Journal of Apicultural Research

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tjar20

Investigation of free-living honey bee colonies in Ireland

Keith A. Browne , Jack Hassett , Michael Geary , Elizabeth Moore , Dora Henriques , Gabriele Soland-Reckeweg , Roberto Ferrari , Eoin Mac Loughlin , Elizabeth O'Brien , Saoirse O'Driscoll , Philip Young , M. Alice Pinto & Grace P McCormack

To cite this article: Keith A. Browne , Jack Hassett , Michael Geary , Elizabeth Moore , Dora Henriques , Gabriele Soland-Reckeweg , Roberto Ferrari , Eoin Mac Loughlin , Elizabeth O'Brien , Saoirse O'Driscoll , Philip Young , M. Alice Pinto & Grace P McCormack (2020): Investigation of free-living honey bee colonies in Ireland, Journal of Apicultural Research, DOI: 10.1080/00218839.2020.1837530

To link to this article: https://doi.org/10.1080/00218839.2020.1837530

Published online: 03 Nov 2020.

Journal of Apicultural Research, 2020
https://doi.org/10.1080/00218839.2020.1837530

ORIGINAL RESEARCH ARTICLE

Investigation of free-living honey bee colonies in Ireland

Keith A. Browne[a], Jack Hassett[b], Michael Geary[b], Elizabeth Moore[b], Dora Henriques[c], Gabriele Soland-Reckeweg[d], Roberto Ferrari[a], Eoin Mac Loughlin[a], Elizabeth O'Brien[a], Saoirse O'Driscoll[a], Philip Young[a], M. Alice Pinto[c] and Grace P McCormack[a]*

[a]*Department of Zoology, Ryan Institute, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland;* [b]*Department of Applied Science, Limerick Institute of Technology, Limerick, Ireland;* [c]*Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Bragança, Portugal;* [d]*Apigenix, Twann, Switzerland*

(Received 23 April 2020; accepted 25 September 2020)

Apis mellifera mellifera (Linnaeus), the Western European honey bee, is considered extinct in the wild over most of its range due largely to hybridisation and replacement by other subspecies, parasitism by *Varroa destructor,* habitat loss, and effects from agricultural pesticides. The purity of the subspecies within the managed cohort is also at risk over much of its range. Here, we investigated if honey bee colonies inhabited locations outside of the apiaries. In those we located, we explored how long the colony persisted and we investigated the genotypes of the bees using multiple markers. We show here that unmanaged free-living honey bee colonies are present and widespread in Ireland, inhabiting a mixture of nesting habitats with some colonies persisting naturally and unaided over multiple years. Molecular data including mitochondrial, microsatellite, and SNPs evidence indicate that the free-living population sampled is largely comprised of pure *A. m. mellifera*. Finally, we discuss the implications of conserving free-living *A. m. mellifera* in Ireland and its possible role in improving the fitness of the managed population both in Ireland and the rest of its European range.

Keywords: *Apis mellifera*; subspecies; *Varroa destructor*; wild bees; feral bees; conservation; molecular data; survival

Introduction

Some subspecies of *Apis mellifera* (Order Hymenoptera, Family Apidae), e.g., *A. m. mellifera*, are currently in a state of near extinction across much of their range due to multiple factors including varroosis caused by the mite *Varroa destructor* (Order Mesostigmata, Family Varroidae) (Anderson & Trueman, 2000), changes in land use and the proliferation of pesticide use. An additional factor creating risk for the continued survival of native subspecies is the replacement of native with imported honey bee strains by beekeepers. The presence of non-native breeding stock and hybrid strains such as *A. m. carnica* and *A. m. ligustica* (C-lineage) and cross-lineage commercial hybrids such as 'Buckfast' has resulted in large-scale introgression between these and native bees reducing the population of pure *A. m. mellifera* and altering its genetic integrity, leading to the strong possibility that genes for locally adapted traits may have been removed from the population (De la Rua et al., 2009; Ellis et al., 2018; Jensen et al., 2005; Parejo et al., 2018; Pinto et al., 2014; Randi, 2008; Soland-Reckeweg, 2006). In the midst of efforts to address such issues mentioned above in the managed honey bee cohort there has been insufficient investigation into the status of wild honey bees leaving considerable uncertainty about their current state (e.g., abundance, distribution, longevity) and conservation need (Moritz et al., 2005; Nieto et al., 2014).

Wild *A. m. mellifera* colonies are thought to be extinct or near extinct in all but a handful of conservation areas and nature reserves (Kohl & Rutschmann, 2018; Moritz et al., 2007). In Ireland, few wild colonies have been seen by experienced beekeepers in recent times (e.g., Micheal MacGiolla Coda, Personal Communication). Though members of the public sometimes reported the presence of bees in chimneys to the authors and to beekeeping associations they were assumed to be escapees from local apiaries and likely hybrids. However, Jaffe et al. (2010) indicated that there were more colonies present in Ireland than could be accounted for by managed colonies only using a molecular approach. For all other countries sampled in that study (apart from one location in Italy), the numbers of colonies estimated from the genotyping approach matched with the number of managed colonies documented, indicating a loss of wild colonies at these locations (Jaffe et al., 2010). We thus sought to investigate if honey bees live under wild conditions in Ireland and if so to determine their status in terms of habitat choice, survival and genetic diversity. Due the difficulty with knowing if bees are truly wild (descended from colonies that never inhabited a beehive) or feral (having swarmed from a nearby apiary) we will use the term 'free-living' to define those bees that have been collected from any

*Corresponding author. Email: Grace.mccormack@nuigalway.ie

habitat excluding a beehive (it includes wild and feral colonies). We considered that this study was essential to inform stakeholders in honey bee conservation both in Ireland and worldwide given that the conservation of native and wild honey bee populations, and local ecotypes, are increasingly considered of high importance (e.g., Requier et al., 2019) particularly those that may be Varroa tolerant/resistant and that pure *A. m. mellifera* has been reported to be common in Ireland (Hassett et al., 2018; Henriques et al., 2018).

Materials and methods

Colony sampling and monitoring

In November 2015 and August 2016 a nationwide appeal for information on the location of free-living honey bee colonies was made via The Irish Times, a national newspaper. Reports were gathered by telephone, email, and social media contact, and sightings determined to be honey bee swarm capture or other bee species were excluded. Samples of honey bees were collected from entrances of all 76 colonies both validated as being free-living honey bee colonies at the time of sampling and accessible for sampling (Figure 1) using a combination of long-handled butterfly nets, clear glass jars, and a proprietary "Bug buster" suction tube. Samples were cooled immediately upon capture before storage at −20 °C until DNA extraction. Colony survival was observed by determining the presence or absence of activity in Spring and late Autumn of each year, and by identifying the cause of death where possible/applicable.

