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

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25 **Compliance with ethical standards**

26 No human participants were involved in the work. Ethical standards were followed for  
27 the sampling of honey bee drones by the removal of antennae by beekeepers. The  
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29 work.  
30

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35 The authors are aware of no potential conflict of interest (financial or non-financial).

### 36 **Abstract**

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

53

### 54 **Keywords:**

55 Introgression, honey bee, subspecies, microsatellite, mitochondrial DNA, conservation

56

57

## 58 **Introduction**

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

77 Honey bees provide an interesting study system to investigate issues arising  
78 from hybridisation and introgression. They are amongst the most important insect  
79 pollinators, especially for the pollination of crop monocultures (Delaplane and Mayer  
80 2000; van Engelsdorp and Meixner 2010). Insect pollination itself has been estimated  
81 as worth €153 billion annually (Gallai et al. 2009) and worth €505 million annually in  
82 the UK (POST 2010). The value of honey bee pollination in the USA alone has been

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

94           In addition to these issues, many beekeepers are now concerned about  
95 the potential loss of locally adapted forms that occur in subspecies, regional varieties  
96 and ecotypes (Meixner et al. 2010). Ten out of twenty-seven subspecies of honey bee  
97 are present in Europe (Meixner et al. 2013). Early morphometric analyses classified  
98 these into M, A, C and O lineages, which owe their origin to the glacial history of  
99 Europe (Ruttner 1988). The M lineage occurs in the west Mediterranean area and  
100 north-western Europe and includes *Apis mellifera mellifera* and *A. m. iberiensis*. The  
101 C lineage occurs in south-eastern Europe and includes the subspecies *A. m. ligustica*,  
102 *A. m. carnica*, *A. m. macedonica*, *A. m. cecropia*, *A. m. cypria* and *A. m. adami* (Ruttner  
103 1988; Meixner et al. 2013). The O lineage occurs in the near East and western Asia  
104 and includes the subspecies *A. m. caucasia*, *A. m. anatolica*, *A. m. syriaca*, *A. m.*  
105 *meda*, *A. m. armeniaca*, *A. m. jemenitica* and *A. m. pomonella* (Meixner et al. 2013;  
106 Ruttner 1988). The A lineage represents a further seven African subspecies (Ruttner

107 1988; Meixner et al. 2013;). There is also a Y lineage in Ethiopia (Franck et al. 2001)  
108 and a Z lineage in Lybia (Alburaki et al. 2013).

109

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

120 As part of the effort to conserve native bee diversity, there is a movement to  
121 protect the dark European honey bee, *A. m. mellifera* (Meixner et al. 2010, 2013). The  
122 range of this subspecies has been much reduced (see Meixner et al. 2010) and for the  
123 purpose of its conservation, the *Societas Internationalis pro Conservazione Apis*  
124 *melliferae melliferae* was established in 1995 (Pinto et al. 2014). Dark European honey  
125 bees can occur in ecotypes with distinct colony population cycles (Louveaux 1966,  
126 cited in Strange et al. 2007) that still persist today (Strange et al. 2007). Genetic  
127 methods can identify these local varieties, although specific ecotypes within these  
128 varieties may not be clearly delineated (Strange et al. 2008; Soland-Reckeweg et al.  
129 2009). In general, local-origin colonies have been shown to have longer colony  
130 survivorship than non-local colonies (Büchler et al. 2014).

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

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

158 Previous studies have examined rates of introgression in *A. m. mellifera*.  
159 Soland-Reckeweg et al. (2009) quantified introgression and hybridization between M  
160 and C lineages of honey bees in Switzerland. Considerable hybridization was  
161 observed, even in colonies managed for pure breeding by apiculturalists interested in  
162 conservation (Soland-Reckeweg et al. 2009). Pinto et al. (2014) examined the integrity  
163 of protected populations using single nucleotide polymorphisms (SNPs) and mtDNA.  
164 Despite their protection, introgression was detected in these populations, although  
165 introgression was higher in unprotected than protected colonies (Pinto et al. 2014).  
166 Honey bees from England and Scotland were included in this analysis. Jensen et al.  
167 (2005) also included English and Scottish samples in their earlier analysis of  
168 introgression in north-west European populations of *A. m. mellifera*. Microsatellite data  
169 and *Dral* tests revealed varying levels of introgression, but also demonstrated the  
170 persistence of this subspecies in northwestern Europe. More recently, Parejo et al.  
171 (2016) examined introgression in Swiss and French populations of *A. m. mellifera*  
172 using whole genome sequence information and were able to detect admixture as well  
173 as population structuring by subspecies and geographic origin.

