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Short title: Haploid drone sequence for population and genome analysis

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Abstract

Honey bee subspecies originate from specific geographic areas in Africa, Europe and the Middle East. The interest of beekeepers in specific phenotypes has led them to import subspecies to regions outside of their original range. The resulting admixture complicates population genetics analyses and population stratification can be a major problem for association studies. As a typical example, the case of the French population is studied here. We sequenced 870 haploid drones for SNP detection and identified nine genetic backgrounds in 629 samples. Five correspond to subspecies, two to isolated populations and two to human-mediated population management. We also highlight several large haplotype blocks, some of which coincide with the position of centromeres. The largest is 3.6 Mb long on chromosome 11, representing 1.6 % of the genome and has two major haplotypes, corresponding to the two dominant genetic backgrounds identified.

Introduction

The honey bee *Apis mellifera* comprises more than 30 subspecies, each of which defined according to morphological, behavioural, physiological and ecological characteristics suited to their local habitat [1–4]. European subspecies broadly group into two evolutionary lineages representing on one side western and northern Europe (M lineage), and on the other eastern and southern Europe (C lineage) [1]. The two European M lineage subspecies are the Dark European or 'black' honey bee *A. m. mellifera* and the Iberian honey bee *A. m. iberiensis*, while the C lineage subspecies include amongst others, the Italian honey bee *A. m. ligustica* and the Carniolan honey bee *A. m. carnica* [4]. Prior to the involvement of apiarists, the Alps are thought to have presented a natural barrier between *A. m. mellifera* to the north, *A. m. carnica* to the southeast, and *A. m. ligustica* to the southwest [5]. Before the turn of the 19th century, French honey bee populations were solely represented by the native *A. m. mellifera*, for which regional ecotypes have previously been described [6,7]. However, during the 20th century much interest arose amongst apiarists in developing hybrids between the endemic *A. m.*

mellifera and other subspecies including *A. m. ligustica*, *A. m. carnica* and the Caucasian *A. m. caucasia* from Georgia [1,8,9]. Apiarists found the hybrids to perform better with regards to the production of honey and royal jelly than the native *A. m. mellifera*, spurring further interest in these subspecies which were also reported to be more docile and easier to manage [1]. *A. m. ligustica* is a very popular subspecies worldwide amongst apiarists because of its adaptability to a wide range of climatic conditions, its ability to store large quantities of honey without swarming, and its docile nature if disturbed [10]. *A. m. ligustica* queens are also frequently exported worldwide, and most of the honey bees imported during the last centuries into the New World were also of Italian origin [10,11]. Apiculture involving *A. m. carnica*, is also very popular among apiarists [12]. *A. m. carnica* became increasingly popular for further selection throughout central and western Europe [13,14] on account of their calm temperament and higher honey yield compared to *A. m. mellifera* [1], to the point where *A. carnica* almost replaced entirely *A.m. mellifera* in Germany [15]. *A. m. caucasia* is a subspecies that was also imported to France, to generate *A. m. ligustica* x *A. m. caucasia* hybrids, that were themselves crossed naturally to the *A. m. mellifera* present in the local environment. Another popular hybrid used in apiculture is the so-called Buckfast, created and bred by Brother Adam of Buckfast Abbey in England [16]. Following the extensive imports of queens from "exotic" subspecies, the genetic makeup of honey bee populations in France became complex, and the genetic pollution of local populations followed with clear phenotypic consequences such as changes in the colour of the cuticle [17]. The increasing admixture of divergent honey bee subspecies has fostered conservationists to protect the native genetic diversity of regional ecotypes, such as *A. m. iberiensis* in Spain and Portugal, *A. m. ligustica* and *A. m. siciliana* in Italy (Fontana et al., 2018), and *A. m. mellifera* in France, Scotland and Switzerland amongst other places [18–22]. As a result of the different breeding practices, the necessity for a study targeted towards *A. m. mellifera* conservatories and French bee breeders specialized in rearing and selling queens arose and in this context the genomic diversity project "SeqApiPop" emerged. Within this project, samples from French conservatories, from

97 individual French breeders and breeder organisations were analysed, including Buckfast samples.
98 Traditionally, such wide diversity studies have been performed using a small number of molecular
99 markers such as microsatellites [23] or limited sets of single-nucleotide polymorphisms (SNPs) [24–
100 27], enabling population stratification, introgression and admixture levels to be characterized.
101 However, to understand complex population admixture events, as has happened for the managed
102 honey bee populations in France and elsewhere, or to identify signatures of natural [25,28–30] or
103 artificial [31,32] selection in the genome, a much higher density of markers is required. As no high-
104 density SNP chip was available for honey bee at the onset of the project, and as the honey bee genome
105 is very small compared to most animal genomes, being only 226.5 Mb long [33], we employed a
106 whole-genome sequencing approach [28,34]. Although the sequencing of honey bee workers has
107 proved successful for detecting selection signatures or admixture events [28,34–36], analysing haploid
108 drones allows to sequence at a lower depth and with greater accuracy in variant detection [20,29,31].
109 An additional advantage of sequencing haploids is that the alleles are phased, which is invaluable for
110 studies investigating genome dynamics such as recombination hotspots and haplotype structure.
111 Although some insights into recombination patterns in the honey bee have been made through the
112 analysis of drones from individual colonies [37,38] and linkage disequilibrium (LD)-based approaches
113 [39,40], a deep understanding of the recombination landscape, essential for fine-scale genetic analyses,
114 requires hundreds of phased genomes. Such 'HapMap' projects have been conducted in humans and
115 cattle, initially using SNP arrays [41,42] and more recently by whole-genome sequencing as in the
116 "1000 genome" projects [43,44].
117 Therefore, as a first step towards a deep understanding of French and Western European managed
118 honey bee populations and of their genome dynamics, we undertook the sequencing of a large dataset
119 of haploid drones. This data comprised samples from French conservatories and commercial breeders
120 in addition to samples from several European countries each representing potentially pure *A. m.*
121 *ligustica*, *A. m. carnica, A. m. mellifera* and *A. m. caucasia* populations typically imported by French

122 breeders. Finally, *A. m. iberiensis*, the Iberian subspecies only separated from the native French *A. m.*
123 *mellifera* by the natural barrier of the Pyrenees was also studied. In total, 870 samples were sequenced
124 for SNP detection and 629 were used for a detailed genetic analysis of present-day honey bee
125 populations in France.
126
127 **Methods**
128 *Sampling and sequencing*
129 For the population genomics analyses, one individual drone per colony was sampled before
130 emergence, from colonies throughout France, Spain, Germany, Switzerland, Italy, the UK, Slovenia,
131 Poland, Denmark, China and from a French beekeeper having imported queens from Georgia,
132 amounting to a total of 642 samples (Supplementary figure 1). To improve the robustness of the
133 primary SNP detection and filtering steps, a further 30 "duplicate" samples were collected from
134 colonies already samples for this study, in addition to 198 samples of similar genetic backgrounds
135 from two other ongoing projects. Thus, although 642 colonies were included for population genomics
136 analyses, in total 870 samples were used for SNP detection (supplementary table 1).
137 DNA was extracted from the thorax of adult bees or from pupae as described in Wragg *et al*. (2016).
138 Briefly, drones were sampled at either the pupae/nymph or larval stage and stored in absolute ethanol
139 at -20°C. DNA was extracted from the thorax or from diced whole larvae. Tissue fragments were first
140 incubated 3 hours at 56° in 1 mL of a solution containing 4 M urea, 10 mM Tris-HCl pH 8, 300 mM
141 NaCl, 1% SDS, 10 mM EDTA and 0.25 mg proteinase K, after which 0.25 mg proteinase K was
142 added for an incubation over-night at 37°C. Four hundred µL of a saturated NaCl solution was added
143 to the incubation, which was then gently mixed and centrifuged for 30 minutes at 15000 g. The
144 supernatant was treated for 5 minutes at room temperature with RNAse (Qiagen) and then centrifuged
145 again, after which the DNA in the supernatant was precipitated with absolute ethanol and re-suspended
146 in 100 µL TE 10/0.1. Pair-end sequencing was performed on Illumina™ HiSeq 2000, 2500 and 3000

147 sequencing machines with 20 samples per lane, or on a NovaSeq machine with 96 samples per lane,
148 following the manufacturer's protocols for library reparations.
149
150 *Mapping and genotype calling*
151 Sequencing reads were mapped to the reference genome Amel_HAv3.1 [33] using BWA-MEM
152 (v0.7.15) [45], and duplicates marked with Picard (v2.18.2;) (http://broadinstitute.github.io/picard/).
153 Libraries that were sequenced in multiple runs were merged with Samtools (v1.8) merge [46] prior to
154 marking duplicates. Local realignment and base quality score recalibration (BQSR) were performed
155 using GATK (v4.1.2.) [47], using single-nucleotide polymorphisms (SNPs) called with GATK
156 HaplotypeCaller as covariates for BQSR. Each drone was processed with the pipeline independently,
157 and genotyped independently with HaplotypeCaller. Although the drones sequenced are haploid,
158 variant calling was performed using a diploid model to allow the detection and removal of SNPs that
159 called heterozygous genotypes in > 1% of samples, which might have arisen for example as a result of
160 short-tandem repeats repeats (STRs) and could highlight copy number variants (CNVs) on the
161 genome. Individual gVCF files were combined with CombineGVCFs, and then jointly genotyped with
162 GenotypeGVCFs, resulting in a single VCF file for the 870 samples containing 14.990.574 raw
163 variants. After removing Indels with GATK SelectVariants, 10.601.454 SNPs remained. Sequencing
164 depth was estimated using Mosdepth [48]. Further details are given in supplementary file
165 SeqApiPop_1_MappingCalling.pdf.
166
167 *Quality filters on SNPs*
168 The first run of filters concerns technical issues related to the sequencing and alignment steps and
169 were therefore used for the total dataset of 870 samples, to benefit from its larger size for SNP
170 detection and validation (supplementary figure 2). These filters included (i) strand biases and mapping
171 quality metrics (SOR ≥ 3; FS ≤ 60 and MQ ≥ 40), (ii) genotyping quality metrics (QUAL > 200 and

QD < 20) and (iii) individual SNP genotyping metrics (heterozygote calls < 1%; missing genotypes < 5%, allele number < 4 and genotypes having individual GQ < 10 < 20%). Distribution and ECDF plots of values for all the filters used on the dataset were used to select thresholds and are shown in supplementary file SeqApiPop_2_VcfCleanup.pdf.

Haplotype block detection, LD pruning, PCA, Admixture, Treemix, RFMix

Haplotype blocks were detected with Plink (v1.9) [49] using the blocks function, "--blocks no-pheno-req no-small-max-span", with the parameter "--blocks-max-kb 5000". LD pruning was performed with Plink using the indep-pairwise function. Principal component analyses were performed with Plink and the contribution of individual SNPs to the principal components were estimated using smartpca from the eigensoft package v7.2.1. Further details are given in supplementary file SeqApiPop_3_LDfilterAndPCAs.pdf. Admixture analysis was performed with the program Admixture v 1.3.0 [50], with values of K ranging from 2 to 16. Fifty runs were performed each time using a unique random seed. The Pong software [51] was used for aligning runs with different K values and for grouping results from runs into clustering modes, setting the similarity threshold to -s = 0.98. Further details are given in supplementary file SeqApiPop_4_Admixture.pdf. Population migration analysis was performed with TreeMix [52], with the option for grouping SNPs set to -k=500, testing between 0 and 9 migrations and performing 100 runs per migration with a unique random seed. The optimum number of migrations was estimated with the R package OptM (Fitak, R. R.: https://github.com/cran/OptM) using the Evanno method provided [53]. Tree summaries for the 100 runs per migration tested were performed with DendroPy [54] and drawn with FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Further details are given in supplementary file SeqApiPop_5_TreeMix.pdf. Local ancestry inference and positioning of haplotype switches were performed with RFMix v2.03-r0 [55]. Three main genetic backgrounds were considered for this analysis, corresponding to the three major groups highlighted in the PCA analysis.