DNA extraction, PCR and sequencing

DNA was extracted from the two hind legs of worker bees using the E.Z.N.A. Forensics DNA extraction kit (Omega Bio-Tek). Mitochondrial DNA consisting of the highly polymorphic intergenic region between the 3′ end of the $tRNA^{leu}$ gene and the 5′ end of the COII subunit gene was amplified using E2 (5′-GGCAGAATAAGTGCATTG-3′) and H2 (5′-CAATATCATTGATGACC-3′) primers (Garnery et al., 1998) with Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare). Polymerase chain reactions (PCR) included an initial denaturation of 5 mins at 95 °C, followed by 35 cycles of 94 °C for 45 secs, 45 °C for 43 secs and 62 °C for 2 mins with a final extension of 20 min at 65 °C (Garnery et al., 1993). PCR products were purified using a GeneJet PCR Purification kit (Thermo scientific) and sequenced by LGC Genomics, Germany. Resulting sequences were manually assessed against their chromatographs in MEGA7 (Kumar et al., 2016) before being imported into a multiple alignment.

Mitochondrial DNA sequence analysis

A total of 99 sequences of satisfactory quality were generated from 49 of the 76 free-living colonies (Table 1). Previous work (Hassett et al., 2018) indicated that all sequences from the free-living bees were of the M lineage. Here we explore further the relationships of the free-living bees using networks as implemented in TCS 1.21 (Clement et al., 2000). Firstly a network was produced from an alignment of the Irish free-living honey bee sequences with related sequences from Genbank and 156 sequences from the Irish managed honey bee cohort (Hassett et al., 2018; Pinto et al., 2014). The extra Q elements present in some haplotypes have a large influence on determining clusters given their length therefore an additional alignment was analysed that included only the informative sites in each Q element (showing some variation between bees). All unique sequences from the free-living honey bee colonies have been deposited into GenBank (accession numbers MT823282-MT823299).

Microsatellites analysis

Genotyping using a twelve microsatellite panel (A273, A43, Ac306, Ap33, B24, Ap226, A76-2p, A007, Ap001, A28, Ap289 and A29) was carried out by Ecogenics, Switzerland. Satisfactory data (ie. low amounts of null alleles) was returned from 59 of the free-living colonies as indicated in Table 1. This includes data from Hassett *et al* (2018) plus 24 additional free-living colonies (total N = 94). Equivalent data (the same loci, and carried out by the same laboratory using the same standards) were included from reference populations of *A. m. ligustica* (Soland-Reckeweg et al., 2009) from Italy (n = 55); *A. m. carnica* from Austria (n = 182) and Slovenia (n = 21), *A. m. mellifera* from Sweden (n = 10), France (n = 24), Norway (n = 18), Switzerland (n = 22) and managed bees from Ireland (n = 171) (Hassett et al., 2018). Bayesian analysis and visualisation of population assignment between C and M lineages was conducted in STRUCTURE V2.3.4 (Pritchard et al., 2000) using the admixture and correlated allele frequency models with the unsupervised option. A total of 750,000 Markov chain Monte Carlo (MCMC) iterations after an initial burn-in of 250,000 were performed for 20 iterations of each of K = 1 to 6. The optimal value of K (Evanno et al., 2005) was calculated using the CLUMPAK (Kopelman et al., 2015) online calculator. Nine populations were designated prior to analysis; seven based on the reference populations as above, and two for the Irish population divided between managed and free-living cohorts (1: Italy ligustica, 2: Austrian carnica, 3: Slovenian carnica 4: Swedish mellifera, 5: French mellifera, 6: Norwegian mellifera, 7: Swiss mellifera 8: Irish managed and 9: Irish free-living). The Q-value threshold used for full assignment to a particular population was $\geq$ 0.900 (Vaha & Primmer, 2006). The populations were

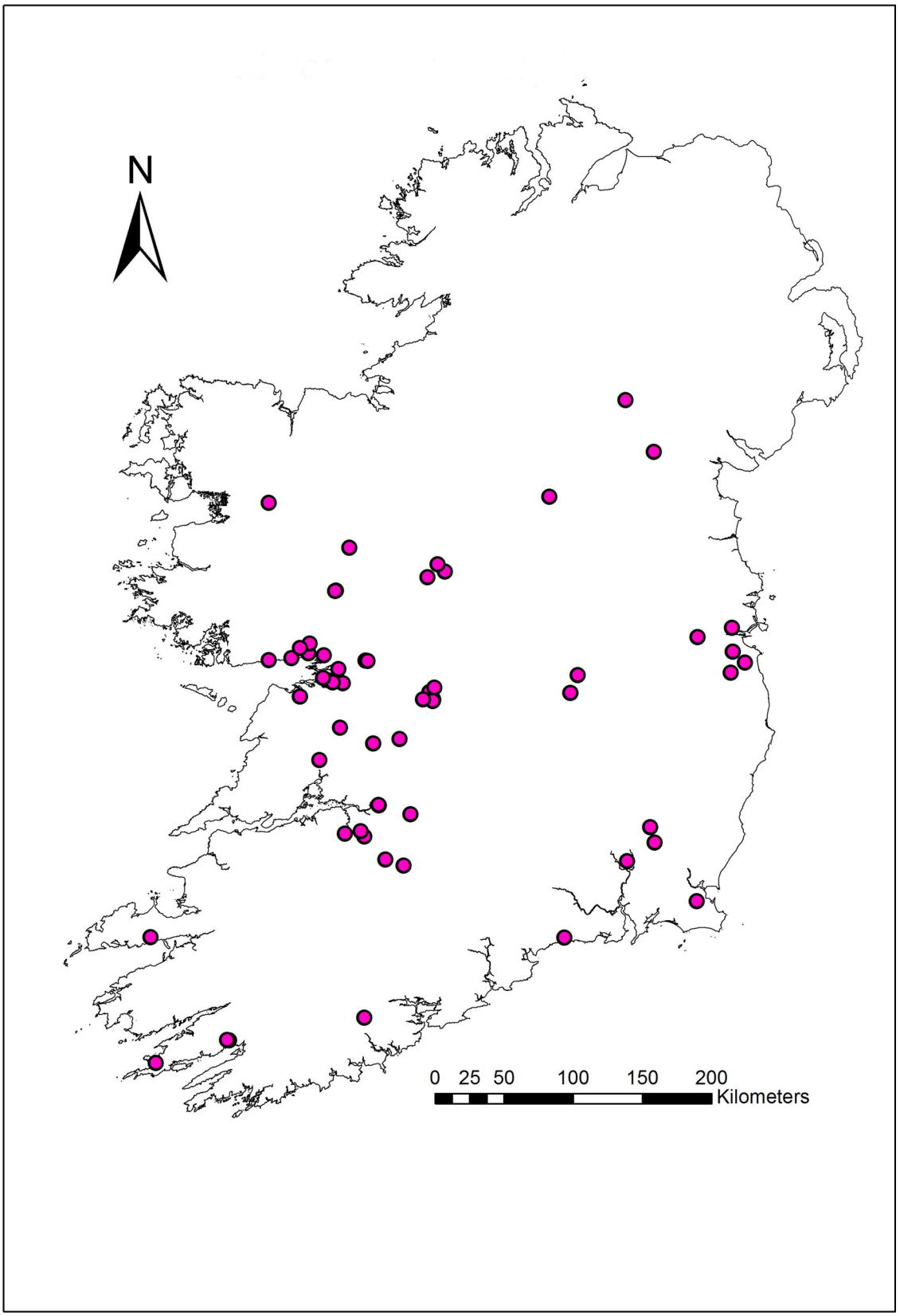

Figure 1. Locations of the 76 free-living colonies sampled in Ireland between 2015 and 2018 from the 182 reported. Some locations contained multiple colonies.

