Introgression in native populations of *Apis mellifera mellifera* L: implications for conservation

Jonathan S. Ellis*[1,2], Gabriele Soland-Reckeweg[3], Victoria G. Buswell[1,2]; J. Vanessa Huml[1,2], Andrew Brown[4], and Mairi E. Knight [1,2]

Affiliations and addresses

1. School of Biological and Marine Sciences, Plymouth University, Drake Circus, Plymouth, PL4 8AA

2. Sustainable Earth Institute, Plymouth University, Drake Circus, Plymouth, PL4 8AA

3. Apigenix, Gaicht 19, 2513 Twann, Switzerland

4. B4, Newton Farm Metherell, Callington, Cornwall, PL17 8DQ

Email addresses and ORCID identifiers:

Corresponding author: Jonathan Ellis, School of Biological and Marine Sciences, Plymouth University, Drake Circus, Plymouth, PL4 8AA, tel: +44(0)1752585946

Jonathan Ellis: jonathan.s.ellis@plymouth.ac.uk; orcid.org/0000-0002-6655-4685

Other authors:

Gabriele Soland: info@apigenix.com

Victoria Buswell: victoria.buswell@plymouth.ac.uk

J. Vanessa Huml: vanessa.huml@plymouth.ac.uk

Andrew Brown: andrew@aglbrown.co.uk

Mairi Knight: mairi.knight@plymouth.ac.uk; orcid.org/0000-0001-9388-4850

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Abstract

Hybridisation and introgression can have negative impacts on regional biodiversity through the potential erosion of locally adapted lineages. The honey bee (*Apis mellifera* L.) occurs in twenty-seven subspecies across Europe, is an extremely economically important insect, yet threatened by multifarious impacts. Transhumance of the most commercially appealing varieties threatens native honey bee diversity by introgression and subsequent loss of locally adapted traits, or even by complete removal of some subspecies from parts of the range. Here levels of admixture and introgression are examined in UK honey bees suspected to be from hives of the dark European honey bee (*Apis mellifera mellifera*). Microsatellite DNA and STRUCTURE analyses reveal that the studied populations are generally admixed, and discriminant analysis of principal components shows them to be intermediate between *A. m. mellifera* and *A. m. carnica* populations. However, examining mitochondrial haplotype data (COI-COII intergenic spacer region) and nuclear DNA reveal that some hives are relatively pure (from four to fifteen hives, depending on the Q-value threshold). Genetic diversity is relatively high in comparison with other European populations. Implications for conservation and management are discussed.

Keywords:
Introgression, honey bee, subspecies, microsatellite, mitochondrial DNA, conservation

Introduction

Hybridisation is widely defined as interbreeding between individuals from distinct lineages, subspecies or species. Globally, rates of hybridisation are increasing due to movement of organisms by humans and habitat alteration (Allendorf et al. 2001). Introgression is the incorporation of genetic material from one lineage into the background of another (Anderson 1949) and can occur following hybridisation and repeated back-crossing. Hybridisation and introgression are often considered to be problematic for conservation because these processes can lead to the loss of combinations of alleles that have resulted from long periods of adaptive evolution. This can disrupt local adaptation, leading to outbreeding depression (Templeton et al. 1986) and can even lead to genomic extinction (the loss of a lineage by introgression or anthropogenic displacement (Epifanio and Philipp 2001; Allendorf et al. 2004)). Overall, hybridisation and introgression may thus be considered to have a negative impact on regional biodiversity (Allendorf et al. 2004). Conversely, hybridisation is sometimes regarded as a positive management option because it augments genetic diversity, conserves evolutionary potential as a consequence, and sometimes the fitness of admixed genotypes is increased (Hamilton and Miller 2016). From this point of view, hybridisation and introgression can increase the overall capacity for adaptation, which is important in a changing environment (Hamilton and Miller 2016).

Honey bees provide an interesting study system to investigate issues arising from hybridisation and introgression. They are amongst the most important insect pollinators, especially for the pollination of crop monocultures (Delaplane and Mayer 2000; van Engelsdorp and Meixner 2010). Insect pollination itself has been estimated as worth €153 billion annually (Gallai et al. 2009) and worth €505 million annually in the UK (POST 2010). The value of honey bee pollination in the USA alone has been

estimated at $14.6 billion (Morse and Calderone 2000). Despite this importance, honey bees face various threats. For example, in Europe between 2008-2012 average winter losses by country varied from 7% to 30% (OPERA 2013). These unexplained winter losses of honey bees may be attributable to interacting underlying factors such as the spread of diseases and parasites (*Varroa destructor, Nosema* spp., bacterial pathogens, deformed wing virus and acute bee paralysis virus), autumn colony strength and winter severity (Genersch 2010; Highfield et al. 2009; Lee et al. 2015; Meixner et al. 2010; OPERA 2013; van Engelsdorp et al. 2012). The increasing use of pesticides and the role of neonicotinoids in particular is another potentially important factor contributing to declines, and is a subject of ongoing debate (reviewed in the OPERA report 2013).

In addition to these issues, many beekeepers are now concerned about the potential loss of locally adapted forms that occur in subspecies, regional varieties and ecotypes (Meixner et al. 2010). Ten out of twenty-seven subspecies of honey bee are present in Europe (Meixner et al. 2013). Early morphometric analyses classified these into M, A, C and O lineages, which owe their origin to the glacial history of Europe (Ruttner 1988). The M lineage occurs in the west Mediterranean area and north-western Europe and includes *Apis mellifera mellifera* and *A. m. iberiensis.* The C lineage occurs in south-eastern Europe and includes the subspecies *A. m. ligustica, A. m. carnica, A. m. macedonica, A. m. cecropia, A. m. cypria* and *A. m. adami* (Ruttner 1988; Meixner et al. 2013). The O lineage occurs in the near East and western Asia and includes the subspecies *A. m. caucasia, A. m. anatolica, A. m. syriaca, A. m. meda, A. m. armeniaca, A. m. jemenitica* and *A. m. pomonella* (Meixner et al. 2013; Ruttner 1988). The A lineage represents a further seven African subspecies (Ruttner

1988; Meixner et al. 2013;). There is also a Y lineage in Ethiopia (Franck et al. 2001) and a Z lineage in Lybia (Alburaki et al. 2013).

Maintaining the diversity distributed across these subspecies is considered necessary to ensure future resilience of honey bees to environmental change (Pinto et al. 2014). Yet, transhumance of commercial varieties (by importation of queens and movement of hives) that are favoured for characteristics that make them amenable to beekeeping, may cause 'genetic pollution' of these varieties by introgression (Garnery et al. 1998a). The subspecies most favoured commercially are *A. m. ligustica* and *A. m. carnica* (van Engelsdorp and Meixner 2010). In some areas, importation of these subspecies has seen complete replacement of local subspecies, e.g. the replacement of *A. m. mellifera* by *A. m. carnica* in Germany (Kauhausen-Keller and Keller 1994; Maul and Hähnle 1994).