Reference samples were selected as having > 95 % pure background. Although most diploid data was removed and data is already phased, shapeit.v2.904 [56] was run to format the vcf files for RFMix. RFMix was run using genetic maps generated from the data of Liu et al. [37]. Briefly, reads from the project SRP043350 were retrieved from Short Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra), aligned to the reference genome for SNP detection and recombinants were detected with the custom script find_crossing_overs.py to produce a genetic map. Further details on the RFMix analysis are given in supplementary file SeqApiPop_6_RFMix.pdf.

Results

Sequencing and genotyping

Sequencing of the honey bee drones for the SeqApiPop diversity project began in 2014 on Illumina HiSeq instruments and some of the first samples had such low coverage that a second run (or even three in the case of OUE8) sequencing was performed. For these samples, the resulting BAM files were merged prior to variant calling. Only four samples of the diversity project were sequenced on Novaseq instruments, for which higher sequencing depths were achieved. Therefore, to improve the robustness of the SNP detection pipeline, we included drone genome sequences from other ongoing subsequent projects using the same genetic types, that were produced with Novaseq instruments. Samples sequenced with the HiSeq and NovaSeq instruments had mean sequencing depths of 12.5 ± 6.1 and 33.5 ± 10.2 respectively (Supplementary Table ST1, Supplementary figures 3 and 4). Genotyping the whole dataset of 870 drones with the GATK pipeline allowed the detection of 10,601,454 raw SNPs (supplementary figure 2). Results of the subsequent filtering steps are shown in the Venn diagrams in supplementary figures 5, 6 and 7. A total of 7,023,976 high-quality SNPs remained after filtering. The 198 samples from the other projects and 30 within-colony duplicate samples from the present diversity project were removed from the dataset for downstream analyses. Although a filter on genotyping rate ≥ 95% was applied in the primary filtering steps, the final filter on

heterozygote calls was set to keep SNPs with up to 1% of heterozygote samples, and these remaining heterozygous genotypes were set to missing (supplementary figure 2). After this, a final filter on missing data in samples was applied and 15 samples were removed due to the fraction of missing genotypes exceeding 10 %. The final diversity dataset comprised 629 drones (supplementary table 1) and 7,012,891 SNPs, and was used for all subsequent analyses unless stated otherwise.

Contribution of SNPs to the variance in PCAs: detection of large haplotype blocks

Principal component analysis was performed on the 629 samples and 7 million SNPs, results in a clear differentiation of three groups of samples. The first principal component, representing 10.8 % of the total variance, broadly differentiates M lineage bees, *A. m. mellifera* and *A. m. iberiensis*, from the *A. m. ligustica*, *A. m. carnica* and *A. m. caucasia* bees. The second principal component, representing 3.1 % of the variance, separates the O lineage *A. m. caucasia* bees from the C lineage *A. m. ligustica* and *A. m. carnica* bees (supplementary figure 8). PC3 represents 1.2 % of the variance and the remaining principal components each represent 0.7 % or less. When looking at the individual contributions of SNPs to the variance, we can see that only a very small proportion of the ~7 million markers contribute significantly to PC1 (red lines on supplementary figure 9) and that this proportion is even much smaller for PCs 2 and 3. Two reasons for such a limited contribution to the variance of the majority of markers is the low informativity of markers of low minor allele frequency (MAF) and the redundancy of markers that are in strong linkage disequilibrium (LD). Therefore, to thin the dataset, we tested the effect of several MAF filters and chose the most pertinent one for subsequent testing of various LD pruning values. The effects of these filters were estimated by inspecting the contributions of the SNPs to the principal components. The MAF filters tested showed clearly that datasets containing only SNPs with MAF > 0.01 or MAF > 0.05 are sufficient to allow a higher proportion of markers contributing to the PCs, with a notable increase of SNPs contributing to PC2 and PC3 (supplementary figure 9). To avoid losing too many potential population-specific markers

247 present at low frequency in the data, we chose to use the lowest MAF threshold tested, leaving a
248 dataset of 3,285,296 SNPs having MAF > 0.01 for subsequent analyses. On inspecting the
249 contributions of individual SNPs to principal components along the genome, a striking feature we
250 observe is that for several large chromosomal regions, five of which being larger than 1 Mb, most
251 SNPs make a significant contribution, with the observed values being amongst the strongest observed
252 genome-wide (Supplementary Figures 10 and 11). Such observations suggest the existence of large
253 haplotype blocks driving differentiation along principal components, in particular principal component
254 1. To explore this further we compared these genomic regions to the haplotype blocks detected with
255 Plink (supplementary table 2) revealing significant overlap by visual inspection (Supplementary
256 Figure 12). The largest of these blocks spans 3.6 Mb on chromosome 11, which is close to 1.6 % of
257 the honey bee genome size, and four others on chromosomes 4, 7 and 9 are larger than 1 Mb (figure 1,
258 supplementary figures 10, 11, 12, supplementary table 2).
259
260 *LD filtering*
261 Population structure and admixture analyses rely largely on the assumption that markers along the
262 genome are independent. Indeed, markers in strong LD such as those in haplotype blocks, can
263 influence genetic structure. Therefore, we sought to investigate the impact of LD pruning on
264 population structure inference. The number of SNPs used in a window for LD pruning was determined
265 such that most windows would correspond to a physical size of 100 kb. To achieve this, we used the
266 mode of the distribution of the number of SNPs in 100 kb bins, which is 1749 for the dataset of
267 3,285,296 SNPs with MAF > 0.01 (supplementary figures 13 and 14). LD pruning was thus performed
268 with a window size of 1749 SNPs and 175 bp (10 %) overlap and various values were tested, spanning
269 between $0.1 \leq$ LD $r^2 \leq 0.9$. PCA following these various thresholds show that with LD $r^2 < 0.3$ the
270 global structure of the dataset is lost, with only one population (*A. m. iberiensis*) contributing strongly
271 to the variance (supplementary figure 15), whereas with LD $r^2 > 0.3$, the contributions to the variance

in PC1 is not so widely distributed (supplementary figure 16). The effect of LD pruning on the haplotype blocks is drastic, with the few SNPs retained having a distribution of their contributions to the variance in PC1 and PC2 similar to that of the rest of the genome (supplementary figure 17). After pruning for LD $r^2 < 0.3$, 601,945 SNPs were left in the dataset which were subsequently used in the analysis of population structure.

Analysis of population structure

The PCA revealed distinct population structure within the data. For instance, some populations from French breeding organisations, such as the Royal Jelly breeder organisation (GPGR: Groupement des Producteurs de Gelée Royale), and the Corsican breeder's organization (AOP Corse), appear quite homogenous (figure 2), with GPGR samples clustering close to the *A. m. ligustica* and *A. m. carnica* reference populations and, while AOP Corse samples appear as a distinct group between the C lineage *A. m. ligustica* and *A. m. carnica* on one side and the M lineage *A. m. mellifera* and *A. m. iberiensis* on the other. Other populations from French breeders appear much less homogenous, with individuals scattered across the whole graph, see for examples Tarn 2 on figure 2, suggesting various degrees of admixture between the three principal genetic groups (supplementary figure 18).

To further investigate the genetic structure and the effects of human-mediated breeding, we performed admixture analyses. Our dataset consists of reference samples from thirteen origins, including two islands, in addition to samples from several commercial breeders and conservatories. The genetic makeup is therefore expected to be complex and the first task was to estimate the optimal number of genetic backgrounds (K). We performed 50 independent runs with the Admixture software for each value of $2 \leq K \leq 16$ on the LD-pruned dataset, totaling 750 independent analyses. Cross-validation (CV) error estimates of the results computed by the software are shown in figure 3A. Results suggest that the most likely number of genetic backgrounds is 8 or 9, with K = 8 having runs with the lowest CV values overall, and K = 9 having the lowest median CV value over its 50 runs. The resulting Q

297 matrices were jointly analyzed using Pong [51], where for each value of K runs are grouped together
298 by similarity into modes and the mode containing the largest number of similar runs is defined as the
299 major mode. As Pong failed to find disjoint modes with the default similarity threshold of 0.97, we
300 increased the stringency of this value to 0.98 for our analyses. Naturally, for low values of K, such as
301 2 or 3, most of the Q matrices are very similar and the major modes contain most runs, if not all.
302 Typically, for K = 2, all 50 runs are in a single mode and for K = 3, the major mode contains 49 out of
303 all 50 runs and reflects the three main groups from the PCA analysis. Amongst the values of K having
304 the lowest CV values (figure 3A), K = 9 stands out as having a major mode containing 33 runs out of
305 50. While K = 8 had the lowest overall mean CV value, its major mode contained only 12 runs,
306 indicating K = 9 to be the better model (figure 3B and supplementary table 3). Interestingly, the
307 pattern observed when considering only K = 3 genetic backgrounds, recapitulates the general pattern
308 observed in the PCAs, in which the reference populations separate into three groups. These groups
309 reflect the main evolutionary lineages present in the dataset, being the M lineage (*A. m. mellifera* and
310 *A. m. iberiensis*), C lineage (*A. m. ligustica*, *A. m. carnica*), and O lineage (*A. m. caucasia*). For K = 2,
311 these *A. m. caucasia* bees are considered as having the same genetic background as the *A. m. ligustica*
312 and *A. m. carnica* samples, also reflecting the results from the PCA (figure 2, supplementary figure
313 19). Our results support the assumption that *A. m. caucasia* bees are assigned to the O lineage by
314 morphometry 22/10/2021 17:15:00 and to the C lineage by mtDNA [57]. Some admixture can be
315 observed for a small proportion of the reference samples. For instance, the reference samples from the
316 Savoy conservatory appear to have a small proportion of genetic background from *A. m. ligustica*
317 and/or *A. m. carnica,* which is consistent with the PCA results (figure 2). Likewise, the *A. m. carnica*
318 samples from Poland have a small proportion of genetic background from *A. m. caucasia*. Finally, the
319 *A. m. carnica* from Switzerland show some proportion of *A. m. mellifera* genetic background.
320 When examining the admixture pattern representing the 33 runs at K = 9 genetic backgrounds, the
321 three main groups are now separated. The M lineage group from the K = 3 backgrounds is now

322 composed of four genetic backgrounds: *A. m. iberiensis* is now separated from *A. m. mellifera* and the
323 *A. m. mellifera* bees are separated in three groups from mainland France, and the two islands of
324 Ouessant and Colonsay. The other three subspecies *A. m. ligustica*, *A. m. carnica* and *A. m. caucasia*
325 each have their own genetic background. An eighth background corresponds to the samples from the
326 bees selected for the production of royal jelly and a ninth appears in the two populations that were
327 noted as Buckfast bees. Although it is a major background in these two populations, a majority of
328 samples have also a large proportion of *A. m. carnica* and, to a lesser extent, of *A. m. ligustica*
329 backgrounds. This ninth background can also be found in other breeder's populations principally in
330 Hérault and Tarn1 (figure 3C). Apart from the royal jelly population, all honey bees from breeders
331 show high levels of admixture. Moreover, there is a great variability in the genetic origins and
332 proportions of backgrounds, even for samples coming from a same location (figure 4). The exception
333 is the population from Corsica, for which all samples show proportions close to 75% - 25% of *A. m.*
334 *mellifera* and *A. m. ligustica* backgrounds respectively.
335
336 *Migrations between populations*
337 Due to the commercial interest expressed by beekeepers for the Buckfast bees and the peculiar genetic
338 composition observed in the Corsican population, we performed a population migration analysis with
339 TreeMix [52]. All samples having more than 80% ancestry from one of the 9 backgrounds detected in
340 the Admixture analysis were selected from one of the K = 9 major mode Q-matrices (supplementary
341 table 4), and the list supplemented with the 43 Corsican samples, making our data set composed of ten
342 representative groups for the European populations.
343 Estimations on the number of migrations (m) between the populations in the dataset, based on the
344 Evanno method [53], return a mode of m = 1, strongly suggesting a single migration, and a relatively
345 high Δm value for m = 2 supports the existence of a second migration. The Δm values for 3 or more
346 migrations are close to zero, suggesting that more than 2 migrations between populations in the dataset

is unlikely (figure 5A). For m = 1 the 100 TreeMix runs indicated a migration from *A. m. ligustica* to the Corsican population. For m = 2 the 100 TreeMix runs show the two migrations as being from *A. m. ligustica* to the Corsica population, and from *A. m. caucasia* to the Buckfast bees (figure 5B). Summaries of the resulting trees with DendroPy [54] are shown in figure 5C, indicating that when the two migrations are taken into account, the Corsican samples are grouped with the *A. m. mellifera* M lineage bees, and the Buckfast bees group with the *A. m. ligustica* and *A. m. carnica* C lineage bees.