Table 1. Details of all free-living honey bee colonies sampled including information on their indicated lineage (M versus C) via mitochondrial, microsatellite and SNP data.

	mtDNA		Msats		SNPs	
Colony ID	**♯bees**	**Lineage**	**♯ bees**	**Prop M**	**♯ bees**	**Prop M**
F1T	nd	nd	nd	Nd	1	0.978
F2L	1	M	2	0.997-8	1	0.989
F3CE	nd	nd	nd	Nd	9	0.999
F4L	2	M	3	0.996-8	1	1
F5LA	nd	nd	3	0.998	1	0.991
F5LB	1	M	nd	nd	nd	nd
F6L	1	M	2	0.996	nd	nd
F7L	1	M	1	0.997	nd	nd
F8KEA	nd	nd	2	0.992-5	1	0.991
F8KEB	1	M	1	0.998	1	0.980
F19DA	1	M	1	0.998	1	1
F19DB	1	M	1	0.981	1	0.976
F10G	1	M	1	0.997	1	1
F11R	1	M	nd	Nd	nd	Nd
F12R	1	M	2	0.998	1	0.980
F13R	1	M	2	0.989-0.993	1	1
F14G	2	M	1	0.998	9	1
F15G	2	M	1	0.989	nd	Nd
F16G	1	M	1	0.997	9	0.983
F17G	nd	nd	nd	nd	nd	Nd
F18G	1	M	nd	nd	1	0.968
F19D	1	M	nd	nd	1	1
F20CE	1	M	2	0.998	9	0.984
F21C	**5**	**M**	**4**	**0.607-0.998**	**1**	**0.728**
F22	1	M	1	0.996	nd	Nd
F23	2	nd	nd	nd	nd	Nd
F24G	2	M	nd	nd	nd	Nd
F25G	1	M	nd	nd	nd	Nd
F26g	nd	nd	nd	nd	nd	Nd
F27G	2	M	2	0.9980	9	1
F28G	1		2	0.9980	1	1
F29G	7	M	5	0.933-997	8	1
F30G	1	M	1	0.9980	9	1
F31G	2	M	1	0.9900	nd	Nd
F32WX	nd	nd	nd	nd	1	0.974
F33KY	nd	nd	1	0.9980	1	1
F34W	2	M	1	0.9960	nd	Nd
F35CW	1	M	nd	nd	nd	Nd
F36C	2	M	1	0.9970	nd	Nd
F37C	nd	nd	1	0.991	1	0.9903
F38C	2	M	1	0.997	nd	Nd
F39G	nd	nd	nd	nd	nd	Nd
F40G	nd	nd	nd	nd	nd	Nd
F41G	3	M	2	0.993-4	9	1
F42G	nd	nd	1	0.997	nd	Nd
F43G	1	M	1	0.998	nd	Nd
F44G	7	M	5	**0.893-0.997**	nd	Nd
F45G	2		2	0.998	9	0.988
F46G	nd	nd	nd	nd	nd	Nd
F47G	3	M	2	0.989-0.998	9	1
F48G	8	M	4	0.997-0.998	9	1
F49R	1	M	1	0.994	nd	Nd
F50R	2	M	1	0.989	nd	Nd
F51MO	1	M	1	0.996	1	0.994
F52D	1	M	1	0.986	1	0.967
F53LO			1	0.997	1	0.985
F54MN	2	M	2	0.986-0.997	1	0.943
F55MN	2	M	2	**0.821, 0.988**	1	0.967
F56LS	2	M	1	0.994	1	0.968
F57OY	2	M	1	0.997-8	1	0.991
F58C	nd	nd	1	0.9960	nd	Nd
F59L	nd	nd	1	0.9980	nd	Nd
F60L	nd	nd	1	0.9920	nd	Nd

(*Continued*)

Table 1. (*Continued*).

	mtDNA		Msats		SNPs	
F61L	5	M	4	0.995-8	nd	Nd
F62L	nd	nd	1	0.9940	nd	Nd
F63WX	nd	nd	1	0.9980	nd	Nd
F64CE	nd	nd	1	0.9740	nd	Nd
F65OY	nd	nd	1	0.9970	nd	Nd
F66G	nd	nd	1	0.9160	nd	Nd
F67G	1	M	1	0.9730	nd	Nd
F68G	1	M	1	0.9980	nd	Nd
F69WW	nd	nd	1	0.9970	nd	Nd
F70CE	nd	nd	1	0.9850	nd	Nd
F71G	1	M	nd	nd	nd	Nd
F72G	1	M	2	0.9970	nd	Nd
F73G	1	M	1	0.9970	nd	Nd
76	**99**	49	**94**		**123**	36

Note: ♯= number of, Msat = microsatellite, SNP = Single Nucleotide Polymorphism, Prob M = probability of individual bee being part of M lineage. Colonies highlighted in grey are those that have results from all three types of data. The three colonies in bold are those where one or more data-type indicate introgression of C into M lineage.

arranged into four groups; European C-lineage (populations. 1, 2 and 3), European mellifera (populations. 4, 5, 6 and 7), Irish managed (population 8) and Irish free-living (population 9). Analysis was carried out both with all sampled bees and also with only one bee per colony to remove bias that may be caused by the presence of related bees. Using the groups above and the reduced dataset of one representative bee per colony for Irish colonies (Irish managed cohort N = 64, free-living bee cohort, N = 56) an analysis of molecular variance (AMOVA) was conducted using Arlequin V3.5.2.2 (Excoffier & Lischer, 2010).

Single nucleotide polymorphisms (SNPs) analysis

As a further approach to investigate the subspecies purity of the free living bees, DNA from 127 free-living bees, representing 39 colonies, were diluted to 10–15 ng/μl and sent to Instituto Gulbenkian de Ciência (Portugal) for genotyping using the Agena BioScience iPLEX chemistry and the MassARRAY® MALDI-TOF platform (Gabriel et al., 2009) using a highly informative 127 SNP assay, designed for reliable introgression estimation of C-into M-lineage (Henriques et al., 2018). After quality control to identify SNPs with missing data, data from 36 colonies were kept in the final analysis (Table 1). While for most colonies one bee was tested for introgression, for 11 colonies nine individual bees were tested and for one colony eight bees were tested (Table 1). Membership proportions (Q-values) were estimated using ADMIXTURE V1.23 (Alexander et al., 2009) for K = 2 with 20 independent runs of 10,000 iterations. The convergence between iterations was examined by comparing log-likelihood scores (LLS) using the default termination criteria set to stop when LLS increases by <0.0001 between iterations. A total of 36 M-lineage and 36 C-lineage individuals were used as reference populations (Henriques et al., 2018). CLUMPAK was used to summarise and visualise the Q-values. A Q-value threshold of Q-value $\geq$ 0.900 was considered full assignment to either lineage.