174 Here, local populations of *A. m. mellifera* from Cornwall in the South-West of  
175 the UK are examined. As mentioned above, subspecies of honey bee, including the  
176 dark European honey bee, may show evidence of local adaptation (Louveaux 1966;  
177 Strange et al. 2007). Populations of dark European honey bee (*A. m. mellifera*) are  
178 likely to have been native to the UK for at least 4000 years (Carreck 2008) and occur  
179 in the South-West of the UK, but have been neglected in previous studies, which have  
180 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.

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199

200

## 201 **Materials and Methods**

202

### 203 ***Sampling***

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

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

233

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 amplification 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).

248

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

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

261

262 After genotyping, MICROCHECKER (van Oosterhout et al. 2004) was used to  
263 investigate the presence of null alleles and other common sources of genotyping error  
264 (e.g. stutter). Samples were grouped by population in this analysis. Estimates of  
265 linkage disequilibrium and departures from Hardy-Weinberg equilibrium were made in  
266 Arlequin 3.5 (Excoffier and Lischer 2010). Again, samples were grouped by population  
267 for these analyses. For pairwise tests of linkage disequilibrium the number of  
268 permutations was 10,000. The selected significance level was  $P=0.05$ , but strict  
269 Bonferroni corrections were applied to pairwise tests by population, thus the revised  
270 level of significance was  $P = 0.0008$  (there were 66 tests per population). For exact  
271 tests of Hardy-Weinberg equilibrium the number of steps in the Markov chain was  
272 1,000,000 and the number of dememorization steps was 100,000. Strict Bonferroni  
273 corrections were again applied to tests done by population (adjusted  $P$  varied as some  
274 loci were monomorphic in some populations, minimum adjusted  $P = 0.004$ ). Some loci  
275 were removed after these steps, prior to downstream analyses (see Results).  
276 Standard measures of genetic diversity were estimated in Arlequin 3.5 (observed and  
277 expected heterozygosity; Excoffier and Lischer 2010) and FSTAT (allelic richness,  
278 Goudet 2001). Allelic richness was calculated across loci per population and was  
279 based on a minimum sample size of ten individuals.

280

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

283 al. 2000) was used. A burn-in period of 50,000 steps was used followed by 500,000  
284 MCMC steps.  $K$  values of 1 to 12 were tested, with three iterations of each  $K$  value. A  
285 correlated allele model (Falush et al. 2003) was applied. The admixture model was  
286 used, but LOCPRIOR was not applied (LOCPRIOR is usually used where the  
287 expected signal is too weak for standard structure models and makes use of location  
288 information with each individual to assist clustering, Pritchard et al. 2010). Iterations  
289 were examined for consistency (by examining similarity of alpha values and 'ln Prob.  
290 of data' across the iterations). The best  $K$  value was investigated using the original  
291 method recommended in STRUCTURE and using the Evanno et al. (2005) method.  
292 The standard method infers the most probable value of  $K$  based on the 'log probability  
293 of the data' (or where this value begins to reach a plateau). The Evanno et al. (2005)  
294 method is based on the rate of change in values of 'log probability of data' for  
295 successive values of  $K$ . *Structure Harvester* was used to generate the relevant plots  
296 for inference of  $K$  (Earl 2012). Although these methods can be used to estimate the  
297 'best'  $K$  value, multiple  $K$  values were interpreted as this is recommended (Janes et al.  
298 2017). Barplots were produced using the online application STRUCTURE PLOT  
299 (Ramasamy et al. 2014). The degree of introgression of sampled colonies was  
300 investigated through inspection of mean  $Q$  values and their standard deviations (from  
301 the three iterations of the analysis) for  $K = 3$  (further explanation in the Results). A  
302 population cluster which included the Cornish honey bee samples and the other *A. m.*  
303 *mellifera* samples was then investigated separately. Analysis parameters and steps  
304 were conducted as described above. Finally, the relationship between degree of  
305 admixture and observed heterozygosity and allelic richness were tested at the  
306 population level. Mean coefficients of admixture (i.e. mean  $Q$  value across individuals)  
307 were calculated for membership to the 'dominant' cluster for each population.