Haplotype conservation in the admixed populations

To investigate further the haplotype blocks detected, we performed a local ancestry inference on our dataset with RFMix. Reference samples were selected as bees having > 95 % ancestry for a given background following the Admixture analysis at K = 3 (figure 3C), resulting in 131 samples for group 1, 148 for group 2 and 17 for group 3, while the remaining 333 samples formed the query dataset. To perform the local ancestry inference, we constructed a genetic map from cross overs identified in the sequence data of 43 males from 3 colonies [37] aligned to the HAv3.1 reference genome. Results indicate that few historical recombination events have occurred in the large haplotype blocks since the admixture between the subspecies. The most notable example is that of the 3.6 Mb haplotype block between positions 3.7 and 7.3 Mb on chromosome 11, in which almost all 333 samples from the query dataset show one continuous stretch for one of the three backgrounds. Only one of the 43 samples from Corsica presents two different ancestral haplotypes within this interval, with a switch from a group 1 to a group 2 haplotype at position ~4.5 Mb on chromosome 11, within the 3.6 Mb haplotype block, whereas numerous switches can be observed on the rest of the chromosome (figure 6A). When counting the haplotype switches detected in all 333 query samples, only 28 are situated within the 3.6 Mb haplotype block on chromosome 11, whereas other regions of the chromosome can have more than 50 switches per 100 kb (figure 6B and see supplementary figure 20 for the other chromosomes). Interestingly, *LOC724287,* which is the largest gene described in the Gnomon annotation set for the

372 genome assembly HAv3.1, is found in this block at position 11:5,292,072-6,161,805. This gene is
373 869,734 bp long and encodes protein rhomboid transcript variant X2, its large size being largely due to
374 intron 4, which is 596,047 bp long. However, on investigating a possible relationship between
375 haplotype block and gene sizes in the honey bee genome no obvious association could be found (data
376 not shown).
377
378 **Discussion**
379 *SNP detection in a haploid dataset*
380 Our complete dataset of haploid drones is composed of 870 samples sequenced using Illumina's HiSeq
381 and NovaSeq technologies. Results clearly show that although a few of the early sequences produced
382 on the HiSeq are of lower depth, only 15 samples were eliminated due to the fraction of missing
383 genotypes exceeding 10%. By contrast, the fraction of missing genotypes over the ~7 million SNPs
384 detected was considerably lower in samples sequenced on the NovaSeq sequencing platform. Having
385 sequenced haploids, the removal of heterozygote SNPs in individual samples is recommended to
386 reduce the likelihood of "pseudo SNPs", as we have shown previously that heterozygote SNPs tend to
387 cluster together [31] and co-locate with repetitive elements (data not shown). This set of ~7 million
388 markers can now be used as a basis for the realization of high-density SNP chips, allowing selections
389 of markers according to optimized spacing and to defined MAF values in the main subspecies of
390 interest. Indeed, an important technical issue in SNP chip design, is that very high SNP densities, such
391 as found in the honey bee, can potentially cause allele dropout when genotyping, due to interference in
392 the probe designs. Deep knowledge of SNP and indel positions will help select candidates flanked by
393 monomorphic sequences. Conversely, for lower density chips, the spacing of markers can be
394 optimized by taking the haplotype structure into account, thus avoiding redundancy while maintaining
395 the highest possible level of genetic information. Another advantage of sequencing haploid samples, is
396 that the whole dataset represents phased chromosomes. Notably, the present dataset will be invaluable

for genotype imputation in future studies using lower density genotyping, such as DNA chips or low-pass sequencing [58–60].

Population structure in managed honey bees

The deep understanding of European honey bee populations and of their recent admixture via imports of genetic stocks by breeders is not a simple task. The analyses of admixture events in complex population structures can be sensitive to a number of parameters and sometimes yields misleading results, especially if one or several populations went through a recent bottleneck [61]. PCA on all ~7 million markers indicate that our dataset is structured into three main genetic types (supplementary figure 8). The first principal component, representing 10.8 % of the variance, separates two major groups corresponding respectively to subspecies from north-western (M lineage) and south-eastern Europe (C lineage). These two groups are represented by several populations, including the Savoy and Porquerolles conservatories from South-East France on one side, and bees that are not so far geographically from Italy or Slovenia on the other. This large genetic distance despite relatively close geographic proximity of the populations supports the hypothesis of the colonization of Europe by honey bees via distinct western and eastern routes [1,24,62,63], and the separation between subspecies due to the Alps forming a natural barrier preventing genetic exchange [5]. Along the second principal component, representing 3.1 % of the variance, the population originating from *A. m. caucasia* separates from the south-eastern European populations (supplementary figure 8). Prior to investigating admixture we pruned SNPs in LD taking care to maximize the removal of redundancy while maintaining the general structure of the data (figure 2 and supplementary figures 15, 16, 17).

We explored a range of K number of genetic backgrounds, running multiple iterations of each, to determine the most likely admixture pattern (figure 3). Our results indicate that this approach is necessary to ensure the results from each K model are stable prior to interpretation. We observe from our Admixture analyses that CV outliers within a K model are common. For instance, at K = 8, the

mode with the lowest CV is only represented by 8 out of 50 Admixture runs, whereas the major mode has 12 runs. On examining the admixture patterns from these two modes, the major mode suggests the *A. m. mellifera* bees from conservatories on mainland France to be hybrids between bees from Ouessant and Spain, with roughly 50% of each genetic background moreover on the same mode, the *A. m. iberiensis* background represents also 50% of the M lineage background in the bees from Corsica (supplementary figure 19). This is extremely unlikely given the geography of Western Europe and our knowledge of the history of the bees of Ouessant. Indeed, Ouessant is a very small island (15.6 km^2) off the coast of western Brittany, isolated from the rest of the French honey bee population since its installation in 1987 and the prohibition of imports since 1991 mostly for sanitary reasons. In contrast, the mode with the 8 runs and lowest CV presents a better separation of *A. m. mellifera* and *A. m. iberiensis,* which is also found in the major mode at K = 9 backgrounds. A smaller level of admixture can still be found between *A. m. mellifera* and *A. m. iberiensis,* that is quite likely due to the shared ancestry between these two subspecies.

The major mode at K = 9 is represented by 33 out of 50 runs and returned the lowest mean CV value. This mode identifies mainland France *A. m. mellifera* samples as having a distinct genetic background and suggests that honey bees from Ouessant may have been re-introduced in the mainland conservatories. This mode also identifies a distinct genetic background in French and Swiss Buckfast bees. Buckfast bees were developed by Brother Adam, and are described on page 14 of "Beekeeping at the Buckfast Abbey" as a cross performed around 1915 between "the leather-coloured Italian bee and the old native English variety" [16]. Brother Adam also notes that the Italian bees that were imported in later years were distinct from the ones used in the development of the Buckfast strain. Our analysis of migrations between populations with TreeMix suggests that the Buckfast in our dataset were subject to introgression with genetic material from *A. m. caucasia* (figure 5B), although the timing of this potential admixture event could not be determined. When the two migrations of *A. m. ligustica* into Corsica and *A. m. caucasia* into the Buckfast are considered, which is a likely scenario

447 suggested by the Evanno analysis, the latter is close to *A. m. carnica*, as seen in figures 5B and 5C.
448 Interestingly, a whole genome sequence study of Italian honey bees, also suggest that the Buckfast
449 bees are closer to *A. m. carnica*, than to *A. m. ligustica* [64] and no proximity of the Buckfast bees
450 with M lineage bees were found neither in this study nor in ours, despite the cross at the origin of the
451 Buckfast including an old native variety. Further investigations including more Buckfast samples and
452 additional honey bee subspecies will be needed to fully elucidate this question. The *A. m. carnica*
453 samples from Slovenia, Germany, France, Switzerland and Poland all share the same genetic
454 background, reflecting their identical origin, probably recent imports.
455 The population of bees from Corsica has the distinct characteristic of being homogenous in
456 composition, despite being admixed, with all samples showing mean proportions of 75% and 25% of
457 *A. m. mellifera* and *A. m. ligustica* backgrounds, respectively (figures 2 and 3). The introgression of
458 Italian bees is confirmed by the TreeMix migration analysis and when this is accounted for, the
459 Corsican samples group with *A. m. mellifera* bees from mainland France instead of being situated
460 between the two main genetic subgroups of western and eastern European bees (figures 2B and 2C).
461 This result likely reflects the fact that Italian bees may have been imported on the Island until the
462 1980's, following which the import of foreign genetic material was prohibited. As beekeepers
463 generally prefer the *A. m. ligustica* Italian bees over *A. m. mellifera*, it is very likely that the latter is
464 the original population, as also suggested by Ruttner [1]. Although the hypothesis of the separation of
465 the two subspecies on the mainland by the Alps seems appropriate [5], the situation of the
466 Mediterranean islands in the region is not so clear. Based on physical geography alone, Corsica being
467 at a closer distance to Italy than to France, the chances would have been greater to have originally M
468 lineage rather than C lineage Italian bees. Moreover, Corsica was under the control of Pisa, then fell to
469 Genoa in 1284 and was only purchased by France in 1768. Further studies including samples from
470 Sardinia would certainly help defining the Mediterranean boundaries between the M lineage and C
471 lineage honey bees and confirm observations based on morphology [1].

Apart from the subspecies references and the royal jelly populations, the honey bees provided by breeders are largely admixed, exhibiting high variability in background proportions - even for samples sourced from the same region. A typical example is that of the Tarn1 and Tarn2 populations, revealing that two breeders situated very close to one another (less than 100 km), have very different genetic management strategies. Tarn1 samples are mainly composed of Buckfast and *A. m. carnica* backgrounds, whereas in Tarn2 a large proportion of *A. m. mellifera* background is also present and the population is far less homogenous (figures 2, 3 and 4). This shows the great heterogeneity of the managed populations found in France and a question that needs further investigation is the influence of the mating strategies used by the breeders, such as artificial insemination, mating stations, with drone producing hives to saturate the environment with the desired genetic strains, or open mating. These strategies influence variable levels of control on the genetic makeup of a breeder's stock. The higher proportion of *A.m. mellifera* background in the Tarn2 population could either be deliberate or due to a lower level of control over the mating of the queens, with a proportion of queens mating to *A. m. mellifera* drones from the environment.

The Royal Jelly population is the inverse: beekeepers from all over France exchange their genetic stock within a selection programme and practice controlled mating. As a result, a specific background with individuals presenting very little admixture, is found for this population at very distant locations. Most of the worldwide production of Royal Jelly comes from China, where high Royal Jelly-producing lineages of honey bees were developed from an imported *A. m. ligustica* lineage [65]. Interestingly, in our dataset, only three *A. m. ligustica* and all of the bees from China have some Royal Jelly genetic background.