Results

Location, health and survival of free-living colonies

Between November 2015 and November 2018, a total of 209 reports of putatively free-living bee colonies were received of which 7.2% (15) were identified as bumble bees, solitary bees, or wasps, and 5.7% (12) were captured swarms of unconfirmed provenance. Colonies reported in early Spring 2016 (prior to expected swarming season), were assumed to have been present since at least late Autumn 2015. The reported habitats of all honey bee colonies (n = 182) consisted primarily of cavities in buildings (68%) where mainly the roof space was occupied by the colony whereas trees formed the second most utilised habitat (10%) (Table 2, Figure 2). Of the 76 colonies that were monitored for survival from Autumn 2015 to Spring 2019, the survival reports on 16 colonies were considered ambiguous (e.g., property containing colony no longer accessible) and removed from further study, 21 colonies (27.63%) survived for 2-2.5 years and 22 (28.95%) survived three or more years (Figure 3). Colony deaths primarily occurred over winter or in early spring. Internal causes of colony death were unknown while external causes of death were extermination by the homeowner and predation by Pine marten (*Martes martes*).

Population structure from mitochondrial data

All 99 COI-COII mitochondrial sequences generated from free-living colonies were designated as *A. m. mellifera* as they contained the P element (Cornuet et al., 1991). After pruning to remove identical sequences, 52 sequences remained; 37 PQQ, 11 PQQQ and four PQQQQ mitotypes (Figure 4). When the entire Q elements were included in the TCS analyses (Clement et al., 2000), unsurprisingly separate networks were formed for sequences that contained different numbers of duplicated Q elements (i.e., PQQ, PQQQ or

Table 2. Reported residency periods (by those who reported them to the authors) and range for 182 honey bee colonies by habitat type.

Habitat	Number (%) of colonies	Mean residency (Years)	Residency range (Years)
Buildings	124 (68%)	4.6	1 to 40
Walls	7 (4%)	6.0	1 to 30
Trees	18 (10%)	5.9	1 to 40
Other	33 (18%)	1.2	1 to 5
All types	**182**	**4.2**	**1 to 40**

"Other" includes, *inter alia*, graves, a statue, a cattle grid and a bird nest box.

PQQQQ). When the Q elements were represented only by one base pair each, plus the seven sites where variation occurred within them, clusters with PQQ, PQQQ or PQQQQ were still evident in the network but the three clusters were now connected (Figure 4) except for a single haplotype (F36C056) and a group of three (F28G267, F35CW055 and F51MO096) which were distinct.

Trimming of the 5′ and 3′ ends of the alignments led to no differentiation between the M4d and M4e, and the M4a and M4m, haplotypes. As shown in Figure 4, just over half of colonies (27, 52%) yielded sequences that were identical to M4e and M4d reference haplotypes and two of the Irish free-living bee sequences were identical to M4f, while the rest of the mitochondrial sequences (23, 44%) were distinct from any available European sequence in GenBank. In a comparison of the data from free-living and managed bees sampled to date from Ireland, seven variants were found only in the free-living bees. Four of these sequences (F31G50, F45G085, F13R036, and F43G080/FKe017) had variations represented by single indels or point mutations. Three variants (F6L011, F36C056 and F28G267/F35CW055/F51MO096) had multi-base deletions of between 6 bp and 10 bp (Figure 4). A fourth variant (F18G104/F47G256), with a significant deletion (6 bp from sites 17 to 22), was found in the Irish managed population but not in any available data from elsewhere in Europe.

Subspecies purity of free-living bees from microsatellite and SNP data

Both microsatellite and SNP data indicate a clear structure between the C-lineage bees and the M-lineage bees for K = 2 with Irish free-living bees showing a high degree of purity with both sets of markers (Table 1 and Figure 5 (for the microsatellite data)). The free-living colony F21CE (Table 1) was located in County Clare in an area known to contain beekeepers that keep 'Buckfast'. This colony contained a mixture of bees with different levels of putative purity, with some bees being assigned to M lineage with Q-value of 0.998 while one bee had a Q-value of 0.607. Colony F44G was collected in the walls of a castle in east Galway and again showed bees with mixed ancestry, some assigned to M lineage with Q-value of 0.997 and others dropping below the 0.900, cut off for confident lineage assignment. Similarly, colony F55MN, collected in the roof of an old cottage in County Monaghan, showed one bee that could be assigned to M lineage with confidence while the other bee could not (Table 1).

SNP data identified only one colony falling below a Q-value threshold of 0.900, and this was colony F21CE, also identified by microsatellites as showing introgression (Table 1). While lineage assignment was not tested for colony F44G via the SNP approach, the bee from colony F55MN had a Q-value of 0.967. Where SNPs data was returned for 8/9 bees per colony, all bees from all 12 tested colonies could be clearly assigned to the M lineage. Unfortunately, colonies F21CE, F44G and F55MN were not included in that experiment.

AMOVA (carried out including only one bee per colony) indicated segregation of the M and C lineages with 33.28% of the genetic variation evident between groups. The greatest proportion of variation occurred within the populations (63.76%), with little distinction between populations within groups. A low pairwise F_{ST} value (0.001), indicated little distinction between the managed and free-living honey bee cohorts in Ireland. Average gene diversity (0.53 ± 0.28) was the same in the two groups of bees whilst observed heterozygosity was slightly lower in the managed honey bee cohort (0.47 compared with 0.51). The numbers of alleles found across populations were not fully comparable due to the differences in population sizes included. While numbers of alleles from the 64 managed bees (128) was higher than the 113 alleles reported from the 54 free-living bees both numbers are far lower than the 240 alleles reported from the 182 *A. m. carnica* bees from Austria but higher than the numbers of alleles returned from all the other populations included.

Pairwise Fst values between the free-living Irish cohort and the *A. m. mellifera* populations from Switzerland and Norway (0.045 and 0.067 respectively) was an order of magnitude lower than pairwise values between Irish bees and those from Sweden and France (0.12 and 0.0.18 respectively). Fst values between the Irish bees and *A. m. ligustica* and *A. m. carnica* were between 0.32 and 0.55 reflecting the subspecies differentiation. Genetic diversity in the Irish free-living cohort was similar but slightly higher than the *A. m. ligustica* population sampled from Italy with similar numbers of bees included in analyses (0.53 versus 0.31). Average gene diversity estimates of the other *A. m. mellifera* populations were similar to those from Ireland (ranging

Figure 2. Images of some locations from where free-living honey bee colonies were collected. A. cavity in a tree showing honey bees at entrance and propolis staining, B. Sampling bees from a house roof fascia with a bee vacuum, C. holes in castle walls are a common location for free-living honey bee colonies (the location of two colonies are indicated by the arrows), D. Tree that hosts a colony of free-living honey bees for over four years (the arrow points to the colony entrance).

from 0.37 France to 0.56 Sweden) despite the higher numbers of bees included in the Irish sample set.