As part of the effort to conserve native bee diversity, there is a movement to protect the dark European honey bee, *A. m. mellifera* (Meixner et al. 2010, 2013). The range of this subspecies has been much reduced (see Meixner et al. 2010) and for the purpose of its conservation, the *Societas Internationalis pro Conservatione Apis melliferae melliferae* was established in 1995 (Pinto et al. 2014). Dark European honey bees can occur in ecotypes with distinct colony population cycles (Louveaux 1966, cited in Strange et al. 2007) that still persist today (Strange et al. 2007). Genetic methods can identify these local varieties, although specific ecotypes within these varieties may not be clearly delineated (Strange et al. 2008; Soland-Reckeweg et al. 2009). In general, local-origin colonies have been shown to have longer colony survivorship than non-local colonies (Büchler et al. 2014).

The identification of native honey bee subspecies and varieties is aided by the study of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) (Cornuet et al. 1991; Cornuet and Garnery 1991; De la Rúa et al. 1998; Garnery et al. 1998a, 1998b; Muñoz et al. 2017). Mitochondrial DNA is ideal as a colony-level marker (Garnery et al. 1998b) as all individuals in a colony share the same haplotype since mtDNA is maternally inherited. Cornuet et al. (1991) outlined the structure of the mitochondrial COI-COII intergenic spacer in honey bees. This is based on copy number variation and sequence variation of 'P' and 'Q' sequences in the intergenic spacer region between these genes (Cornuet et al. 1991). Haplotypes are named similarly to the morphometric lineages, but there is not complete consistency between the systems, for example *A. m. iberiensis* is in the M morphometric lineage, but can have M and A mtDNA haplotypes (Meixner et al. 2013). Dark European honey bees (*A. m. mellifera*) are in the M morphopmetric lineage and have M haplotypes (Meixner et al. 2013). A comprehensive review and description of COI-COII haplotypes in *A. m. mellifera* has been published by Rortais et al. (2011). This diversity may be interrogated by the use of restriction fragment length polymorphism analyses known as the *Dra*I test, (validated by Garnery et al. 1993). Nuclear markers like microsatellites are also useful as they may demonstrate different levels of introgression to those inferred from mtDNA (Ballard and Whitlock 2004; Garnery et al. 1998a). For example, Garnery et al. (1998a) observed asymmetrical levels of introgression for mtDNA versus nDNA markers in parts of France and the Iberian peninsula. Mitochondrial DNA is most commonly inherited uniparentally and generally does not undergo recombination (Ballard and Whitlock 2004). In haplodiploid and diploid taxa the mtDNA effective population size is usually smaller than for nDNA, and mtDNA also represents only a small proportion of the whole genome (Ballard and Whitlock 2004). Consequently it is prudent to utilise

information from both DNA sources when assessing the history of a species using molecular data.

Previous studies have examined rates of introgression in *A. m. mellifera*. Soland-Reckeweg et al. (2009) quantified introgression and hybridization between M and C lineages of honey bees in Switzerland. Considerable hybridization was observed, even in colonies managed for pure breeding by apiculturalists interested in conservation (Soland-Reckeweg et al. 2009). Pinto et al. (2014) examined the integrity of protected populations using single nucleotide polymorphisms (SNPs) and mtDNA. Despite their protection, introgression was detected in these populations, although introgression was higher in unprotected than protected colonies (Pinto et al. 2014). Honey bees from England and Scotland were included in this analysis. Jensen et al. (2005) also included English and Scottish samples in their earlier analysis of introgression in north-west European populations of *A. m. mellifera*. Microsatellite data and *Dra*I tests revealed varying levels of introgression, but also demonstrated the persistence of this subspecies in northwestern Europe. More recently, Parejo et al. (2016) examined introgression in Swiss and French populations of *A. m. mellifera* using whole genome sequence information and were able to detect admixture as well as population structuring by subspecies and geographic origin.

Here, local populations of *A. m. mellifera* from Cornwall in the South-West of the UK are examined. As mentioned above, subspecies of honey bee, including the dark European honey bee, may show evidence of local adaptation (Louveaux 1966; Strange et al. 2007). Populations of dark European honey bee (*A. m. mellifera*) are likely to have been native to the UK for at least 4000 years (Carreck 2008) and occur in the South-West of the UK, but have been neglected in previous studies, which have sampled elsewhere in the UK or continental Europe (Costa et al. 2012; Ilyasov et al.

2016; Jensen et al. 2005; Muñoz et al. 2015; Pinto et al. 2014). However, local beekeepers believe that relict hives occur in the region and that these show local adaptations including winter hardiness, a maritime brood cycle, longevity of workers and queens, activity in cold weather, and possible hardiness against *Varroa* (see http://www.b4project.co.uk/). These beekeepers have initiated the 'B4 project: bringing back black bees' for beekeepers interested in conserving local diversity of the dark bee, *A. m. mellifera* in this region. We emphasise that the focus of our study is at the regional level because of a real need to identify introgression for the practical conservation of dark European honey bees by beekeepers in the 'B4' organisation. These beekeepers suspect their colonies to be dark European bees and have set up a voluntary reserve in the area where only dark European hives are to be kept. It is not possible to identify relatively pure hives or hybrid individuals confidently on the basis of morphometric data, thus there is a practical conservation need on the part of these beekeepers to accurately identify and know the state of introgression in their hives. Our research therefore uses genetic techniques and modern analytical methods to bridge a gap between scientific research and the practical conservation of insects, an approach which is especially important for sound conservation practice.