Large haplotype blocks in the honey bee genome, specific to the M and C lineages

When investigating the contribution of SNPs to variance in the PCA, we noted several large genomic regions, up to 3.5 Mb long, in which almost all markers contributed very strongly to the first principal

497 component, separate bees from north-western (M lineage) and south-eastern Europe (C lineage). These
498 regions were noted to coincide with haplotype blocks detected with Plink. To investigate the matter
499 further, we performed local ancestry inference in the admixed samples with RFMix, using samples
500 exhibiting 95% ancestry for the three main genetic backgrounds as references. A low recombination
501 rate is confirmed by the observation of very few switches between the three main genetic backgrounds
502 within these haplotype blocks. Interestingly, some of our regions, including the largest one detected on
503 chromosome 11, coincide with regions of low recombination rate detected in other studies. These
504 include a LD map produced with 30 diploid sequences from African worker bees [39], ancestry
505 inference in an admixed population [35], low resolution genetic maps produced by Rad or ddRAD
506 sequencing, with microsatellite or SNP markers, ddRAD sequencing [66,67] or higher resolution
507 genetic maps produced by whole genome sequencing of European [37] and African subspecies [38].
508 Most of these regions coincide with the position of the centromeres such as described in the reference
509 genome assembly, which is primarily based on the combination of the location of *Ava*I repeats, that
510 were previously assigned to centromeres by cytogenetic analysis, and of a low GC content [33,68].
511 However, the *Ava*I repeats only represent a very small fraction of the centromeric regions described,
512 with the largest one only covering 14 kb [33], whereas the estimation of the extent of the centromeres,
513 based on a GC content lower than the genome average is much larger although imprecise and supposes
514 a similar organization as for the AT-rich alpha-satellite repeats in vertebrates, such as human [69].
515 Whereas in some cases the boundaries of our regions of low recombination rate coincide with the
516 actual positioning of the centromere on the genome assembly [33], such as in chromosomes 5 or 8, in
517 other instances, such as in chromosome 12, the region defined is much narrower. Due to the
518 difficulties in interpreting banding patterns in honey bee chromosomes, the position of the centromeres
519 is not well defined. Some evidence based on G- and C- banding suggests there are four metacentric
520 and 12 submetacentric or subtelocentric chromosomes [70], whereas other evidence based on the
521 fluorescent *in situ* hybridization of a centromere probe suggests there are two metacentric, four

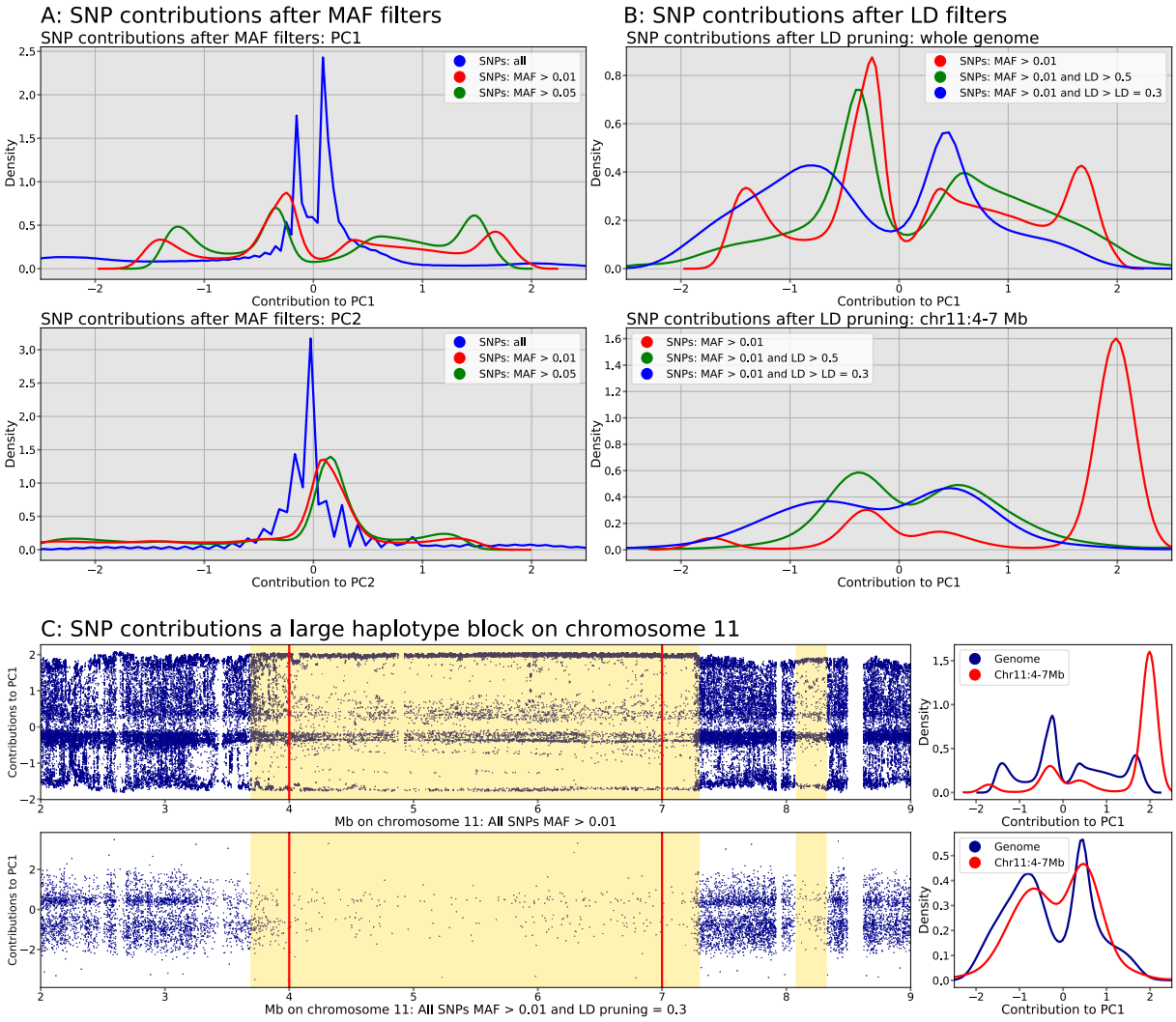
522 submetacentric, two subtelocentric and eight telocentric chromosomes [71]. Our evidence suggests at
523 least six chromosomes that could be telocentric or acrocentric: chromosomes 3, 5, 6, 9, 14 and 15.
524 Some of the haplotype blocks/regions of low recombination can seem very large, such as representing
525 up to 21 % in the case of chromosome 11 (figure 6). This may seem a lot, but recent findings in a
526 complete sequencing of the human genome give a similar proportion for chromosomes 9, in which 40
527 Mb of satellite arrays represent 20 % of the chromosome [72]. One important difference, however, is
528 that the block on honey bee chromosome 11 contains some genes, except in the central region,
529 whereas the satellite array described on human chromosome 9 does not. This reaffirms that our
530 understanding of the centromere positions in the honey bee chromosomes requires refinement. The
531 specific case of the acrocentric chromosomes in terms of gene content (supplementary figure 20)
532 seems to compare better to the situation described in human, as the sequencing of the p-arm of the five
533 human acrocentric chromosomes has allowed the discovery of novel genes within the satellite repeat-
534 containing regions [69].
535 Some haplotype blocks may have another origin than centromeric DNA. For instance, some may have
536 maintained genetic divergence by limiting recombination via the presence of structural variants such
537 as inversions. Indeed, two of the blocks described here, between positions 4.0 - 5.1 Mb and 5.8 - 6.9
538 Mb on chromosome 7, seem to coincide at least partially with two regions of haplotype divergence
539 possibly due to inversions, detected between positions 3.9 – 4.3 and 6.3 – 7.3 Mb on the same
540 chromosome, in a highland versus lowland study of East African bees [36]. The slight differences in
541 coordinates found between the two studies could be due to the fact that different version of the HAv3
542 assembly were used. However, if confirmed, this finding suggests that haplotype blocks differing
543 between M lineage and C lineage bees such as found here, might coincide with blocks found in other
544 subspecies in Africa. Another study identifying the thelytoky locus (*Th*) in the South African Cape
545 honey bee *Apis mellifera capensis* showed it was in a non-recombining region over 100 kb long on
546 chromosome 1, although long-read mapping failed to detect any inversion [73].

Given the current hypotheses on the colonization of Europe by honey bees via distinct western and eastern routes [1,24,62,63], it is not surprising that the haplotype blocks described here, whether or not representing centromeric regions, tend to separate mainly the M and C lineage bees. Further analyses will be necessary to define the centromeric regions more precisely and study their implication, together with the other haplotype blocks, in the sub species structure of the honey bee populations.

Conclusion

The sequencing of close to 900 haploid honey bee drones, was shown here to be an invaluable approach for variant detection and for understanding the fine genetic makeup of a complex population having gone through multiple events of admixture. In addition, the extent of regions of extremely low recombination rate could be defined with a higher precision than previously. The dataset generated here is a solid base for future research involving other honey bee populations and for any analyses requiring a reference set for phasing or imputation.