Discussion

This study provides the first fully documented evidence, since the discovery of *V. destructor* in Ireland in 1998, that free-living honey bees exist in the country, primarily using the cavities provided in new and historic buildings to house their colonies. While the majority of colonies have been monitored as part of this study for only three years, some of these colonies had already been in place for a period prior to the study commencing. Very few sampled free-living colonies showed signs of introgression from introduced subspecies and hybrids and we provide sequences of novel mitochondrial haplotypes from this cohort.

In contrast to our findings where 68% of colonies were located in buildings, in the UK there was no significant difference between the use of trees, houses and walls for colony sites (Thompson, 2012). In Ireland approximately 11% land cover is woodland, the majority of which is commercially grown coniferous species (D.A.F.M., 2018). A high turnover of trees in managed forests combined with loss of mature deciduous woodland is likely to produce a low density of trees with cavities of sufficient size for colonisation by honey bees. Conversely, although the UK has a

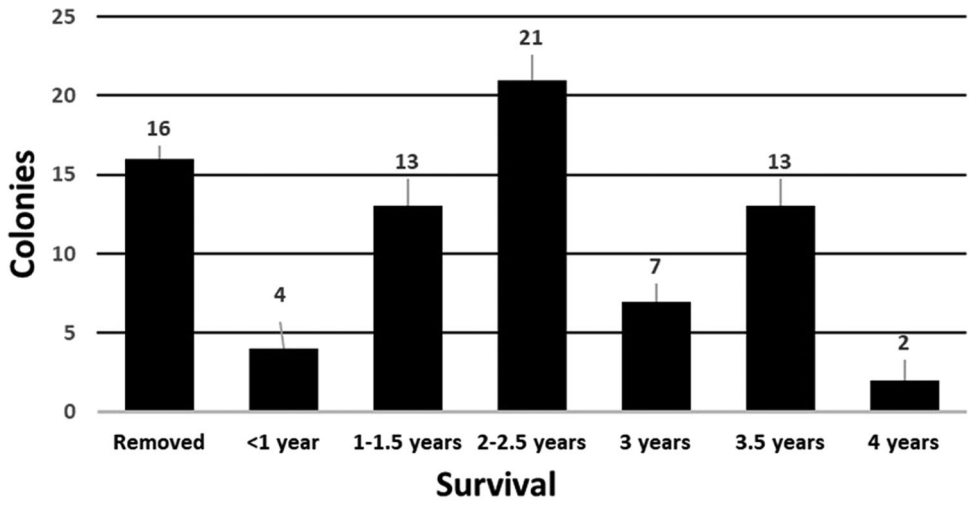

Figure 3. Survival of colonies (n = 76) monitored between Autumn 2015 and Spring 2019. Sixteen colonies were removed from the study due to incomplete records or ambiguity regarding survival as reported by the custodian, 22 have survived at least three winters of which two have survived four. In some cases, custodians reported that locations have been housing colonies for decades.

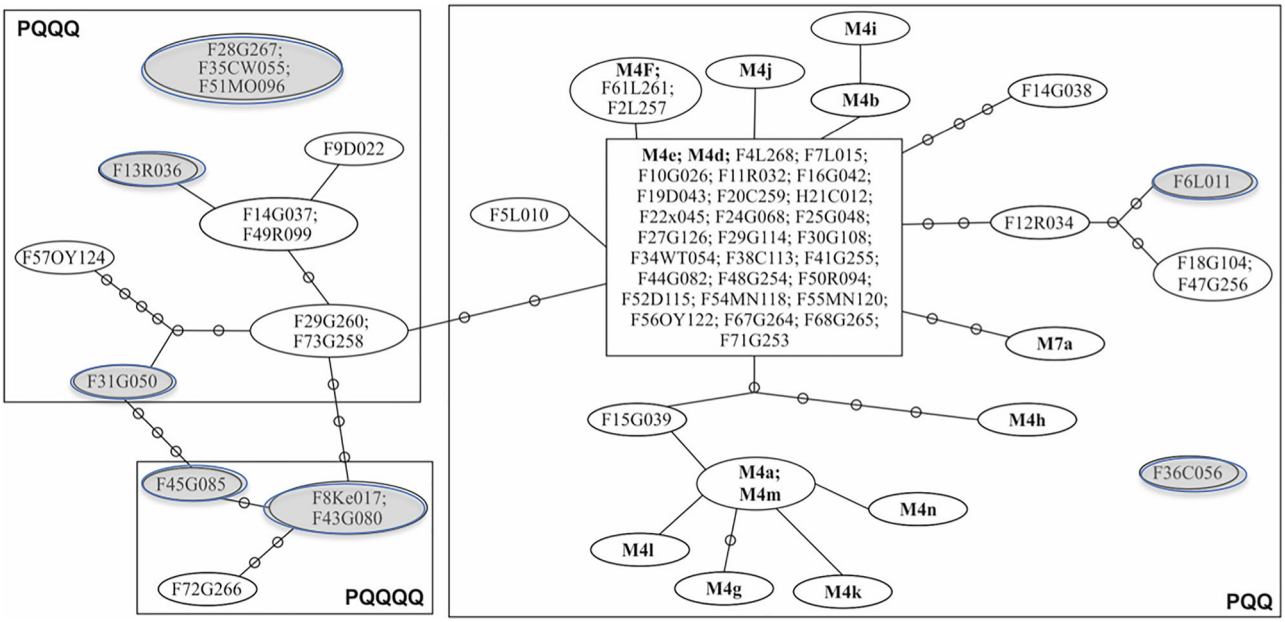

Figure 4. Statistical parsimony network of the 19 different mitotypes from Irish free-living bees. These have been included with European haplotypes downloaded from Genbank. Each circle (o) on a branch represents a single indel or point mutation. Branch lengths are not representative of distance. The variants in grey are those variants that were only present in the free-living population in Ireland, being absent from the managed population sampled so far. PQQ: All sequence types in the PQQ box contain the intergenic P motif and two Q repeat elements, PQQQ: all sequences included contain the intergenic P motif and three Q repeat elements, PQQQ: all sequences included contain the intergenic P motif and four Q repeat elements.

similar relative woodland cover (13%), it consists of roughly even areas of coniferous and broadleaved woodland, which creates a higher age profile than that found in Ireland (Forest Research UK, 2018) and this may help explain the relatively greater use of trees by UK colonies. The difference may also be related to sample design with colonies in houses being easier to spot by members of the public. Searches for wild honey bees in old Irish woodland should be made to expand this dataset. However, colonies in house cavities obtain the benefits of a long lasting, insulated space, giving them the time needed to expand both individual colonies and the dynasty of the queen, an arrangement which may be a benefit over the use of tree cavities. It maybe that honey bees have learnt to exploit the increase in building numbers given the decline in other suitable habitat.