Materials and Methods

Sampling

Bees were sampled from forty-three hives across thirty-four apiaries managed by ten beekeepers in Cornwall, England, during summer 2015 in the vicinity of Truro and to the west of Plymouth. Colonies were chosen by the beekeepers where they suspected an unhybridized dark bee, thus sampling aimed to detect remaining population

fragments of *A. m. mellifera*. Members of the B4 network were supplied with 5mL sterile sample tubes and ~2 mL absolute ethanol. Queens were indirectly sampled using a pool of antennae of 30 drones. DNA can be efficiently extracted from antennae (Issa et al. 2013). Drone brood were sampled by removing the cell lid with a clean sharp tool. Beekeepers were instructed to sample thirty individuals of the drone brood that were quite well-developed with antennae. The right antenna of each of the thirty drones was then removed using college pliers and placed in a 1.5mL centrifuge tube in absolute ethanol. Samples were posted to Apigenix (Biel, Switzerland) for genetic analysis. Pools of drones from each hive sampled were genotyped to establish the queen genotype. DNA was isolated from the pools. In the authors' experience it is better to ask beekeepers to supply drone antennae because it is easy to then use a standard amount of tissue per individual when extracting the DNA. The use of larvae gives variously sized tissue samples from the individuals sampled. Furthermore, the use of drone antennae makes it more probable that worker-produced individuals have been removed by this stage. This means the estimation of the queen genotype is not 'contaminated' by alleles from the patrilines that would be present if the worker offspring were accidentally included. Regarding whether pools of 30 drones per hive are sufficient to establish the queen's genotype at a given heterozygous locus, the probability a haploid male has either one of the queen's alleles is 0.5, on average. The probability of only detecting a single one of these alleles can therefore be modelled as a binomial distribution where the probability of success is 0.5 and the number of trials equals the number of males sampled, in this case 30. In this case, the probability of all trials detecting a single allele at a given locus is 9.3×10^{-8}. This assumes an equal contribution to the DNA pool across males and the absence of null alleles. All mtDNA sequencing and genotyping was conducted by Apigenix (Switzerland).

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234 ***Investigation of admixture***

235 DNA was isolated from the drone samples using a Qiagen DNEasy Blood and Tissue
236 kit following the manufacturer's protocol. PCR amplificiation of 12 microsatellite loci
237 was performed in two multiplex reactions in a 10 µl reaction volume containing 2-10 ng
238 of genomic DNA, 5 µl HotStarTaq Master Mix, double distilled water, and 10 µM of
239 forward and reverse primers each. (Multiplex 1: FAM A43, FAM A273, FAM AC306,
240 FAM Ap33, ATTO565 Ap226, ATTO565 B24; multiplex 2: FAM A76; ATTO550 A28,
241 ATTO550 Ap289, ATTO532 A007, ATTO532 AP1, ATTO565 A29, Solignac et al.
242 2003). The following cycling protocol on a TC-412 programmable thermal controller
243 (Techne) was used: 40 cycles with 94°C for 30 s, 56°C for 90 s, and 72°C for 60 s.
244 Before the first cycle, a prolonged denaturation step (95°C for 15 min) was included
245 and the last cycle was followed by a 30 min extension at 72°C. Fragments were run
246 on a ABI3730 Prism Genetic Analyser (Applied Biosystems) using GeneScan TM-500
247 LIZ size standard. Fragments were scored using the software GeneMarker 3.0 (ABI).

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249 Additional samples from Italy for *A. m. ligustica*, Austria and Slovenia for *A. m. carnica*
250 and Sweden, France, Norway, Switzerland and Ireland for *A. m. mellifera* were
251 included for testing admixture and introgression in Cornish bees (see Soland-
252 Reckeweg et al. 2009 for more information including a map of sampling locations).
253 These sample locations include *A. m. mellifera* conservation areas in Norway and
254 Sweden, and areas where least introgression is expected. Hybrids have been
255 previously removed from this reference dataset of genotypes (Soland-Reckeweg et al.
256 2009). Microsatellite genotyping was also conducted using a set of pre-typed
257 individuals of known genotypes to create an allele ladder across the size range of

alleles at each locus. This approach allows to confidently assign microsatellite genotypes and avoid errors due to size shifts which can problematic, especially if different genotyping equipment is used (e.g. Ellis et al. 2011)

After genotyping, MICROCHECKER (van Oosterhout et al. 2004) was used to investigate the presence of null alleles and other common sources of genotyping error (e.g. stutter). Samples were grouped by population in this analysis. Estimates of linkage disequilibrium and departures from Hardy-Weinberg equilibrium were made in Arlequin 3.5 (Excoffier and Lischer 2010). Again, samples were grouped by population for these analyses. For pairwise tests of linkage disequilibrium the number of permutations was 10,000. The selected significance level was P=0.05, but strict Bonferroni corrections were applied to pairwise tests by population, thus the revised level of significance was P = 0.0008 (there were 66 tests per population). For exact tests of Hardy-Weinberg equilibrium the number of steps in the Markov chain was 1,000,000 and the number of dememorization steps was 100,000. Strict Bonferroni corrections were again applied to tests done by population (adjusted P varied as some loci were monomorphic in some populations, minimum adjusted P = 0.004). Some loci were removed after these steps, prior to downstream analyses (see Results). Standard measures of genetic diversity were estimated in Arlequin 3.5 (observed and expected heterozygosity; Excoffier and Lischer 2010) and FSTAT (allelic richness, Goudet 2001). Allelic richness was calculated across loci per population and was based on a minimum sample size of ten individuals.

To investigate admixture, two complementary approaches were used, as has been recommended (Janes et al. 2017). For the first approach, STRUCTURE (Pritchard et

al. 2000) was used. A burn-in period of 50,000 steps was used followed by 500,000 MCMC steps. *K* values of 1 to 12 were tested, with three iterations of each *K* value. A correlated allele model (Falush et al. 2003) was applied. The admixture model was used, but LOCPRIOR was not applied (LOCPRIOR is usually used where the expected signal is too weak for standard structure models and makes use of location information with each individual to assist clustering, Pritchard et al. 2010). Iterations were examined for consistency (by examining similarity of alpha values and 'ln Prob. of data' across the iterations). The best *K* value was investigated using the original method recommended in STRUCTURE and using the Evanno et al. (2005) method. The standard method infers the most probable value of *K* based on the 'log probability of the data' (or where this value begins to reach a plateau). The Evanno et al. (2005) method is based on the rate of change in values of 'log probability of data' for successive values of *K*. *Structure Harvester* was used to generate the relevant plots for inference of *K* (Earl 2012). Although these methods can be used to estimate the 'best' *K* value, multiple *K* values were interpreted as this is recommended (Janes et al. 2017). Barplots were produced using the online application STRUCTURE PLOT (Ramasamy et al. 2014). The degree of introgression of sampled colonies was investigated through inspection of mean Q values and their standard deviations (from the three iterations of the analysis) for $K = 3$ (further explanation in the Results). A population cluster which included the Cornish honey bee samples and the other *A. m. mellifera* samples was then investigated separately. Analysis parameters and steps were conducted as described above. Finally, the relationship between degree of admixture and observed heterozygosity and allelic richness were tested at the population level. Mean coefficients of admixture (i.e. mean Q value across individuals) were calculated for membership to the 'dominant' cluster for each population.

Correlations between mean Q-value and mean observed heterozygosity (calculated in Arlequin 3.5) and mean allelic richness (calculated in FSTAT 2.9.3.2 (Goudet 2001)) were tested using Spearman's rank method in R 3.4.1 (R Foundation for Statistical Computing).