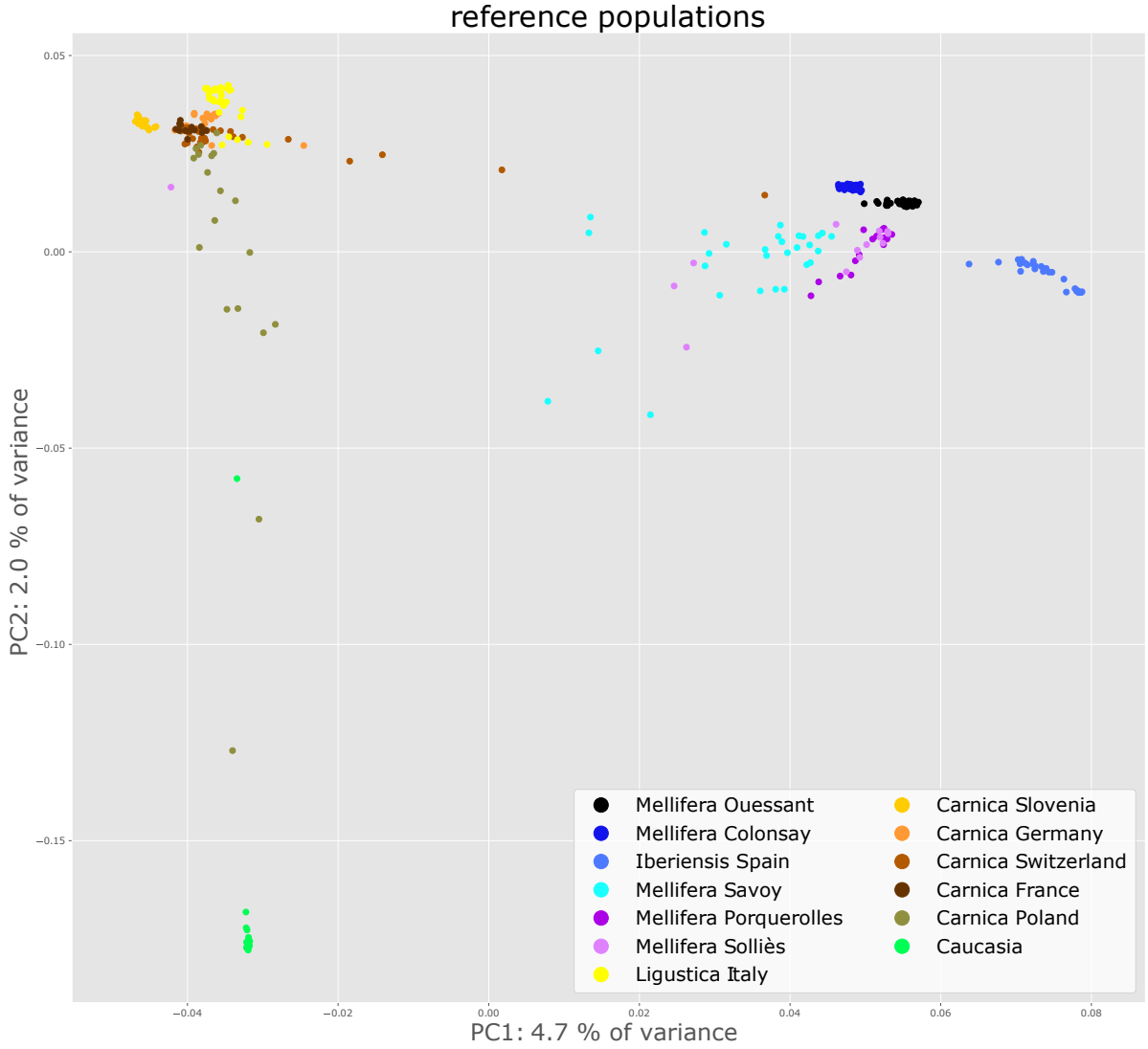
Figures

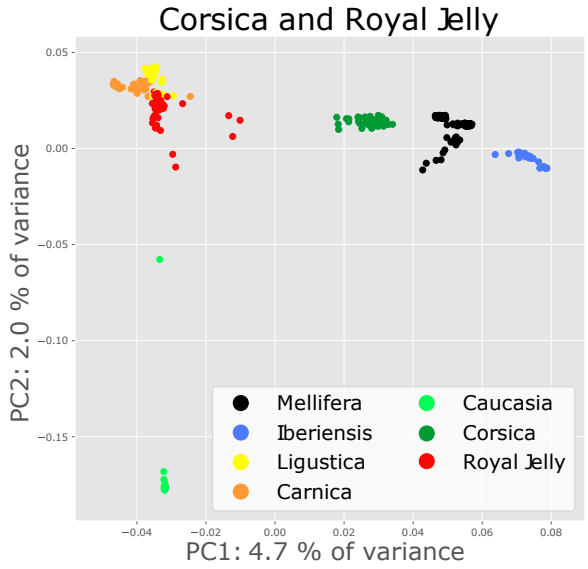


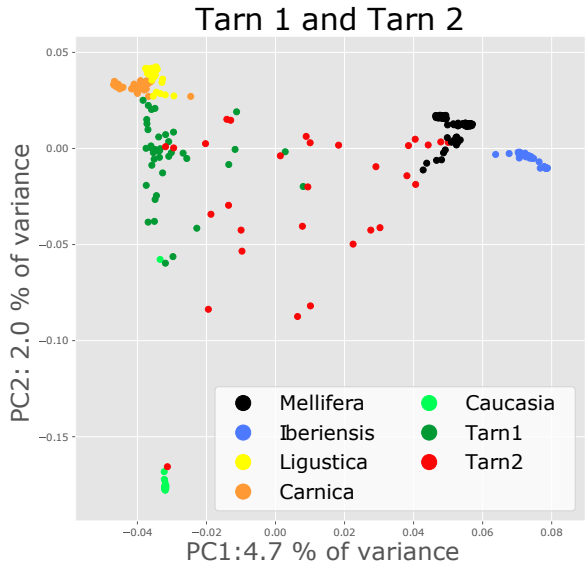
562

563 **Figure 1: Contribution of SNPs to the principal components, MAF and LD filters and detection**
564 **of large haplotype blocks.** A: contribution to PC1 (top) and PC2 (bottom). When all 7 million SNPs
565 are analysed simultaneously, the majority share a small contribution to PC1 and have no contribution
566 to PC2 (blue). When retaining only markers with MAF > 0.01 or 0.05, (3,285,296 and 2,525418 SNPs;
567 green and red lines, respectively), markers retained have a stronger contribution to PC1 and a higher
568 proportion of markers contribute to PC2. B: LD pruning on the 3,285,296 SNPs with MAF > 0.01 (red
569 line). Top: out of the 1,011,918 and 601,945 SNPs retained after pruning at LD = 0.5 (green line) or
570 0.3 (blue line), the distribution of the contribution of the markers is more even. Bottom: contribution
571 of SNPs to PC1 in a 3 Mb region of chromosome 11. Almost all markers in this region show

572 contributions are among the highest genome-wide (red line). The distribution of these contributions is
573 improved by LD pruning (green and blue lines). C: blue points show the contribution of individual
574 SNPs along a 6 Mb region of chromosome 11 containing two haplotype blocks of > 3 Mb and ~200 kb
575 (yellow backgrounds) before (top) and after (bottom) LD pruning. The LD pruning eliminates
576 successfully the markers in the haplotype blocks and the distribution of marker approaches that of the
577 rest of the genome, as shown in the corresponding density plots on the right.
578

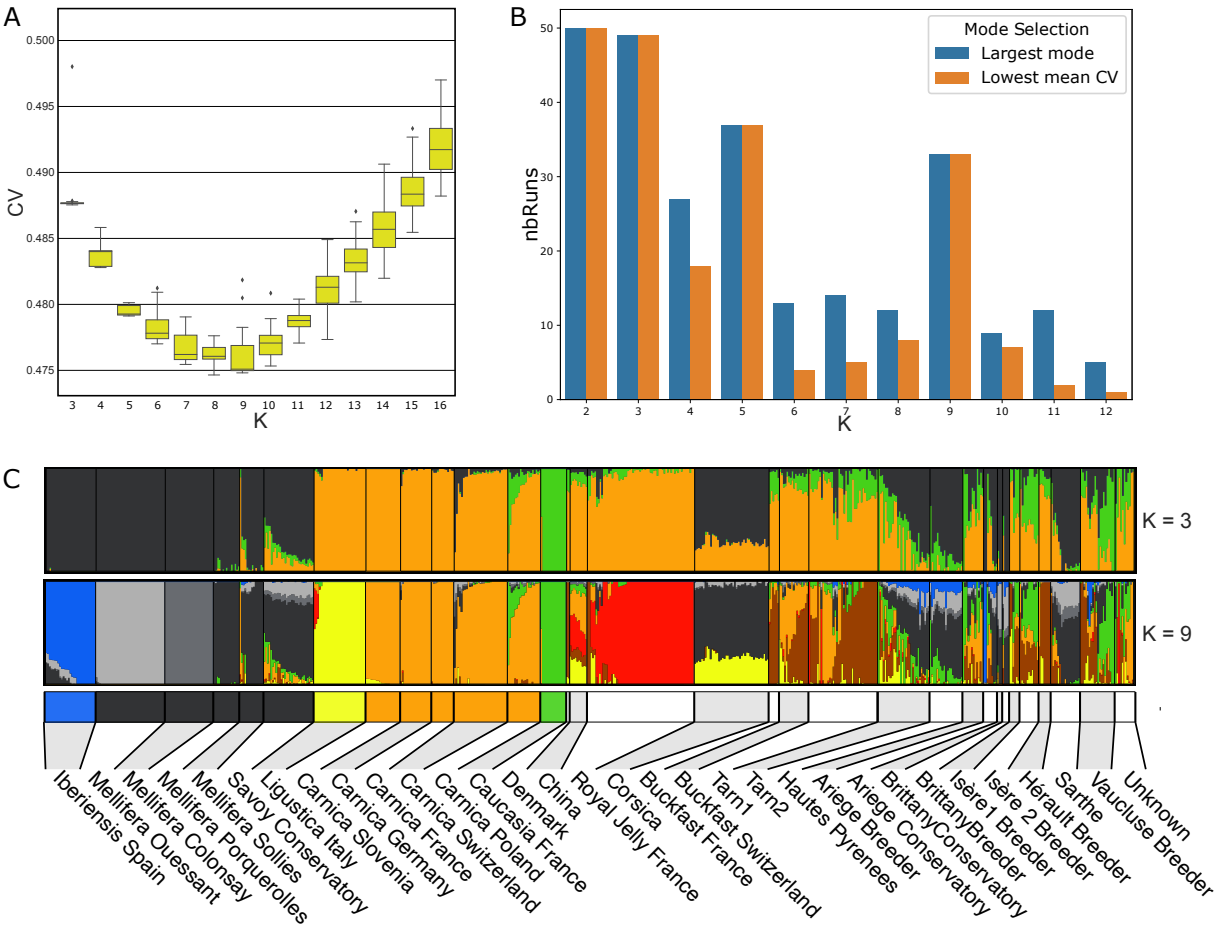






579

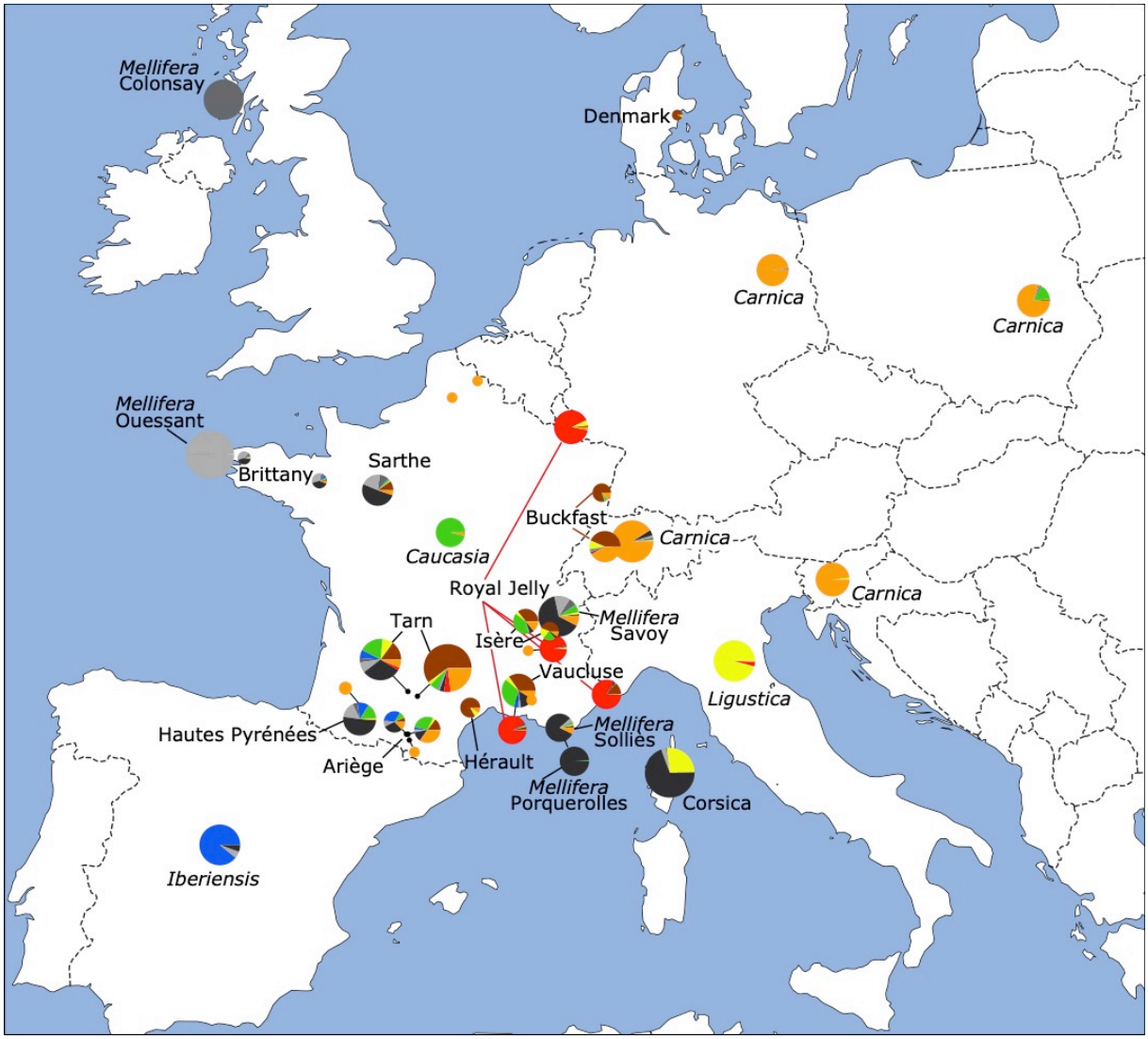
580 **Figure 2: PCA on the reference populations and on a sample of representative breeder**
581 **populations.** The 601,945 SNPs obtained after MAF filtering and LD pruning were used. Left:
582 reference populations only, with a colouring scheme according to their origin. Middle and left: only
583 the reference populations with a high proportion of pure background individuals, as observed after
584 Admixture analysis, were kept and coloured according to the five subspecies. Some breeder
585 populations appear homogeneous, such as the honey bees selected for Royal Jelly or those from
586 Corsica. Others are heterogeneous, such as populations Tarn1 and Tarn2, from breeders.
587