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

312

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

330

331 ***Assignment of mtDNA haplotypes***

332 The COI-COII region was sequenced using the primers E2 (GGC AGA ATA AGT GCA  
333 TTG) and H2 (CAA TAT CAT TGA TGA CC) (Garnery et al. 1993). The following  
334 cycling protocol on a TC-412 Programmable Thermal Controller (Techne) was used:  
335 35 cycles with 94°C for 60 s, 54°C for 45 s, and 62°C for 120 s. Before the first cycle,  
336 a prolonged denaturation step (95°C for 15 min) was included and the last cycle was  
337 followed by a 10 min extension at 72°C. Sanger sequencing was then conducted  
338 (Sanger et al. 1977) using fluorescent dyes (Ansorge et al. 1987; Middendorf et al.  
339 1992), specialized DNA polymerases (Taq-polymerase; Carothers et al. 1989) and  
340 modified nucleotides to avoid problems with DNA secondary structure (Frederick  
341 1999). Capillary electrophoresis was performed on an ABI3730 using Dye Chemistry  
342 Software Data Collection Version 3; Sequencing Analysis 5.2 (Applied Biosystems).  
343 Sequences were aligned using ClustalW (Thompson 1994) in Bioedit (Hall 2004).  
344 Mitochondrial haplotypes were identified on the basis of the presence of P and Q  
345 repeats (Cornuet et al. 1991). *A. m. mellifera* and *A. m. iberiensis* are in the M lineage  
346 and are indicated by the presence of a P repeat and one or more Q repeats (Cornuet  
347 et al. 1991, Achou et al. 2015) although *A. m. iberiensis* can also have A haplotypes  
348 and present two types of P sequence (P0 and P). The common C lineage commercial  
349 subspecies, *A. m. ligustica* and *A. m. carnica* lack a P repeat and have only a single  
350 Q repeat (Cornuet et al. 1991).

351

352

## 353 **Results**

### 354 ***Quality control***

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

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

367

### 368 ***Investigation of admixture***

369 STRUCTURE analysis of all populations showed  $K = 3$  and  $5$  as numbers of clusters  
370 likely to be useful to describe the population structure (one should be careful with  
371 interpreting the 'correct'  $K$  (Pritchard et al. 2000; Janes et al. 2017) hence results for  
372 both are presented; see supplementary data figures 1 and 2).  $K = 3$  clearly delineates  
373 all three subspecies, with admixture shown for the Cornish population (Figure 1a).  $K$   
374 =  $5$  again separates *A. m. ligustica* from the other species; *A. m. carnica* show  
375 membership to two clusters and *A. m. mellifera* again show a separate signal for the  
376 Cornish sample in comparison with other populations of this subspecies (Figure 1b).

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



381           There was a significant negative relationship between mean Q-value and mean  
382 observed heterozygosity at the population level ( $\rho = -0.68$ ,  $n = 12$ ,  $P < 0.05$ ; Figure  
383 3a). There was also a significant negative relationship between mean Q-value and  
384 mean allelic richness (corrected for sample size) at the population level ( $\rho = -0.62$ ,  
385  $n=12$ ,  $P < 0.05$ ; Figure 3b)