In the second approach, investigation of admixture was carried out using the R package *adegenet* 1.3-0 (Jombart 2008) using the *dapc* functions (discriminant analysis of principal components, DAPC, Jombart 2011). Preliminary analysis showed that Italian bees were distant from the other samples, so this analysis was performed only for *A. m. carnica* and *A. m. mellifera* samples (however, analysis of all samples is included in the supplementary material). First the *find.clusters* function was used to identify the most likely number of population clusters. A test DAPC analysis (for the most likely number of population clusters identified using *find.clusters*) was then run retaining all principal components and linear discriminants. The *a.score* function was used to select the ideal number of principal components (PCs) to avoid overfitting. The *a.score* function was run four times to examine convergence in the recommended number of PCs to retain. The DAPC analysis was then repeated with the reduced set of twenty PCs and four linear discriminants, for the most likely number of population clusters identified in the first step. Scatter plots were produced for visual inspection of clusters. Group memberships of individuals across source populations to the identified clusters were tabulated. Membership probability of individual Cornish bees to the identified clusters was plotted using the *compoplot* function.

Assignment of mtDNA haplotypes

The COI-COII region was sequenced using the primers E2 (GGC AGA ATA AGT GCA TTG) and H2 (CAA TAT CAT TGA TGA CC) (Garnery et al. 1993). The following cycling protocol on a TC-412 Programmable Thermal Controller (Techne) was used: 35 cycles with 94°C for 60 s, 54°C for 45 s, and 62°C for 120 s. Before the first cycle, a prolonged denaturation step (95°C for 15 min) was included and the last cycle was followed by a 10 min extension at 72°C. Sanger sequencing was then conducted (Sanger et al. 1977) using fluorescent dyes (Ansorge et al. 1987; Middendorf et al. 1992), specialized DNA polymerases (Taq-polymerase; Carothers et al. 1989) and modified nucleotides to avoid problems with DNA secondary structure (Frederick 1999). Capillary electrophoresis was performed on an ABI3730 using Dye Chemistry Software Data Collection Version 3; Sequencing Analysis 5.2 (Applied Biosystems). Sequences were aligned using ClustalW (Thompson 1994) in Bioedit (Hall 2004). Mitochondrial haplotypes were identified on the basis of the presence of P and Q repeats (Cornuet et al. 1991). *A. m. mellifera* and *A. m. iberiensis* are in the M lineage and are indicated by the presence of a P repeat and one or more Q repeats (Cornuet et al. 1991, Achou et al. 2015) although *A. m. iberiensis* can also have A haplotypes and present two types of P sequence (P0 and P). The common C lineage commercial subspecies, *A. m. ligustica* and *A. m. carnica* lack a P repeat and have only a single Q repeat (Cornuet et al. 1991).

Results

Quality control

Three loci (A76, Ap001, A29) were not genotyped in Italian and French population samples. Locus A43 was implicated twice as showing evidence of null alleles in

MICROCHECKER. Loci Ap226, A76, Ap289 were implicated once in showing evidence of null alleles. Departure from Hardy-Weinberg equilibrium was shown for loci A43 (Austria 2015), Ap001 (Austria) and for A76 and A28 (Austria Würm). Pairwise linkage disequilibrium was not consistent for loci across populations apart from loci Ac306 and Ap226 in the Swedish and Slovenian samples. Consequently loci A43 and A76 (showed null alleles and departure from Hardy-Weinberg equilibrium), Ap001 (showed departure from Hardy-Weinberg equilibrium and had poor coverage across populations) and A29 (poor coverage across all populations) were removed from the dataset prior to downstream analyses. Standard estimates of genetic diversity are shown in Table 1.

Investigation of admixture

STRUCTURE analysis of all populations showed *K* = 3 and 5 as numbers of clusters likely to be useful to describe the population structure (one should be careful with interpreting the 'correct' *K* (Pritchard et al. 2000; Janes et al. 2017) hence results for both are presented; see supplementary data figures 1 and 2). *K* = 3 clearly delineates all three subspecies, with admixture shown for the Cornish population (Figure 1a). *K* = 5 again separates *A. m. ligustica* from the other species; *A. m. carnica* show membership to two clusters and *A. m. mellifera* again show a separate signal for the Cornish sample in comparison with other populations of this subspecies (Figure 1b).

For *A. m. mellifera* examined separately in STRUCTURE, *K* = 2 and *K* = 3 are useful descriptions of the population structure (supplementary data figures 3 and 4). Both analyses show the Cornish population showing some distinction from the other *A. m. mellifera* populations (Figure 2a and b).

There was a significant negative relationship between mean Q-value and mean observed heterozygosity at the population level (rho = -0.68, n = 12, P<0.05; Figure 3a). There was also a significant negative relationship between mean Q-value and mean allelic richness (corrected for sample size) at the population level (rho = -0.62, n=12, P<0.05; Figure 3b)

Discriminant analysis of principal components showed five clusters as providing a useful description of the population structure (supplementary figure 5). Twenty PCs were retained after inspecting four iterations of *a.score* optimisation (the range of recommended PCs to retain was 14-26; supplementary data figure 6). In addition, four linear discriminants were used to model the population structure, visualised in a scatterplot (Figure 4). Examination of membership of individuals to the identified clusters (Table 2) shows that clusters 2, 3 and 5 mostly consist of *A. m. carnica* individuals, cluster four consists mostly of Cornish *A. m. mellifera* samples and cluster one represents other populations of *A. m. mellifera.* Individuals of Cornish samples that did not group with cluster four were assigned to clusters two and five (four of forty-three samples [9.3%]; Table 2, Figures 4 and 5). Analysis including the Italian bees can be seen in the supplementary material (supplementary figure 7 and Table S1) and also shows *A. m. mellifera* from Cornwall to be intermediate between *A. m. mellifera* from continental Europe and *A. m. carnica*.

Admixture and mtDNA haplotype assignment

Examination of Q-values from the STRUCTURE analysis of all populations for K = 3 in combination with mtDNA haplotype assignment give an indication of the degree of introgression across the Cornish hives included in the analyses (Table 3; Table 4).

Interpretation is considered for Q threshold values of 80%, 90%, 95% and 99%; i.e for a threshold of 0.99 an individual has to meet or exceed this value to be deemed 'pure' (Table 4, see discussion). When lower values of Q-threshold are specified to indicate a 'pure' bee, agreement between nuclear and mtDNA signal improves (i.e. more of the M lineage samples are deemed to be 'pure' *A. m. mellifera* samples). Applying the strictest threshold to deem a queen as 'pure' reveals only four individuals to be so and also have an M haplotype (Table 4). Applying the lowest threshold, fifteen bees are deemed to be pure on the basis of agreement between nDNA and mtDNA data (Table 4). All mtDNA sequences are available in GenBank (accession numbers MF197320-197363).