588

589 **Figure 3: Admixture analysis.** A: estimation of Cross validation error for 50 runs of Admixture for 3
590 ≤ K ≤ 16. B: Major modes and modes with the lowest mean cross-validation (CV) error for Admixture
591 runs. For each value of K ranging between 2 and 12, Q matrices from Admixture runs were grouped

592 by similarity in modes by using the Pong software (Behr et al. 2016). Blue: number of runs in the
593 major mode; orange: number of runs in the major or minor mode having the lowest mean CV value.
594 Amongst the values of K having the lowest CV values from Admixture runs (see figure 12), K = 9
595 stands out as having a major mode containing 33 runs out of 50, which is also the mode having the
596 lowest mean CV value from the Admixture runs. For other values of K, such as 4, 6, 7, 8, the major
597 modes do not have the lowest mean CV values. C: Admixture plots for all 629 samples for K = 3
598 (major mode containing 49 out of 50 runs) and K = 9 (major mode containing 33 out of 50 runs).
599 Reference populations on the left have a colour code under the admixture plot that recapitulates their
600 colour on the PCA plots of figure 2; other populations are indicated with alternating grey and white
601 colours.
602



603

604 **Figure 4: Admixture proportions and location of sample populations used in the diversity study.**

605 The size of the pie charts indicates the number of samples from a given location, with the number

606 ranging from 2 samples (e.g. Denmark) to 43 samples (Corsica). Positions in France indicate the

607 coordinates of the breeder or honey bee conservatory sampled. In other countries, reference samples

608 are all grouped together, unless two genetic types were sampled (e.g. Switzerland). Colours in the pie

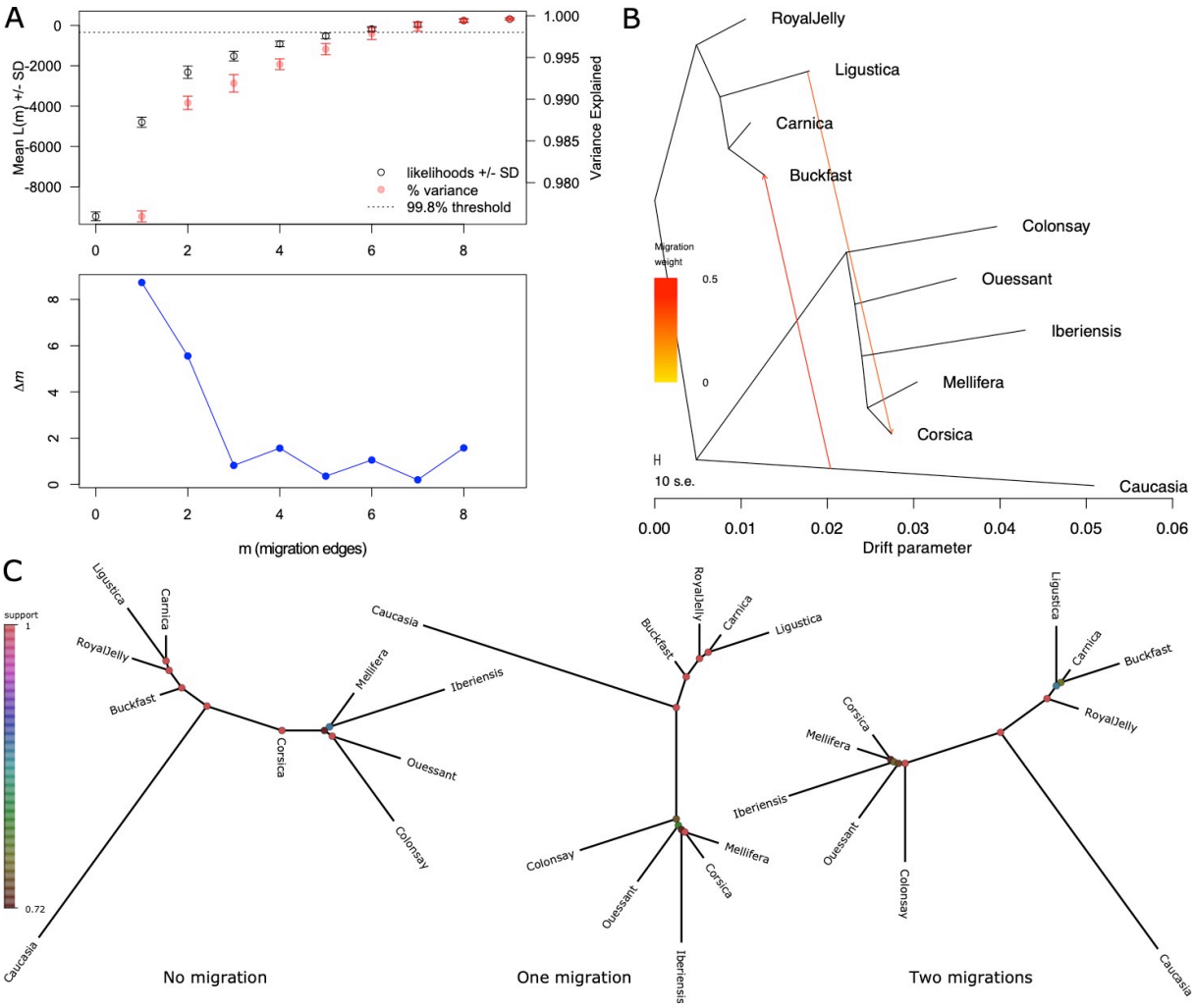
609 charts correspond to the backgrounds found in the admixture analysis for K = 9, as presented in figure

610 3. Reference populations for the five subspecies are indicated in italics. Two Buckfast populations in

611 France and Switzerland are indicated, so as the four breeders from the Royal Jelly breeder organisation

612 (GPGR: Groupement des Producteurs de Gelée Royale) having provided samples.

613



614

615 **Figure 5: Analysis of migrations with Treemix.** A: the OptM package was used to determine the

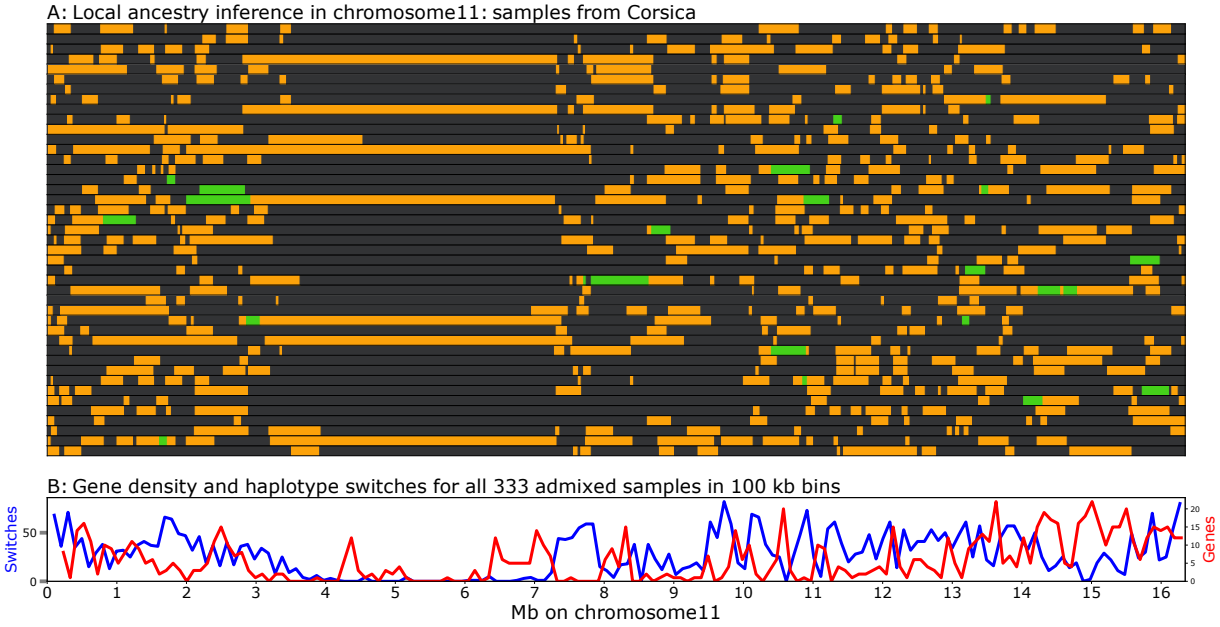
616 optimal number of migrations between populations and backgrounds. The Δm values suggest one or

617 two migrations. B: TreeMix graph selected amongst the 100 runs showing the two migrations

618 identified. C: summaries of trees from TreeMix, estimated from 100 runs per migration with

619 DendroPy.

620



621

622 **Figure 6: Local ancestry inference on chromosome 11 in the admixed samples from Corsica.** A:

623 each horizontal line represents the ancestry inference on one of the 43 individual samples from

624 Corsica. Grey: *A. m. mellifera* and *A. m. iberiensis* backgrounds; yellow: *A. m. ligustica* and *A. m.*

625 *carnica* backgrounds; green: *A. m. caucasia* background. B: haplotype switches in all 412 admixed

626 samples analysed. The 3 Mb haplotype block at positions 4-7 Mb on chromosome 11 shows very little

627 historical recombination.

628

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Data Accessibility

DNA Sequences for this project have been deposited in the Sequence Read Archive (SRA) at www.ncbi.nlm.nih.gov/sra under the BioProject accessions PRJNA311274 as part of the SeqApiPop French honey bee diversity project dataset and PRJEB16533 as part of the Swiss honey bee population and conservation genomics project dataset. Individual SRA run and BioSample accessions for all samples are given in supplementary table 1. A vcf file with the filtered 7 million SNP and 870 samples is available at https://doi.org/10.5281/zenodo.5592452 for download, together with the list of the 629 unique samples.

Authors' contributions

YLC, J-PB, BB and AV designed the experiment. BB, YLC, and AV coordinated colony selection and sampling and samples were provided by KB, MB, CC, AG, PK, MP and AP. KC-T, EL and OB performed DNA extraction, library preparation and sequencing. DW, AV, SE and BS performed the

654 bioinformatic analyses and co-wrote the manuscript.