As we reported in Hassett et al. (2018) for the Irish honey bee population generally, over 50% of the

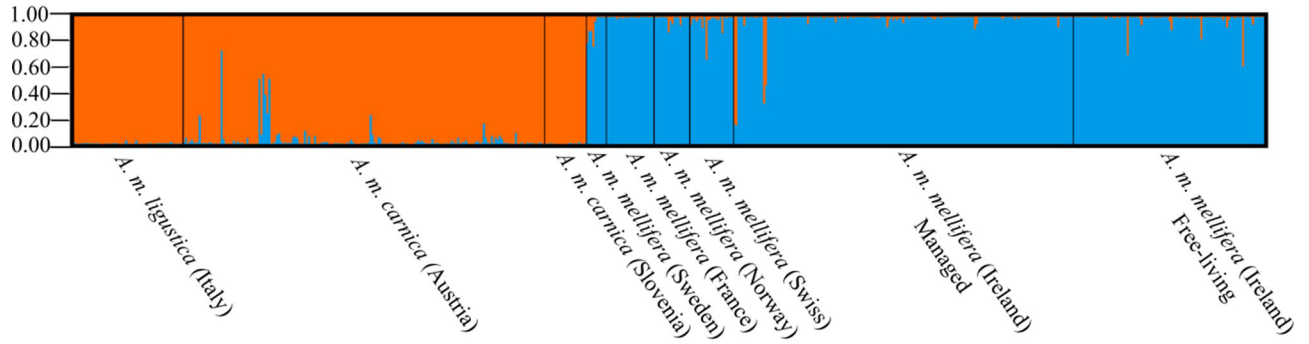

Figure 5. Structure K = 2 assignments of 598 individuals from 9 putative populations (1: Italian *A. m. ligustica*, 2: Austrian *A. m. carnica*, 3: Slovenian *A. m. carnica* 4: Swedish *A. m. mellifera*, 5: French *A. m. mellifera*, 6: Norwegian *A. m. mellifera*, 7: Swiss *mellifera* 8: Irish managed and 9: Irish free-living) including 95 individual honey bees from 50 free-living colonies. Each bar represents an individual bee with assignment apportioned between orange (C-lineage) and blue (M-lineage). Assignment values are from 0.000 to 1.000.

mitochondrial haplotypes identified in the Irish free-living colonies were identical to Dutch haplotypes and are likely descendants from the significant imports that were brought in from Holland. Remarkably none of the free-living bees showed haplotypes from elsewhere in Europe (i.e., not identical to any of the sequences from Rortais et al. (2011) or Pinto et al. (2014)). Hassett et al. (2018) revealed two Irish bees with identical mitochondrial sequences to a French haplotype and one bee showed identity to bees from Colonsay. These bees were from the managed cohort and together the results so far indicate little influence in Ireland of *A. m. mellifera* from mainland Europe, apart from a major influence from the importations from Holland. Most of the mitochondrial variants found in the free-living bees could also be found within the managed Irish population (Hassett et al., 2018), meaning Ireland has a free-living population that appears fundamentally undifferentiated from the managed one, also confirmed by AMOVA of microsatellite data. That said, seven mitochondrial variants were found exclusively in the Irish free-living bees, which might indicate unique genetic variation present in free-living bees. However, this is a relatively small study and additional sampling may identify the putatively exclusive free-living variants in the managed population.

Interestingly, the retention of clusters that are essentially defined by the number of Q elements present may provide some evidence that each duplication occurs as a single event such as the AT-rich homologous motifs suggested by Cornuet et al. (1991). If the duplication event is a synapomorphy, then the Q element architecture represents separate M lineages, creating a problem with the current naming system for mitochondrial haplotypes. Putative mitochondrial lineages as evident in Figure 4 and in Hassett et al. (2018) and more recently in Henriques et al. (2018) will need to be confirmed with other data. Highly significant is that both microsatellite and SNP data indicate that, in keeping with the Irish population as a whole, the free-living population sampled consists mostly of bees that can be assigned to *A. m. mellifera* with high confidence.

Genetic diversity estimates from data generated from the free-living honey bee population in Ireland indicates a healthy population with no evidence of a genetic bottleneck. While results are not directly comparable (given that only some of the same microsatellites were used and were performed via a different laboratory), the levels of heterozygosity and gene diversity reported here are higher than those for France and Sweden reported by Estoup et al. (1995) and for Spain (De la Rúa et al., 2003).

Evidence now hints at colony survival past the stage where Varroosis related death would be expected (Korpela et al., 1992). In periods as short as ten years, experimental survivorship tests in isolated areas have indicated that a balanced host-parasite relationship can develop in colonies from a small population (Fries et al., 2006; Le Conte et al., 2007). The high rate of genetic recombination in honey bees (Beye et al., 2006) may produce a sufficiently diverse population from only a few survivors to allow genes linked to resistance/tolerance mechanisms to quickly proliferate in a small population. Under natural conditions, colony density can return to the levels present before the arrival of Varroa (Mikheyev et al., 2015; Seeley, 2007) and given the low levels of commercialised beekeeping in Ireland, combined with the existence of a putatively large free-living population, it seems plausible that colonies with Varroa resistance/tolerance mechanisms have emerged in Ireland in the 20 years since Varroa was first discovered. Colonies with traits that allow survival in the presence of Varroa probably existed in Irish apiaries, as well as in the wild population, providing the resources on which natural selection could act. The lack of differentiation between the managed and free-living populations sampled so far may not be considered a negative condition if it leads to the discovery of Varroa resistant/tolerant genotypes. Sampling brood from free-living colonies for disease screening is remarkably difficult. Development of methods to access and study pathogens and parasites in resident free-living colonies are now

required to elucidate variability in disease loads and mechanisms of survival/tolerance.

Given the interactions between genotype and environment that have been clearly shown in honey bees (Büchler et al., 2014; Meixner et al., 2014), and the fact that local ecotypes seem to do better than bees translocated from different microclimates (Costa et al., 2012), there is also a need to protect local adaptations. Research on free-living bees in Ireland now requires expansion to allow an accurate indication of colony density on a national scale, together with observation of survivorship and associated mechanisms, as well as characterisation of local strains. We believe that the results presented here, combined with those of Hassett et al. (2018), and the observations of beekeepers, require the immediate application of the precautionary principle of conservation practice (Finnoff et al., 2007), for the protection of Ireland's locally adapted free-living honey bee population. We join with other researchers in requesting legal protection for local adaptations in *A. mellifera* (Fontana et al., 2018) through stricter control on the movement of live bees and the banning of imports. Inter-country movement of *A. m. mellifera,* even where it is the indigenous subspecies, needs to be given careful consideration in each case to avoid out-breeding locally adapted gene complexes. However, having been the welcome recipient of Dutch honey bees following the collapse of Irish beekeeping at the beginning of the 20th century, Ireland may yet be able to return the favour by returning bees of Dutch haplotypes home to the Netherlands from a free-living population.