Discussion

STRUCTURE analyses and a discriminant analysis of principal components both indicate that samples of *A. m. mellifera* from beekeepers involved in the B4 project in the south-west of the UK are clearly distinct to other *A. m. mellifera* populations. This is most likely a consequence of admixture with imported lines rather than these apiaries representing a naturally differentiated lineage of *A. m. mellifera.* The bees sampled showed admixture from *A. m. carnica* introgression (STRUCTURE analyses) and were intermediate between clusters of *A. m. mellifera* and *A. m. carnica* (DAPC plots). This result is hardly surprising given the history of beekeeping in the UK. Local beekeepers report that after the First World War and the 'Isle of Wight disease' (when widespread losses of bees were attributed (incorrectly) to a single infectious disease (Bailey 1964)), bees were brought into Cornwall from the Netherlands (dark European honey bees), but also from Italy. Since this time, there have also been many imports of other subspecies of honey bee to the UK. Cornwall is not far from Buckfast in Devon

where Brother Adam developed the hybrid line that became known as “the Buckfast bee™”. Imports of honey bee into the UK increased in the period 2013-2016 (Learner 2017) and current advice to beekeepers from the National Bee Unit is that importing bees ‘is neither difficult nor a chore’ (Learner 2017). Previous studies examining *A. m. mellifera* have shown that there is admixture in unprotected English populations and that English samples showed both M and C lineages (Jensen et al. 2005; Pinto et al. 2014). Scottish samples from protected areas showed only M lineages (Jensen et al. 2005; Pinto et al. 2014). Elsewhere in Europe, and for other subspecies, hybrids have been found in populations of dark bees in Poland (Oleksa et al. 2011), admixed ancestry is reported in Serbian bees between *A. m. carnica* and *A. m. macedonica* (Nedić et al. 2014), but there are also places where lineages are relatively pure, e.g. *A. m. mellifera* in parts of the Urals and Volga region (Ilyasov et al. 2016) and *A. m. carnica* in Hungary (Péntek-Zakar et al. 2015). Clearly, transhumance of colonies frequently leads to introgression, but there are also places where *A. m. mellifera* remains relatively intact (Byatt et al. 2015)

Regarding the identification of relatively pure hives for conservation efforts in South-West England, the power in the dataset to effectively detect hybrids and the effect of threshold values on the designation of ‘pure’ individuals needs to be considered. Vähä and Primmer (2006) investigated the use of STRUCTURE and NEWHYBRIDS to do so and show that the number of loci for efficient and accurate determination of hybrids depends on the amount of genetic differentiation between the parental populations. F_{ST} values between the subspecies studied here are quite large (e.g. in the range 0.3-0.6 for *Aml* and *Amm*, 0.2-0.6 for *Amc* and *Amm*, and 0.3-0.4 for *Aml* and *Amc* for the loci used here, data not shown), so the use of relatively few loci will still be suitable for

identification of hybrids. However, it should be remembered that here, we are not detecting hybrid individuals between two parent lines where *K*=2, but rather trying to identify the degree of admixture from populations with a long history of crosses and back-crosses, where the useful number of clusters to describe the populations is probably from 3-5. Consequently, caution should be drawn when considering the relative purity of individuals using the approach described here. Vähä and Primmer (2006) showed that a stricter Q-value reduced the misclassification of back-crossed individuals as purebred individuals in their simulations. These authors note that as Q-value thresholds are increased there is a trade-off between the efficiency of detecting hybrids (proportion of individuals in a group correctly identified as hybrids) and the accuracy (proportion of an identified group truly belonging in that category). More stringent thresholds improve the accuracy of identifying hybrids, but decrease the efficiency (Vähä and Primmer 2006). Essentially, what needs to be decided is whether accurate hybrid identification (all the individuals in the hybrid group are hybrids, but some of the individuals in the purebred group are hybrids) or accurate purebred identification (all the individuals in the purebred group are purebred, but some of the individuals in the hybrid group are purebreds) is required. To conserve dark European honey bees, the purity of the dark European honey bee stock is of course the most desired outcome. However, to be certain of purity, the founding stock will be small (Table 4). Only four hives sampled showed an M lineage and a Q-value of >99% to *A. m. mellifera* cluster when *K* = 3. This value increases to 12 for a Q-value of 0.9. Further work investigating the phenotypic traits of Cornish bees for hives of differing admixed ancestry will help elucidate what is a useful threshold Q-value. We also note here that samples are limited and only eight microsatellites are analysed so the results should be interpreted with caution.

Depending on the Q-value (Table 4), some hives were observed to have an M lineage haplotype and show nuclear introgression. This pattern would be expected in a controlled population threatened by hybridization. Between one to three samples showed limited nuclear introgression, but had C haplotypes. This suggests an historical intake of foreign queens. This could be from swarms of unknown origin or purchase of queens from uncontrolled breeding programmes. Recurrent backcrosses with native bees subsequent to this historical introgression would give rise to a situation where foreign nDNA cannot be detected with the applied marker set. No samples were classified as pure *A. m. carnica* or *A. m. ligustica* (for Q>0.9, one individual was assigned to *A. m. carnica* at Q>0.8) but the sampling method used here particularly targeted beekeepers believing they likely had dark European honey bees. This was intentional as we aimed to investigate the level of admixture in bees of this type and identify potential hives for conservation management of dark European honey bees in the South-West. Sampling was also limited to mostly East and West Cornwall. It is likely that other keepers of dark European honey bees in the area could have been missed in the current study; our research was conducted through the local organisation ‘B4’ and only included the dark Europen honey bee beekeepers known to this organisation.

The conservation implications of these findings are either to accept a degree of foreign introgression, or to look to set up breeding programmes with other UK hives in order to ‘stock’ reserves for South-West dark European honey bees. Although four samples could be classified as pure *A. m. mellifera,* clearly, breeding from a founding stock of only four colonies would lead to inbreeding and significant loss of genetic diversity

which may increase extinction risk (Frankham 2005). Much broader sampling of hives in the region needs to be undertaken to identify other dark European honey bee hives in the area (we specifically sampled hives from beekeepers involved in the B4 project, but in total in the region in the year of sampling there were 4966 hives registered on Beebase. In April 2018, there were 1140 beekeepers registered in Cornwall, with 5538 colonies). Although it is possible that much of the genetic load can be purged by selection on the haploid sex in haplodiploids (Henter 2003), female sex-limited traits may not be affected (Tien et al. 2015) and there is evidence that haplodiploids can still show inbreeding depression (Henter 2003). This is especially important in systems where single locus complementary sex determination exists (Whitehorn et al. 2009). In honey bees, within-colony genetic diversity is also known to be important for disease resistance (Brown and Schmid-Hempel 2003).