386

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

401

#### 402 ***Admixture and mtDNA haplotype assignment***

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

406 Interpretation is considered for Q threshold values of 80%, 90%, 95% and 99%; i.e for  
407 a threshold of 0.99 an individual has to meet or exceed this value to be deemed 'pure'  
408 (Table 4, see discussion). When lower values of Q-threshold are specified to indicate  
409 a 'pure' bee, agreement between nuclear and mtDNA signal improves (i.e. more of the  
410 M lineage samples are deemed to be 'pure' *A. m. mellifera* samples). Applying the  
411 strictest threshold to deem a queen as 'pure' reveals only four individuals to be so and  
412 also have an M haplotype (Table 4). Applying the lowest threshold, fifteen bees are  
413 deemed to be pure on the basis of agreement between nDNA and mtDNA data (Table  
414 4). All mtDNA sequences are available in GenBank (accession numbers MF197320-  
415 197363).

416

## 417 **Discussion**

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

431 where Brother Adam developed the hybrid line that became known as “the Buckfast  
432 bee<sup>TM</sup>”. Imports of honey bee into the UK increased in the period 2013-2016 (Learner  
433 2017) and current advice to beekeepers from the National Bee Unit is that importing  
434 bees ‘is neither difficult nor a chore’ (Learner 2017). Previous studies examining *A. m.*  
435 *mellifera* have shown that there is admixture in unprotected English populations and  
436 that English samples showed both M and C lineages (Jensen et al. 2005; Pinto et al.  
437 2014). Scottish samples from protected areas showed only M lineages (Jensen et al.  
438 2005; Pinto et al. 2014). Elsewhere in Europe, and for other subspecies, hybrids have  
439 been found in populations of dark bees in Poland (Oleksa et al. 2011), admixed  
440 ancestry is reported in Serbian bees between *A. m. carnica* and *A. m. macedonica*  
441 (Nedić et al. 2014), but there are also places where lineages are relatively pure, e.g.  
442 *A. m. mellifera* in parts of the Urals and Volga region (Ilyasov et al. 2016) and *A. m.*  
443 *carnica* in Hungary (Péntek-Zakar et al. 2015). Clearly, transhumance of colonies  
444 frequently leads to introgression, but there are also places where *A. m. mellifera*  
445 remains relatively intact (Byatt et al. 2015)

446

447 Regarding the identification of relatively pure hives for conservation efforts in South-  
448 West England, the power in the dataset to effectively detect hybrids and the effect of  
449 threshold values on the designation of ‘pure’ individuals needs to be considered. Vähä  
450 and Primmer (2006) investigated the use of STRUCTURE and NEWHYBRIDS to do  
451 so and show that the number of loci for efficient and accurate determination of hybrids  
452 depends on the amount of genetic differentiation between the parental populations.  
453  $F_{ST}$  values between the subspecies studied here are quite large (e.g. in the range 0.3-  
454 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  
455 loci used here, data not shown), so the use of relatively few loci will still be suitable for

456 identification of hybrids. However, it should be remembered that here, we are not  
457 detecting hybrid individuals between two parent lines where  $K=2$ , but rather trying to  
458 identify the degree of admixture from populations with a long history of crosses and  
459 back-crosses, where the useful number of clusters to describe the populations is  
460 probably from 3-5. Consequently, caution should be drawn when considering the  
461 relative purity of individuals using the approach described here. Vähä and Primmer  
462 (2006) showed that a stricter Q-value reduced the misclassification of back-crossed  
463 individuals as purebred individuals in their simulations. These authors note that as Q-  
464 value thresholds are increased there is a trade-off between the efficiency of detecting  
465 hybrids (proportion of individuals in a group correctly identified as hybrids) and the  
466 accuracy (proportion of an identified group truly belonging in that category). More  
467 stringent thresholds improve the accuracy of identifying hybrids, but decrease the  
468 efficiency (Vähä and Primmer 2006). Essentially, what needs to be decided is whether  
469 accurate hybrid identification (all the individuals in the hybrid group are hybrids, but  
470 some of the individuals in the purebred group are hybrids) or accurate purebred  
471 identification (all the individuals in the purebred group are purebred, but some of the  
472 individuals in the hybrid group are purebreds) is required. To conserve dark European  
473 honey bees, the purity of the dark European honey bee stock is of course the most  
474 desired outcome. However, to be certain of purity, the founding stock will be small  
475 (Table 4). Only four hives sampled showed an M lineage and a Q-value of >99% to *A.*  
476 *m. mellifera* cluster when  $K=3$ . This value increases to 12 for a Q-value of 0.9. Further  
477 work investigating the phenotypic traits of Cornish bees for hives of differing admixed  
478 ancestry will help elucidate what is a useful threshold Q-value. We also note here that  
479 samples are limited and only eight microsatellites are analysed so the results should  
480 be interpreted with caution.