Acknowledgements

We particularly thank the custodians of the free-living honey bee colonies and the Native Irish Honey Bee Society (NIHBS) for their assistance. KAB is a recipient of an Irish Research Council postgraduate fellowship (GOIPG/2015/2767) and a Tony Ryan Postgraduate fellowship. Additional funding was gratefully received from the Department of Agriculture, Food and the Marine [grant number GRGAS 16/GR/09], the Federation of Irish Beekeeping Associations, the Eva Crane Trust [grant number ECTA20160303] and The Native Irish Honey Bee Society. Financial support for DH was provided through the program COMPETE 2020 – POCI (Programa Operacional para a Competividade e Internacionalização) and by Portuguese funds through FCT (Fundação para a Ciência e a Tecnologia) in the framework of the project BeeHappy (POCI-01-0145-FEDER-029871).

Disclosure statement

The authors declare that there are no conflicts of interest or disputes over ownership of the data and all contributions to this work have been attributed appropriately.

ORCID

Keith A. Browne http://orcid.org/0000-0001-6666-9821
Roberto Ferrari http://orcid.org/0000-0003-4015-8104
Grace P McCormack http://orcid.org/0000-0003-4960-5185

References

Alexander, D. H., Novembre, J., & Lange, K. (2009). Fast model-based estimation of ancestry in unrelated individuals. *Genome Research*, *19*(9), 1655–1664. https://doi.org/10.1101/gr.094052.109

Anderson, D. L., & Trueman, J. W. H. (2000). Varroa jacobsoni (Acari: Varroidae) is more than one species. *Experimental & Applied Acarology*, *24*(3), 165–189. https://doi.org/10.1023/A:1006456720416

Beye, M., Gattermeier, I., Hasselmann, M., Gempe, T., Schioett, M., Baines, J. F., Schlipalius, D., Mougel, F., Emore, C., Rueppell, O., Sirvio, A., Guzman-Novoa, E., Hunt, G., Solignac, M., & Page, R. E. (2006). Exceptionally high levels of recombination across the honey bee genome. *Genome Research*, *16*(11), 1339–1344. https://doi.org/10.1101/gr.5680406

Büchler, R., Costa, C., Hatjina, F., Andonov, S., Meixner, M. D., Conte, Y. L., Uzunov, A., Berg, S., Bienkowska, M., Bouga, M., Drazic, M., Dyrba, W., Kryger, P., Panasiuk, B., Pechhacker, H., Petrov, P., Kezić, N., Korpela, S., & Wilde, J. (2014). The influence of genetic origin and its interaction with environmental effects on the survival of *Apis mellifera* L. colonies in Europe. *Journal of Apicultural Research*, *53*(2), 205–214. https://doi.org/10.3896/IBRA.1.53.2.03

Clement, M., Posada, D., & Crandall, K. A. (2000). TCS: A computer program to estimate gene genealogies. *Molecular Ecology*, *9*(10), 1657–1659. https://doi.org/10.1046/j.1365-294x.2000.01020.x

Cornuet, J. M., Garnery, L., & Solignac, M. (1991). Putative origin and function of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA. Genetics, *128*(2), 393–403.

Costa, C., Lodesani, M., & Bienefeld, K. (2012). Differences in colony phenotypes across different origins and locations: Evidence for genotype by environment interactions in the Italian honeybee (*Apis mellifera ligustica*)? Apidologie, *43*(6), 634–642. https://doi.org/10.1007/s13592-012-0138-9

De la Rúa, P., Galián, J., Serrano, J., & Moritz, R. F. A. (2003). Genetic structure of Balearic honeybee populations based on microsatellite polymorphism. *Genetics, Selection, Evolution*, *35*(3), 339–350. https://doi.org/10.1186/1297-9686-35-3-339

De la Rua, P., Jaffe, R., Dall'Olio, R., Munoz, I., & Serrano, J. (2009). Biodiversity, conservation and current threats to European honeybees. Apidologie, *40*(3), 263–284. https://doi.org/10.1051/apido/2009027

D.A.F.M. (2018). (Department of Agriculture Fisheries and Marine) website. Retrieved November 23. www.agriculture.gov.ie/forestservice/

Ellis, J. S., Soland-Reckeweg, G., Buswell, V. G., Huml, J. V., Brown, A., & Knight, M. E. (2018). Introgression in native populations of Apis mellifera mellifera L: Implications for conservation. *Journal of Insect Conservation*, *22*(3-4), 377–390. https://doi.org/10.1007/s10841-018-0067-7

Estoup, A., Garnery, L., Solignac, M., & Cornuet, J.-M. (1995). Microsatellite variation in the honeybee (Apis mellifera L.) populations: Hierarchical genetic structure and test of the infinite allele and stepwise mutation models. *Genetics*, *140*, 679–695.

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology*, *14*(8), 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Excoffier, L., & Lischer, H. E. L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, *10*(3), 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Finnoff, D., Shogren, J. F., Leung, B., & Lodge, D. (2007). Take a risk: Preferring prevention over control of biological invaders. *Ecological Economics*, *62*(2), 216–222. https://doi.org/10.1016/j.ecolecon.2006.03.025

Fontana, P., Costa, C., Di Prisco, G., Ruzzier, E., Annoscia, D., Battisti, A., Caoduro, G., Carpana, E., Contessi, A., Dal Lago, A., Dall 'Olio, R., De Cristofaro, A., Felicioli, A., Floris, I., Fontanesi, L., Gardi, T., Lodesani, M., Malagnini, V., Manias, L., … Segrè, A. (2018). Appeal for biodiversity protection of native honey bee subspecies of *Apis mellifera* in Italy (San Michele all'Adige declaration). *Bulletin of Insectology*, *71*(2), 257–271.

Forest Research UK. (2018). *National forest inventory*. Retrieved November 26, 2018, from www.forestresearch.gov.uk/tools-and-resources/national-forest-inventory/

Fries, I., Imdorf, A., & Rosenkranz, P. (2006). Survival of mite infested (Varroa destructor) honey bee (Apis mellifera) colonies in a Nordic climate. Apidologie, *37*(5), 564–570. https://doi.org/10.1051/apido:2006031

Gabriel, S., Ziaugra, L., & Tabbaa, D. (2009). SNP genotyping using the Sequenom MassARRAY iPLEX platform. *Current Protocols in Human Genetics*, *60*(1), 2.12.1–2.12.18 https://doi.org/10.1002/0471142905.hg0212s60

Garnery, L., Franck, P., Baudry, E., Vautrin, D., Cornuet, J.-M., & Solignac, M. (1998). Genetic diversity of the west European honey bee (Apis mellifera mellifera and A. m. iberica). I. Mitochondrial DNA. *Genetics Selection Evolution*, *30*(Suppl 1), S31–S47. https://doi.org/10.1186/1297-9686-30-S1-S31

Garnery, L., Solignac, M., Celebrano, G., & Cornuet, J. M. (1993). A simple test using restricted PCR-amplified mitochondrial-DNA to study the genetic structure of Aois-mellifera L. Experientia, *49*(11), 1016–1021. https://doi.org/10.1007/BF02125651

Hassett, J., Browne, K. A., McCormack, G. P., Moore, E., Society, N. I. H. B., Soland, G., Geary, M., & Native Irish Honey Bee Society. (2018). A significant pure population of the dark European honey bee (Apis mellifera mellifera) remains in Ireland. *Journal of Apicultural Research*, *57*(3), 337–350. https://doi.org/10.1080/00218839.2018.1433949