The argument for conservation of locally adapted varieties makes sense from a viewpoint that maintaining a network of locally adapted forms (i) maximises genetic variation across the species as a whole; (ii) maintains co-adapted locus complexes and allows the persistence of locally adapted forms which already exist and are assumed to be most resilient to local environmental stochasticity; (iii) maintains the possibility of allowing human-mediated migration of particularly resistant forms (e.g. in the event of disease outbreaks or climate change). Nevertheless, and as already mentioned, action should be taken to minimise erosion of genetic diversity from these local populations through inadequate breeding population sizes and consequent genetic drift. In contrast, counter arguments could be made (Harpur et al. 2012) in the sense that admixture will increase within population genetic variance. Selection is also more efficient in large populations (because low frequency advantageous *de novo*

mutations are less likely to be lost by drift (see Olson-Manning et al. 2012 for an up-to-date review)). Although a preliminary analysis included here shows a significant negative correlation between Q-values and observed heterozygosity and allelic richness (more admixed populations are more genetically diverse), our sample sizes were small in several populations and only twelve population samples were included. In contrast, de la Rua et al. (2013) in their critique of Harpur et al. (2012), found that even where ongoing introduction of foreign queens takes place, genetic diversity is not necessarily increased (Muñoz et al. 2014). In Italian honey bee populations, large-scale queen breeding has reduced genetic diversity (see Dall'Olio et al. 2007). The argument (de la Rua et al. 2013 cf. Harpur et al., 2012) about which scenario best maximises evolutionary potential depends on the relative importance of increased within population variation (resulting from hybridization/introgression) versus loss of between population variance (that conservation of locally adapted forms seek to minimize).

Currently, legislation regarding honey bees in England and Wales relates to screening of colonies for diseases and parasites (Bee Diseases and Pests Control (England) Order 2006; Bee Diseases and Pests Control (Wales) Order 2006), health certification (Council Directive 92/65/EEC) and regards countries from outside the EU whence bees may be imported (Commision regulation (EU) 206/2010) as well as foods standards laws regarding the composition of honey for sale. There is an argument that the National Pollinators Strategy (DEFRA) should be extended to give greater protection to the native honey bee diversity that exists. Strict protection would be necessary to avoid hybridization of native colonies, as has occurred in other protected

areas in the past (Jensen et al. 2005). Urgent action is needed to characterise local adaptation before further erosion of these forms occurs.

Considering all results, immediate action is recommended to (i) more extensively sample both the South-West population and the UK populations to detect any pure uncompromised breeding stock; (ii) obtain more accurate assessment of introgression using ancestry informative SNPs which are known to outperform microsatellites (Muñoz et al. 2017); (iii) measure local adaptation of dark European honey bee colonies across the UK using genome-wide data aiming to detect recent and historical selection; (iv) start conservation actions to protect locally adapted varieties identified; (v) bring together networks of *A. m. mellifera* beekeepers from across the UK at the appropriate geographic scales identified.

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Figure legends

Fig. 1

Group membership to clusters identified using STRUCTURE with inference based on all populations (*A. m. ligustica* = 1 Italy, *A. m. carnica* = 2 Austria, 3 Austria Würm, 4 Slovenia, 11 Austria 2015, *A. m. mellifera* = 5 Sweden, 6 France, 7 Norway, 8 Switzerland Glarus, 9 Switzerland Schistal, 10 Ireland, 12 Cornwall), **(a)** K = 3, **(b)** K = 5. (Note that the coloured bar at the bottom of the figure illustrates the population of origin only)

Fig. 2 Group membership to clusters identified using STRUCTURE with inference based on *A. m. mellifera* clusters only (numbering of populations is retained as in Fig 2 for comparison), **(a)** K = 2 **(b)** K = 3. (Note that the coloured bar at the bottom of the figure illustrates the population of origin only)

Fig. 3 (a) Correlation between Q-values (lower values indicate increased admixture) and observed heterozygosity across populations, **(b)** correlation between Q-values and allelic richness (Ar)

Fig. 4 Discriminant analysis of principal components for all populations sampled, apart from *A. m. ligustica* (Italy; see text). Group four represents the putative dark European honey bees from Cornwall. Clusters 2, 3 and 5 mostly consist of *A. m. carnica* individuals. Cluster one represents continental European populations of *A. m. mellifera.* (Membership of individuals from each population to each group is

937 shown in table 2). Analysis is based on retention of 20 principal components (Fig S6)
938 and all linear discriminants (four)
939
940 **Fig. 5** Group membership to clusters identified using the *find.clusters* function, for
941 Cornish honey bees only, based on the proportion of successful assignments to the
942 identified clusters. Groups 2,3 and 5 represent *A. m. carnica* populations (see Table
943 2) and group 4 *A. m. mellifera* populations
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Tables

Table 1 Genetic diversity of population samples included in the study. Populations for each subspecies are: *A. m. ligustica* – Italy; *A. m. carnica* – Austrian and Slovenian samples; *A. m. mellifera* all other samples

Population	**Sample size**	**Allelic richness** (± standard deviation)	**Observed heterozygosity** mean	standard deviation	standard error	**Expected heterozygosity** mean	standard deviation	standard error
Italy	55	3.5±2.7	0.35	0.35	0.12	0.38	0.37	0.13
Austria	44	4.4±2.0	0.54	0.24	0.08	0.57	0.20	0.07
Austria Würm	36	3.9±0.57	0.57	0.15	0.05	0.59	0.12	0.04
Austria 2015	102	4.3±1.9	0.51	0.22	0.08	0.52	0.24	0.08
Slovenia	212	3.6±1.8	0.45	0.26	0.09	0.44	0.27	0.09
Sweden	10	3.0±1.4	0.38	0.27	0.10	0.46	0.33	0.12
France	24	3.8±3.6	0.38	0.31	0.11	0.39	0.34	0.12
Norway	18	3.6±2.8	0.41	0.38	0.13	0.40	0.35	0.12
Switzerland Glarus	10	3.9±3.0	0.41	0.34	0.12	0.43	0.33	0.12
Switzerland Schistal	12	3.6±2.4	0.43	0.33	0.12	0.40	0.32	0.11
Ireland	22	3.7±2.6	0.36	0.26	0.09	0.39	0.32	0.11
Cornwall	43	5.1±2.8	0.60	0.26	0.09	0.63	0.21	0.07

953 **Table 2** Membership of individuals from each sampled population to the clusters
954 inferred in adegenet 1.3-0 using the *find.clusters* function. Numbers are the number
955 of individuals assigned to each cluster. Five clusters were inferred on the basis of
956 BIC estimates (Supplementary data, Figure 4). Subspecies are indicated in
957 superscript (*c = A. m. carnica*; *m = A. m. mellifera*)

958

Populations	**Inferred cluster**				
	1	**2**	**3**	**4**	**5**
Austria[c]	3	7	17	1	16
Austria Wurm[c]	0	29	2	0	5
Austria 2015[c]	0	12	43	0	44
Slovenia[c]	0	1	15	0	5
Sweden[m]	10	0	0	0	0
France[m]	24	0	0	0	0
Norway[m]	18	0	0	0	0
Switzerland Glarus[m]	9	0	0	1	0
Switzerland Schistal[m]	12	0	0	0	0
Ireland[m]	22	0	0	0	0
Cornwall[m]	0	1	0	39	3

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Table 3 Membership of Cornish honey bees to the clusters identified in STRUCTURE for K = 3. The Q-values are the mean admixture coefficients from three iterations of each K value. Standard deviations are also indicated. Blank cells under 'mtDNA haplotype' were not sequenced.