481

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

500

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

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

518

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

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

545

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

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

557

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

567

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571

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913 **Figure legends**

914

915 **Fig. 1**

916 Group membership to clusters identified using STRUCTURE with inference based  
917 on all populations (*A. m. ligustica* = 1 Italy, *A. m. carnica* = 2 Austria, 3 Austria  
918 Würm, 4 Slovenia, 11 Austria 2015, *A. m. mellifera* = 5 Sweden, 6 France, 7 Norway,  
919 8 Switzerland Glarus, 9 Switzerland Schistal, 10 Ireland, 12 Cornwall), **(a)** K = 3, **(b)**  
920 K = 5. (Note that the coloured bar at the bottom of the figure illustrates the population  
921 of origin only)

922

923 **Fig. 2** Group membership to clusters identified using STRUCTURE with inference  
924 based on *A. m. mellifera* clusters only (numbering of populations is retained as in Fig  
925 2 for comparison), **(a)** K = 2 **(b)** K = 3. (Note that the coloured bar at the bottom of  
926 the figure illustrates the population of origin only)

927

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

931

932 **Fig. 4** Discriminant analysis of principal components for all populations sampled,  
933 apart from *A. m. ligustica* (Italy; see text). Group four represents the putative dark  
934 European honey bees from Cornwall. Clusters 2, 3 and 5 mostly consist of *A. m.*  
935 *carnica* individuals. Cluster one represents continental European populations of *A.*  
936 *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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947 **Tables**948 **Table 1** Genetic diversity of population samples included in the study. Populations949 for each subspecies are: *A. m. ligustica* – Italy; *A. m. carnica* – Austrian and950 Slovenian samples; *A. m. mellifera* all other samples

951

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

952

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 <sup>c</sup>	3	7	17	1	16
Austria Wurm <sup>c</sup>	0	29	2	0	5
Austria 2015 <sup>c</sup>	0	12	43	0	44
Slovenia <sup>c</sup>	0	1	15	0	5
Sweden <sup>m</sup>	10	0	0	0	0
France <sup>m</sup>	24	0	0	0	0
Norway <sup>m</sup>	18	0	0	0	0
Switzerland Glarus <sup>m</sup>	9	0	0	1	0
Switzerland Schistal <sup>m</sup>	12	0	0	0	0
Ireland <sup>m</sup>	22	0	0	0	0
Cornwall <sup>m</sup>	0	1	0	39	3

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961

**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.

Individual	<i>Apis mellifera mellifera</i>		<i>Apis mellifera ligustica</i>		<i>Apis mellifera carnica</i>		mtDNA haplotype
	Q	s.d	Q	s.d.	Q	s.d.	
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

