honey bee population is an important population of a threatened subspecies given its relatively low levels of introgression. When comparing the populations of Irish and Swiss *A. m. mellifera* honey bees, the model-based cluster approach assigned the majority of the Irish colonies to the M-lineage with very high probability (0.96–1) whereas every Swiss colony in this dataset had some level of probability that they belonged to another lineage. The close proximity of Switzerland to the native range of the C-lineage subspecies could be a reason for this as well as the continued influx of C lineage alleles through introgression past and present. The lack of introgression in much of the Irish honey bee population is now something that is rare and worth preserving and the lower levels of introgression in the Swiss conservation area are also very promising. Regarding Ireland, there is no natural way that C-lineage honey bees can enter Ireland, unlike the natural movement between Switzerland and C-lineage native ranges such as Italy. There are also only two jurisdictions to consider, the Republic of Ireland and Northern Ireland, meaning that an all-Ireland solution to the protection of *A. m. mellifera* could be possible.

There are lots of studies to suggest that importation of honey bee queens results in introgression affecting the genetic integrity of local and native stocks [e.g.[2–6,11–15,71,72]]. The most promising approach to prevent continued introgression is to remove the source of the introgression from the environment, in this case it requires the ban of invasive subspecies from being imported to the island, or the introduction of strictly enforced regulations around where an imported colony can be placed[72]. Introduction of legislation may be challenging because honey bees are classed as a domesticated livestock by the European Union (even though they are not truly domesticated) and thus may be traded between all EU member states regardless of which genetic lineage they belong to. However, legally protected areas are more successful at limiting introgression than voluntary ones [e.g.[73]]. The removal of the source of introgression is, however, especially necessary for honey bees as research has shown that only partial reproductive isolation occurs between queens of lineages M and C and largely depends on environmental conditions[75,76]. To date, we have no information on mating/fertilisation of Irish *A. m. mellifera* queens but differential fitness maybe one reason why the Irish *A. m. mellifera* population has remained in the face of low levels of imports since the 1920s. In times of wet windy weather in July *A. m. mellifera* virgin queens and drones are likely to be relatively fitter than those of other subspecies. A combination of sunny summers with higher numbers of imports could have detrimental impacts on local populations of *A. m. mellifera*.

Despite the limitations of the study, which include a non-random sampling of available honey bee colonies and the inclusion of many samples from Donegal, potential batch effects from combining data from multiple sources and inclusion of only lineage M and C reference data, this study is important to highlight the current levels of introgression in the Irish population using a pool-seq genomics approach and to indicate the potential distinctiveness of the population. This information, in combination with what we already know about this population suggest to us that the Irish honey bee population could be regarded as an *A. m. mellifera* ecotype and an important island population deserving of protection. The data will be further interrogated for additional population genetic patterns and has been made available for others to use.

Conclusion

Ireland still contains a majority of non introgressed *A. m. mellifera*, however, introgressed honey bee colonies can be found throughout Ireland. Rates of introgression here provide further evidence of a higher level of introgression than has been found previously. The Irish *A. m. mellifera* is distinct from other European *A. m.*

mellifera populations supporting the existence of an ecotype. The Irish population was further proven to have higher levels of non introgressed *A. m. mellifera* when compared to Swiss colonies. The results from the two Swiss conservation efforts revealed that voluntary conservation areas are inferior to enforced ones with clearly defined legal boundaries, suggesting that Ireland should move to pass legislation to remove the threat of extinction to this important population through introgressive hybridisation.

Data availability

Whole genome pool-seq data generated and used here were deposited into the whole genome sequence database at ncbi.nlm.gov database under accession number PRJNA1162726. The custom-made workflow can be found within the following GitHub repositories: https://github.com/SSmith-NUIG/Colony_Analysis, https://github.com/SSmith-NUIG/Pool-seq_analysis_hybrid_detection_methods).

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Author contributions
S Smith : Data generation, data curation, methodology, analysis, writing original draft & review and editing. **E Mac Loughlin** : Sample collection **, A. Valentine** : Sample collection, data generation, writing. **M. Verspuij** : Sample collection, data interpretation. **A. Moro** : Data curation, methodology, data interpretation. **K. Browne** : Laboratory supervision, data generation, **C. Binetti** : Data generation, **M. Neuditschko** : Conceptualisation supervision, methodology, analysis, writing—review and editing and **GP McCormack** : Conceptualisation, funding acquisition, data interpretation, supervision, writing—original draft, review and editing.

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Declarations

Competing interests
The authors have no relevant financial or non-financial interests to disclose.

Ethical approval
Ethical approval was not required for this research. However live honeybees were treated as humanely as possible, and with respect, to minimise stress during collection.

Additional information
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