	Apis mellifera mellifera		*Apis mellifera ligustica*		*Apis mellifera carnica*		
Individual	Q	s.d	Q	s.d.	Q	s.d.	**mtDNA haplotype**
15-001	0.94	0.000	0.04	0.001	0.02	0.000	
15-002	0.97	0.000	0.01	0.000	0.01	0.000	
15-003	0.91	0.002	0.04	0.001	0.05	0.001	
15-004	0.98	0.000	0.01	0.000	0.01	0.000	
15-1321	0.94	0.001	0.04	0.000	0.03	0.000	
15-1401	0.41	0.001	0.03	0.000	0.57	0.001	
15-1407	0.99	0.000	0.00	0.000	0.01	0.000	
15-1409	0.99	0.000	0.01	0.000	0.01	0.000	
15-1410	0.99	0.000	0.01	0.000	0.01	0.000	
15-1411	0.97	0.000	0.01	0.000	0.01	0.000	
15-1137	0.94	0.001	0.03	0.001	0.03	0.000	M
15-1188	0.91	0.001	0.00	0.000	0.09	0.001	M
15-1204	0.91	0.001	0.01	0.000	0.08	0.001	M
15-1310	0.82	0.001	0.01	0.000	0.17	0.001	M
15-1311	0.99	0.000	0.00	0.000	0.01	0.000	M
15-1312	0.56	0.001	0.03	0.001	0.41	0.002	C
15-1313	0.93	0.001	0.01	0.000	0.07	0.001	M
15-1315	0.37	0.002	0.01	0.000	0.62	0.002	C
15-1317	0.98	0.000	0.01	0.000	0.01	0.000	
15-1322	0.62	0.003	0.04	0.001	0.34	0.004	
15-1323	0.99	0.000	0.00	0.000	0.01	0.000	M
15-1327	0.95	0.001	0.01	0.000	0.04	0.000	M
15-1330	0.86	0.002	0.01	0.000	0.14	0.002	M
15-1332	0.55	0.000	0.01	0.000	0.45	0.000	C
15-1334	0.63	0.003	0.01	0.000	0.36	0.003	M
15-1335	0.99	0.000	0.00	0.000	0.01	0.000	C
15-1336	0.43	0.001	0.02	0.000	0.55	0.000	C
15-1345	0.99	0.000	0.00	0.000	0.01	0.000	
15-1348	0.98	0.000	0.01	0.000	0.01	0.000	C
15-1349	0.94	0.000	0.03	0.000	0.03	0.000	M
15-1351	0.16	0.001	0.01	0.000	0.84	0.001	C
15-1352	0.83	0.000	0.02	0.000	0.15	0.001	C
15-1355	0.99	0.000	0.00	0.000	0.00	0.000	M
15-1356	0.57	0.001	0.01	0.000	0.42	0.001	C

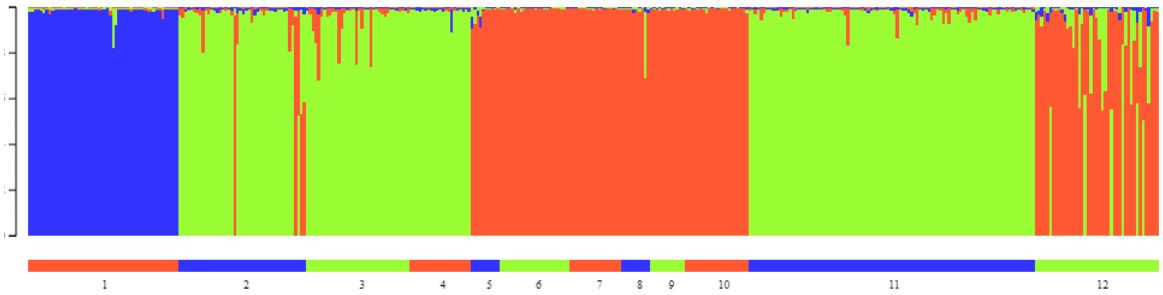
15-1359	0.86	0.000	0.02	0.000	0.13	0.000	M
15-1360	0.35	0.001	0.07	0.001	0.58	0.000	C
15-1364	0.74	0.002	0.02	0.001	0.24	0.001	M
15-1372	0.49	0.001	0.00	0.000	0.51	0.001	C
15-1377	0.99	0.000	0.01	0.000	0.01	0.000	M
15-1422	0.58	0.001	0.08	0.001	0.34	0.002	C
15-1423	0.94	0.000	0.01	0.000	0.06	0.000	M
15-1426	0.98	0.000	0.01	0.000	0.01	0.000	C
15-1427	0.98	0.000	0.01	0.000	0.02	0.000	M

Table 4 Assignment of mtDNA haplotypes (M or C lineage) and nuclear introgression considered together. Individuals are assigned as 'pure' based on differing Q-values of >99%, >95%, >0.90% and >0.80% for *K* = 3.