Henriques, D., Browne, K. A., Barnett, M. W., Parejo, M., Kryger, P., Freeman, T. C., Munoz, I., Garnery, L., Highet, F., Jonhston, J. S., McCormack, G. P., & Pinto, M. A. (2018). High sample throughput genotyping for estimating C-lineage introgression in the dark honeybee: An accurate and cost-effective SNP-based tool. *Scientific Reports*, *8*(1), 8552. https://doi.org/10.1038/s41598-018-26932-1

Jaffe, R., Dietemann, V., Allsopp, M. H., Costa, C., Crewe, R. M., Dall'olio, R., de la Rua, P., El-Niweiri, M. A. A., Fries, I., Kezic, N., Meusel, M. S., Paxton, R. J., Shaibi, T., Stolle, E., & Moritz, R. F. A. (2010). Estimating the density of honeybee colonies across their natural range to fill the gap in pollinator decline censuses. *Conservation Biology*, *24*(2), 583–593. https://doi.org/10.1111/j.1523-1739.2009.01331.x

Jensen, A. B., Palmer, K. A., Boomsma, J. J., & Pedersen, B. V. (2005). Varying degrees of Apis mellifera ligustica introgression in protected populations of the black honeybee, Apis mellifera mellifera, in northwest Europe. *Molecular Ecology*, *14*(1), 93–106. https://doi.org/10.1111/j.1365-294X.2004.02399.x

Kohl, P., & Rutschmann, B. (2018). The neglected bee trees: European beech forests as a home for feral honey bee colonies. *PeerJ*, *6*(3), e4602.

Kopelman, N. M., Mayzel, J., Jakobsson, M., Rosenberg, N. A., & Mayrose, I. (2015). Clumpak: A program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, *15*(5), 1179–1191. https://doi.org/10.1111/1755-0998.12387

Korpela, S., Aarhus, A., Fries, I., & Hansen, H. (1992). Varroa jacobsoni OUD in cold climates: Population growth, winter mortality and influence on survival of honey bee colonies. *Journal of Apicultural Research*, *31*(3-4), 157–164. https://doi.org/10.1080/00218839.1992.11101278

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Le Conte, Y., De Vaublanc, G., Crauser, D., Jeanne, F., Rousselle, J.-C., & Becard, J.-M. (2007). Honey bee colonies that have survived Varroa destructor. Apidologie, *38*(6), 566–572. https://doi.org/10.1051/apido:2007040

Meixner, M. D., Büchler, R., Costa, C., Francis, R. M., Hatjina, F., Kryger, P., Uzunov, A., & Carreck, N. L. (2014). Honey bee genotypes and the environment. *Journal of Apicultural Research*, *53*(2), 183–187. https://doi.org/10.3896/IBRA.1.53.2.01

Mikheyev, A. S., Tin, M. M. Y., Arora, J., & Seeley, T. D. (2015). Museum samples reveal rapid evolution by wild honey bees exposed to a novel parasite. *Nature Communications*, *6*(1), 7991. https://doi.org/10.1038/ncomms8991

Moritz, R. F. A., Bernhard Kraus, F., Kryger, P., & Crewe, R. M. (2007). The size of wild honeybee populations (Apis mellifera) and its implications for the conservation of honeybees. *Journal of Insect Conservation*, *11*(4), 391–397. https://doi.org/10.1007/s10841-006-9054-5

Moritz, R. F. A., Hartel, S., & Neumann, P. (2005). Global invasions of the western honeybee (Apis mellifera) and the consequences for biodiversity. Écoscience, *12*(3), 289–301. https://doi.org/10.2980/i1195-6860-12-3-289.1

Nieto, A., Roberts, S. P. M., Kemp, J., Rasmont, P., Kuhlmann, M., García Criado, M., Biesmeijer, J. C., Bogusch, P., Dathe, H. H., De la Rúa, P., De Meulemeester, T., Dehon, M., Dewulf, A., Ortiz-Sánchez, F. J., Lhomme, P., Pauly, A., Potts, S. G., Praz, C., Quaranta, M., … Michez, D. (2014). *European Red List of bees*. Publication Office of the European Union.

Parejo, M., Henriques, D., Pinto, M. A., Soland-Reckeweg, G., & Neuditschko, M. (2018). Empirical comparison of microsatellite and SNP markers to estimate introgression in Apis mellifera mellifera. *Journal of Apicultural Research*, *57*(4), 504–506. https://doi.org/10.1080/00218839.2018.1494894

Pinto, M. A., Henriques, D., Chávez-Galarza, J., Kryger, P., Garnery, L., van der Zee, R., Dahle, B., Soland-Reckeweg, G., de la Rúa, P., Dall' Olio, R., Carreck, N. L., & Johnston, J. S. (2014). Genetic integrity of the Dark European honey bee (Apis mellifera mellifera) from protected populations: A genome-wide assessment using SNPs and mtDNA sequence data. *Journal of Apicultural Research*, *53*(2), 269–278. https://doi.org/10.3896/IBRA.1.53.2.08

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945–959.

Randi, E. (2008). Detecting hybridization between wild species and their domesticated relatives. *Molecular Ecology*, *17*(1), 285–293. https://doi.org/10.1111/j.1365-294X.2007.03417.x

Requier, F., Garnery, L., Kohl, P. L., Njovu, H. K., Pirk, C. W. W., Crewe, R. M., & Steffan-Dewenter, I. (2019). The conservation of native honey bees is crucial. *Trends in Ecology & Evolution*, *34*(9), 789–798. https://doi.org/10.1016/j.tree.2019.04.008

Rortais, A., Arnold, G., Alburaki, M., Legout, H., & Garnery, L. (2011). Review of the Dral COI-COII test for the conservation of the black honeybee (Apis mellifera mellifera). *Conservation Genetics Resources*, *3*(2), 383–391. https://doi.org/10.1007/s12686-010-9351-x

Seeley, T. D. (2007). Honey bees of the Arnot Forest: A population of feral colonies persisting with Varroa destructor in the northeastern United States. Apidologie, *38*(1), 19–29. https://doi.org/10.1051/apido:2006055

Soland-Reckeweg, G. (2006). *Genetic differentiation and hybridization in the honeybee (Apis mellifera L.) in Switzerland* [Thesis]. University of Bern.

Soland-Reckeweg, G., Heckel, G., Neumann, P., Fluri, P., & Excoffier, L. (2009). Gene flow in admixed populations and implications for the conservation of the Western honey bee. *Journal of Insect Conservation*, *13*(3), 317–328. https://doi.org/10.1007/s10841-008-9175-0

Thompson, C. E. (2012). *The health and status of the feral honeybee (Apis mellifera sp) and the Apis mellifera mellifera population of the UK* [Thesis]. University of Leeds.

Vaha, J. P., & Primmer, C. R. (2006). Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Molecular Ecology*, *15*(1), 63–72. https://doi.org/10.1111/j.1365-294X.2005.02773.x