655

656

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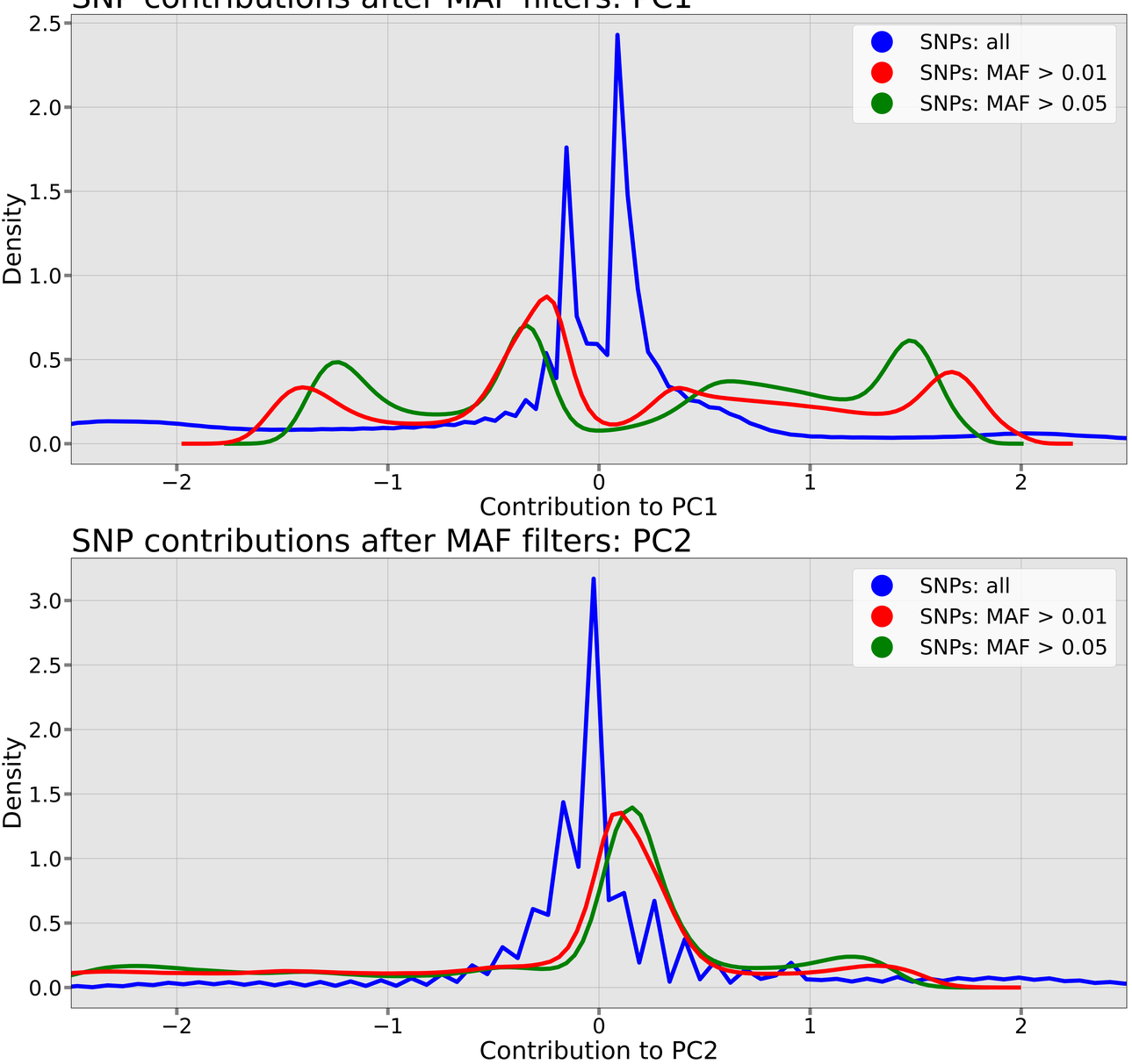
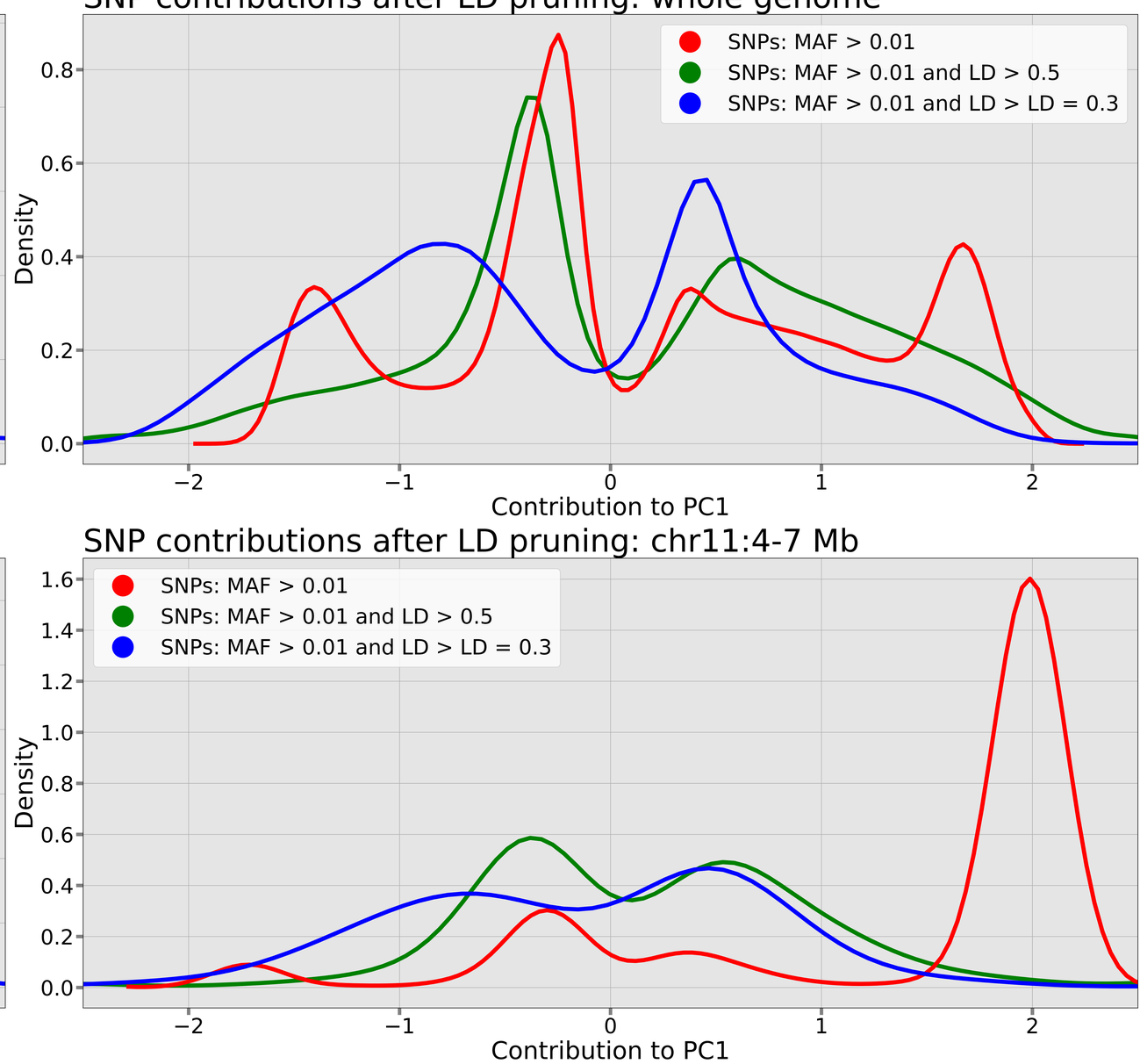
841
842