Q-threshold	nDNA	mtDNA M	mtDNA C	Not sequenced
0.99	'pure'	4	1	4
	introgressed	13	12	9
0.95	'pure'	6	3	8
	introgressed	11	10	5
0.90	'pure'	12	3	11
	introgressed	5	10	2
0.80	'pure'	15	3	11
	introgressed	2	10	2

Fig 1

(a)



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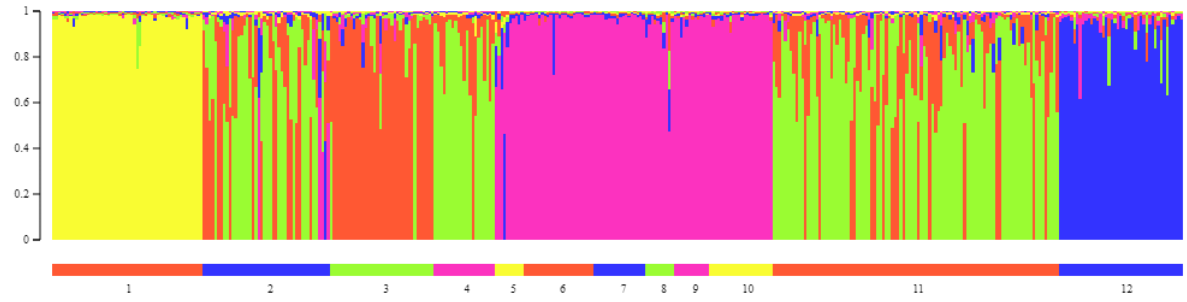
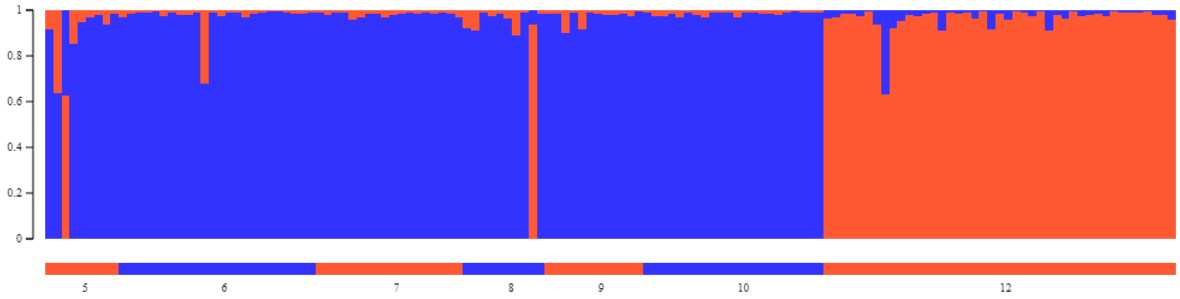


Fig 2

(a)



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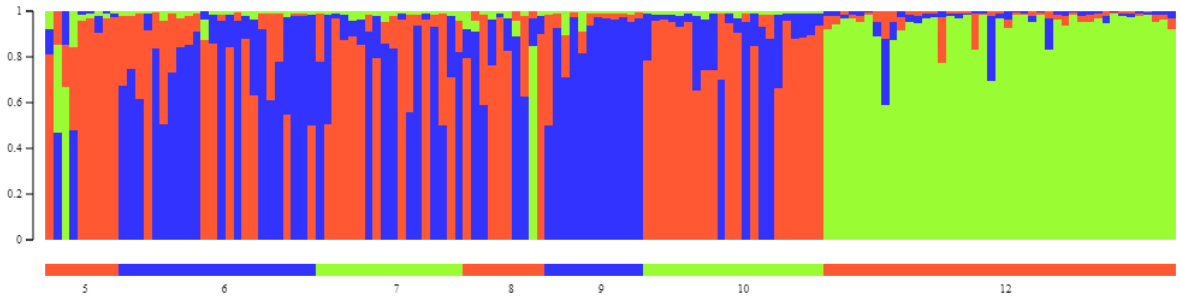
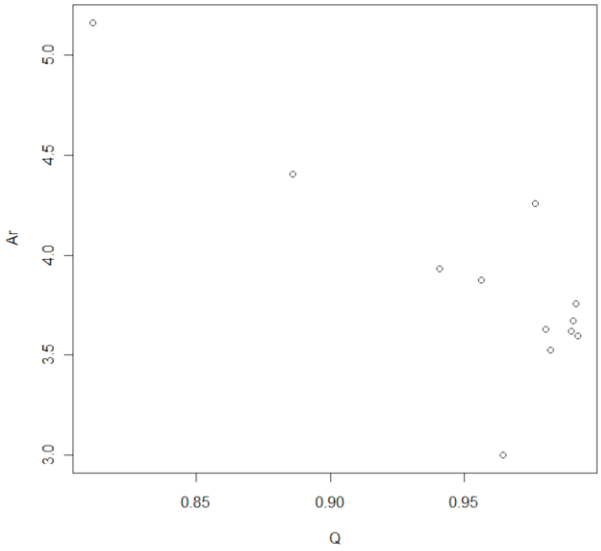


Fig 3
(a)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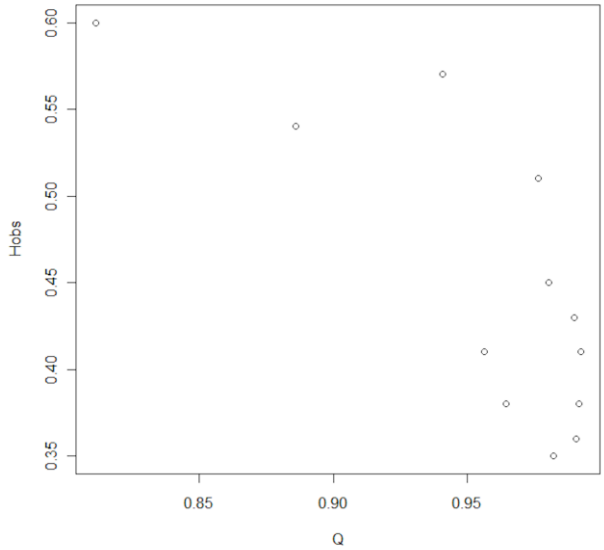
4.5
4.0
3.5
3.0
0.85
0.90
0.95
Q

(b)


Hobs
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0.50
0.45
0.40
0.35
0.85
0.90
0.95
Q

Fig 4

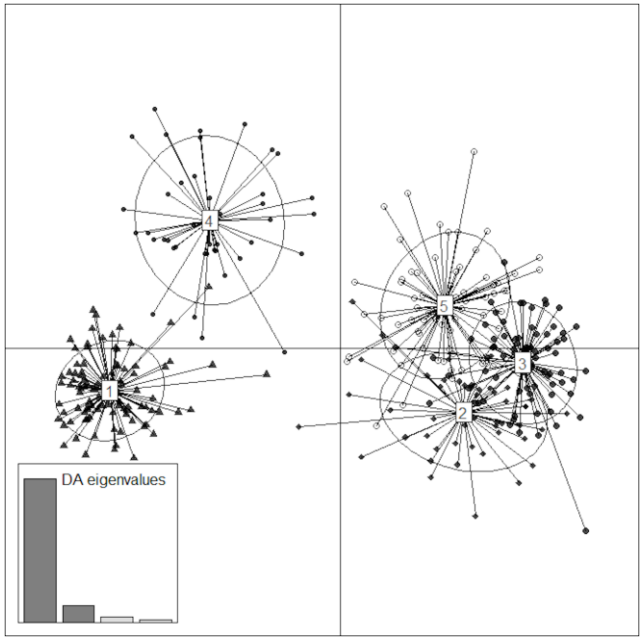

Fig 5