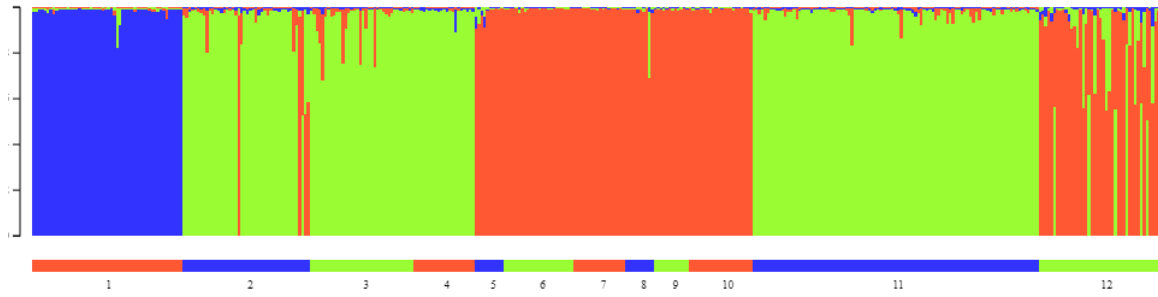
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**Table 4** Assignment of mtDNA haplotypes (M or C lineage) and nuclear introgression considered together. Individuals are assigned as ‘pure’ based on differing Q-values of >99%, >95%, >0.90% and >0.80% for  $K = 3$ .

Q- threshold	nDNA	mtDNA		Not sequenced
		M	C	
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)



(b)

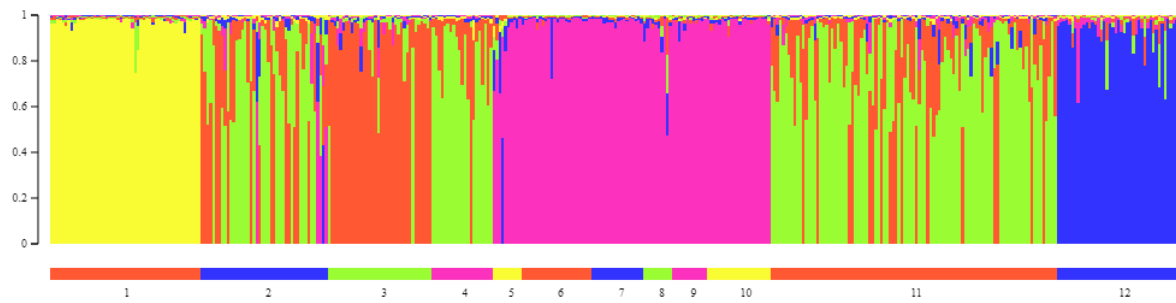
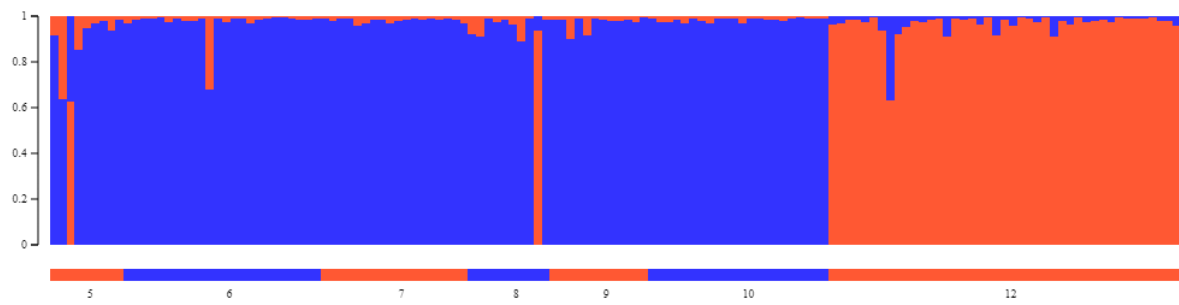
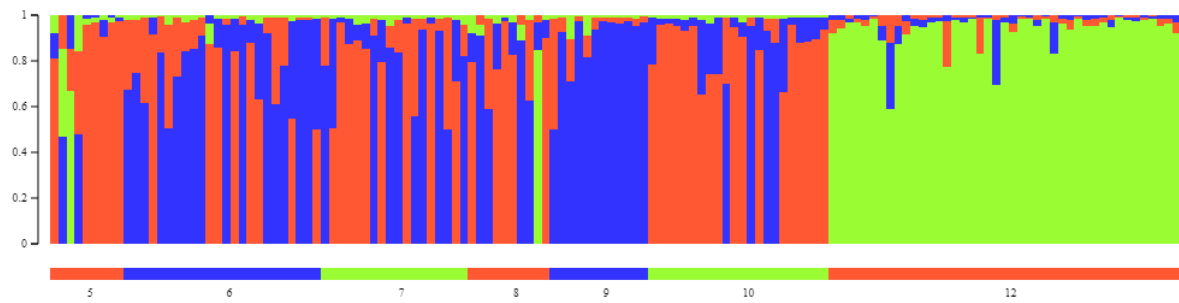


Fig 2

(a)



(b)



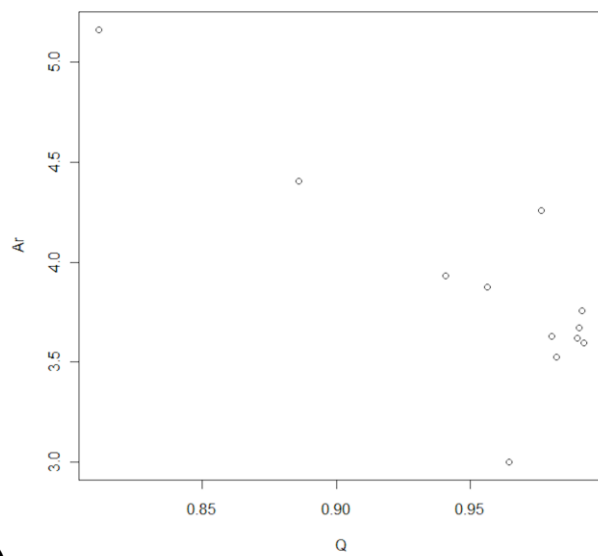
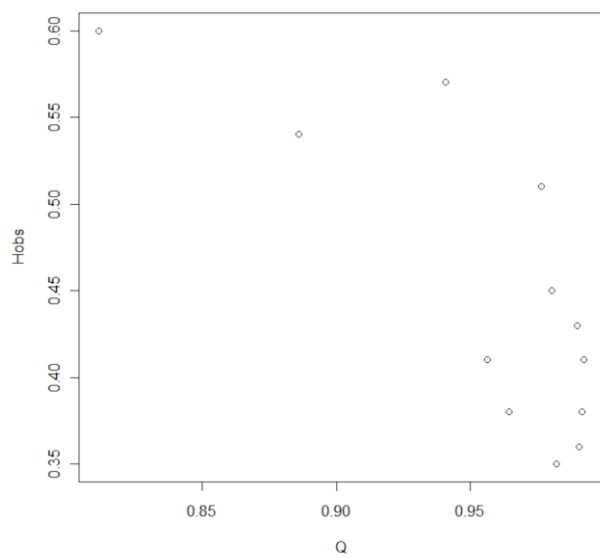
**Fig 3**  
**(a)****(b)**



Fig 4

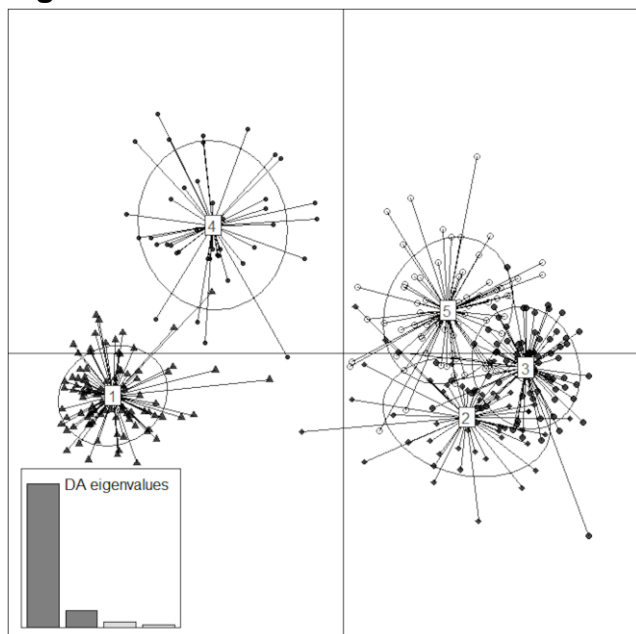


Fig 5