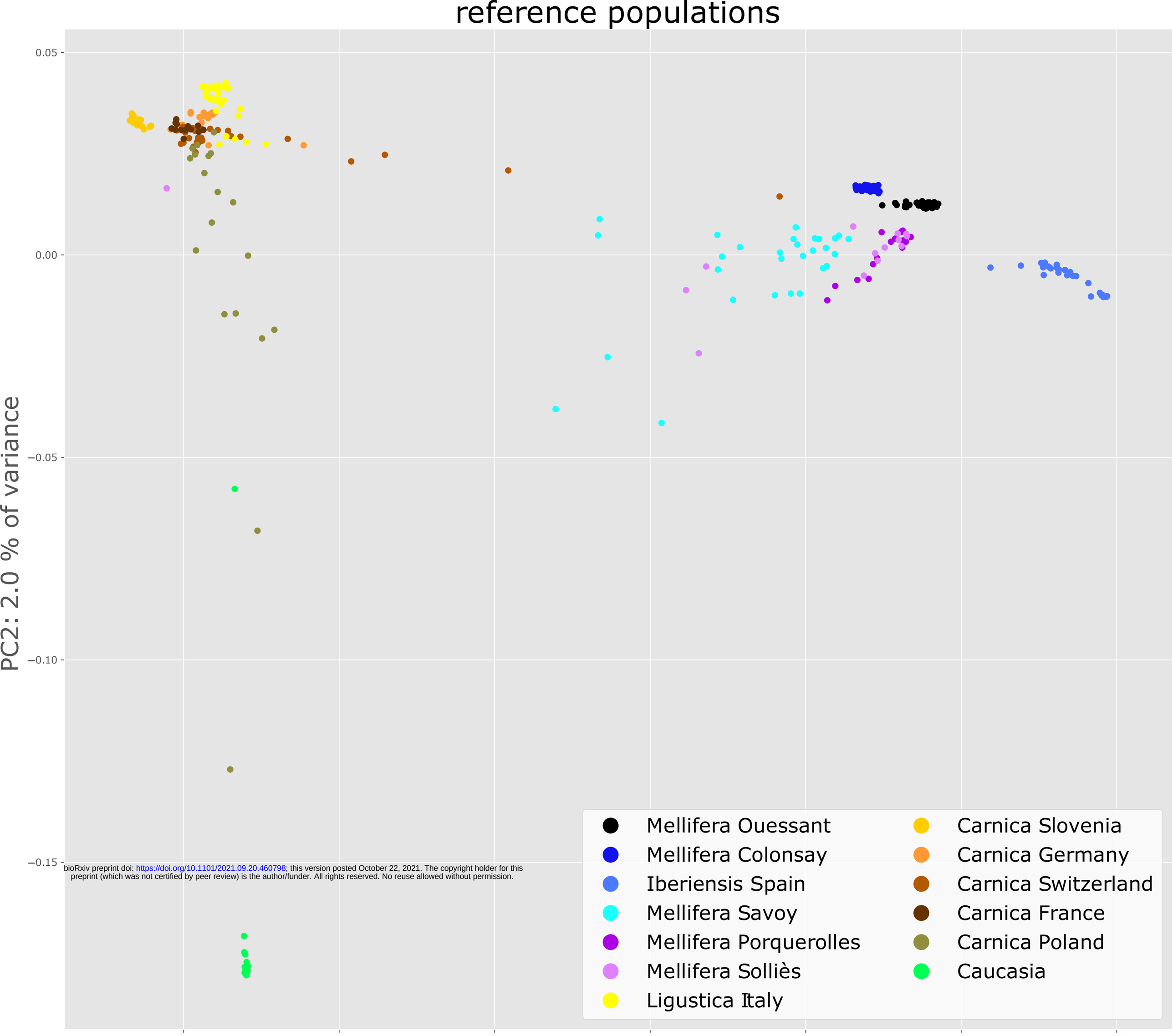
A: SNP contributions after MAF filters

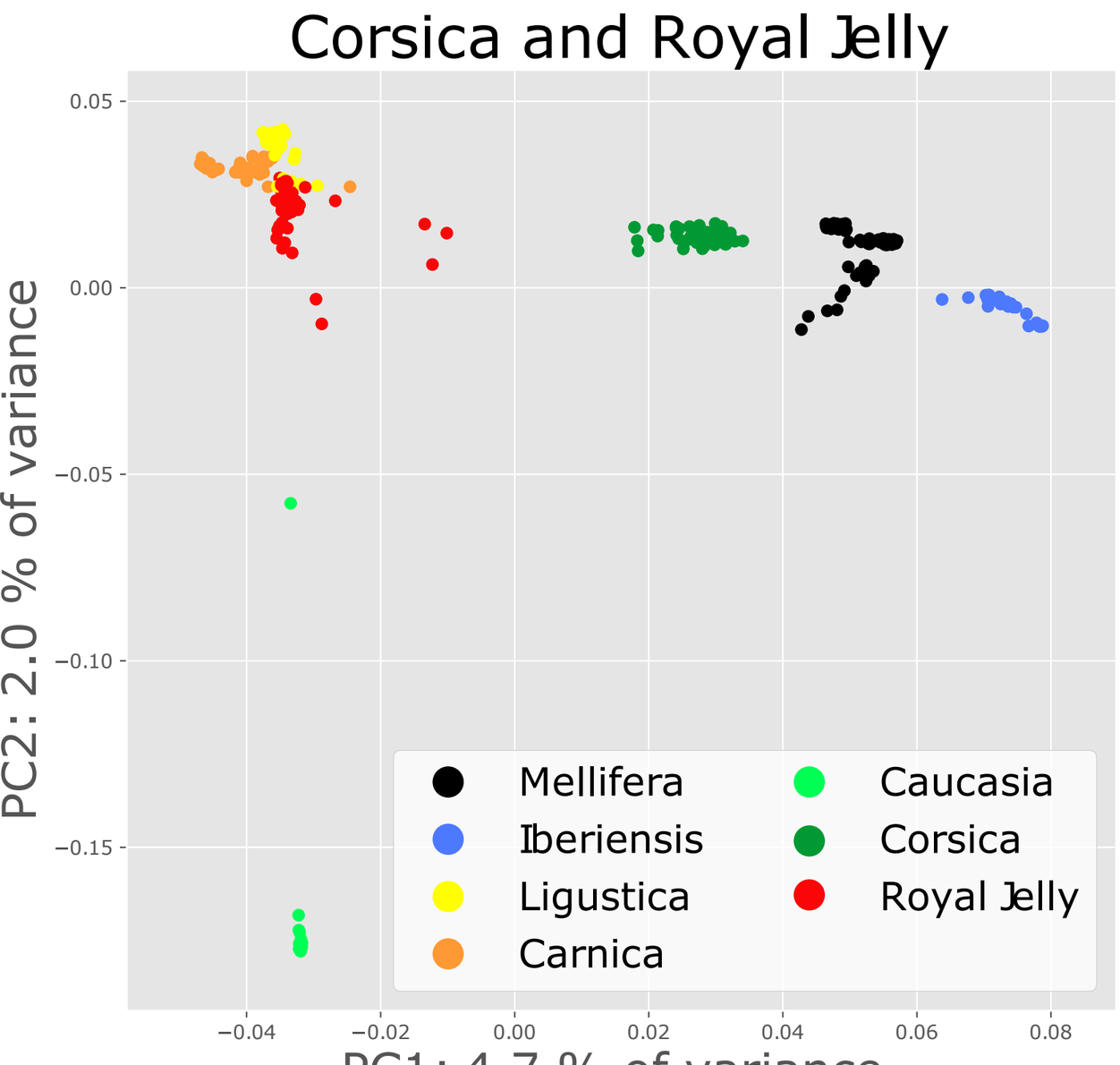


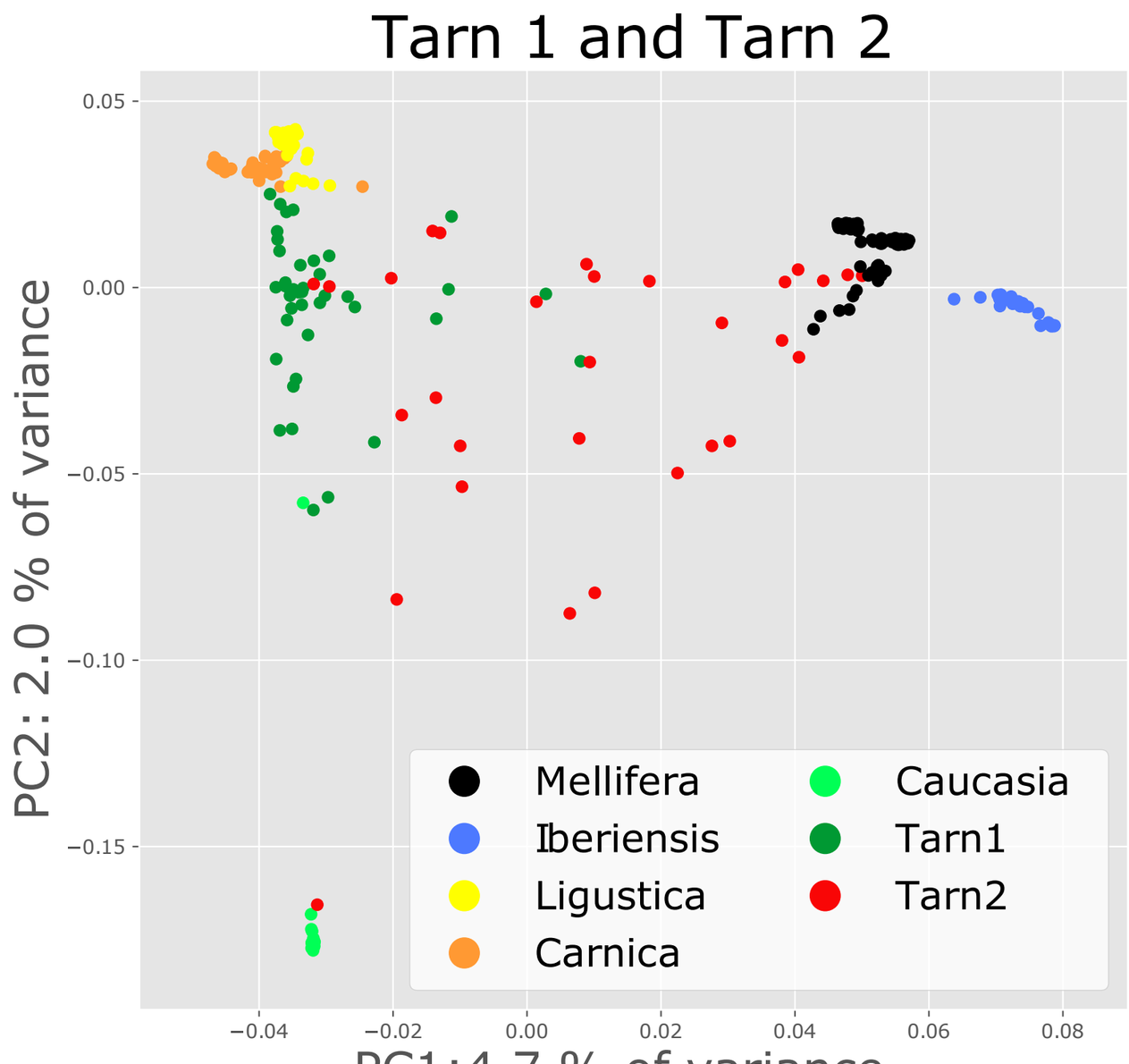
B: SNP contributions after LD filters



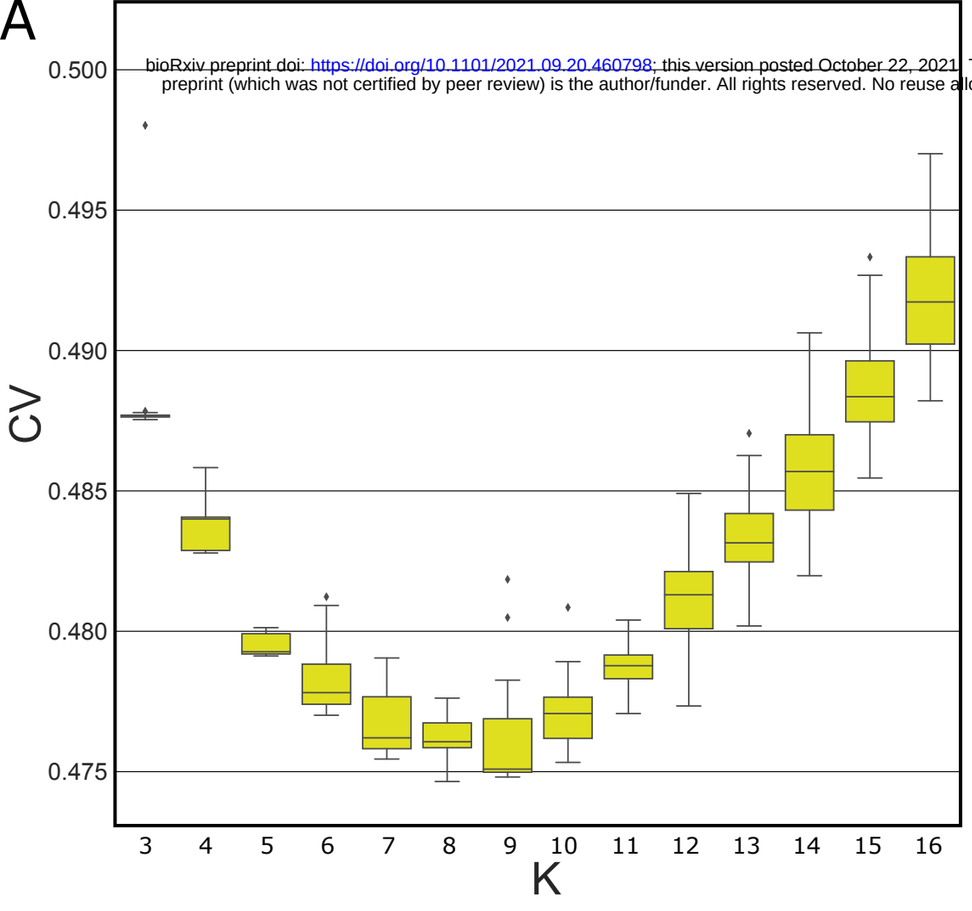
C: SNP contributions a large haplotype block on chromosome 11



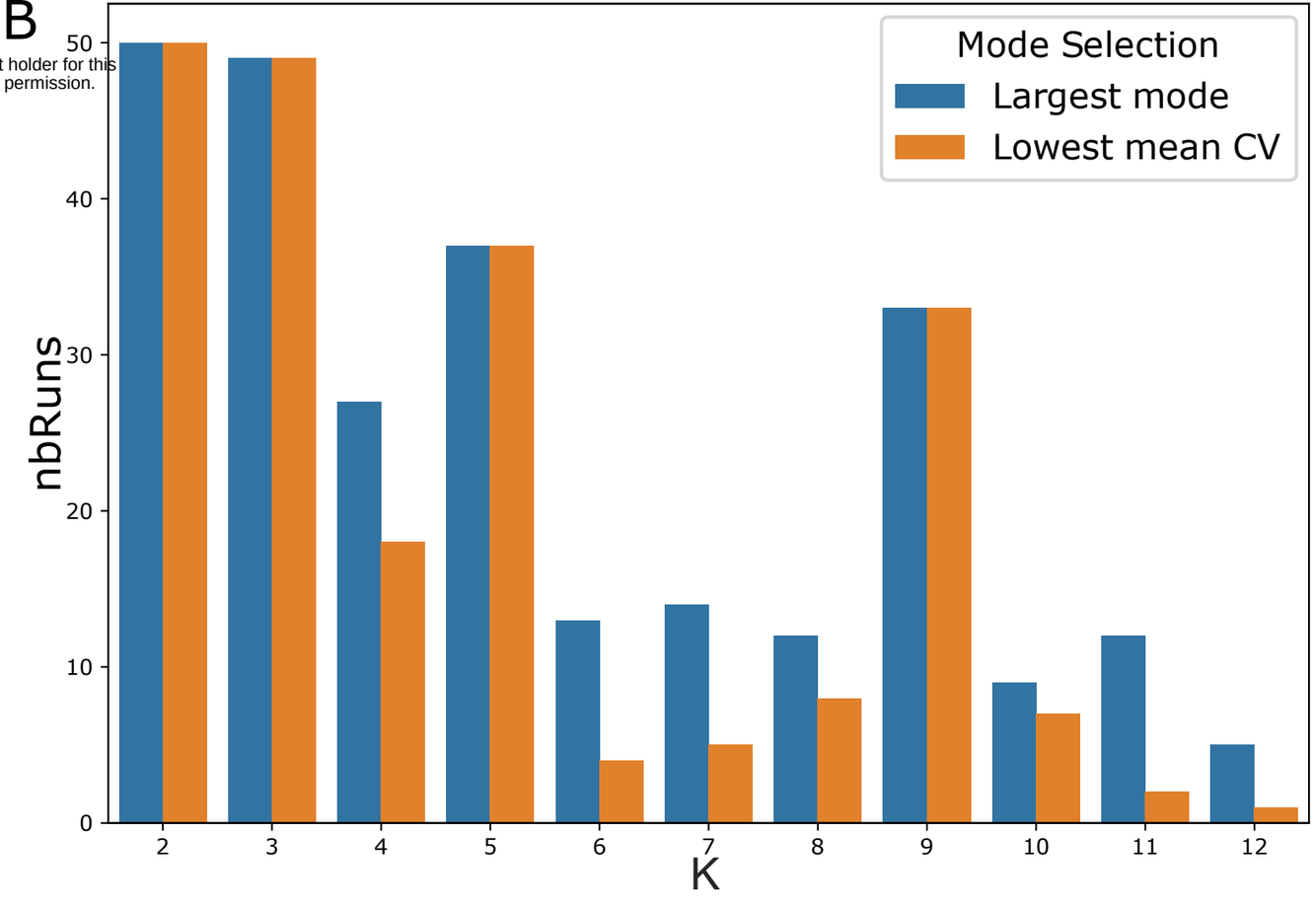




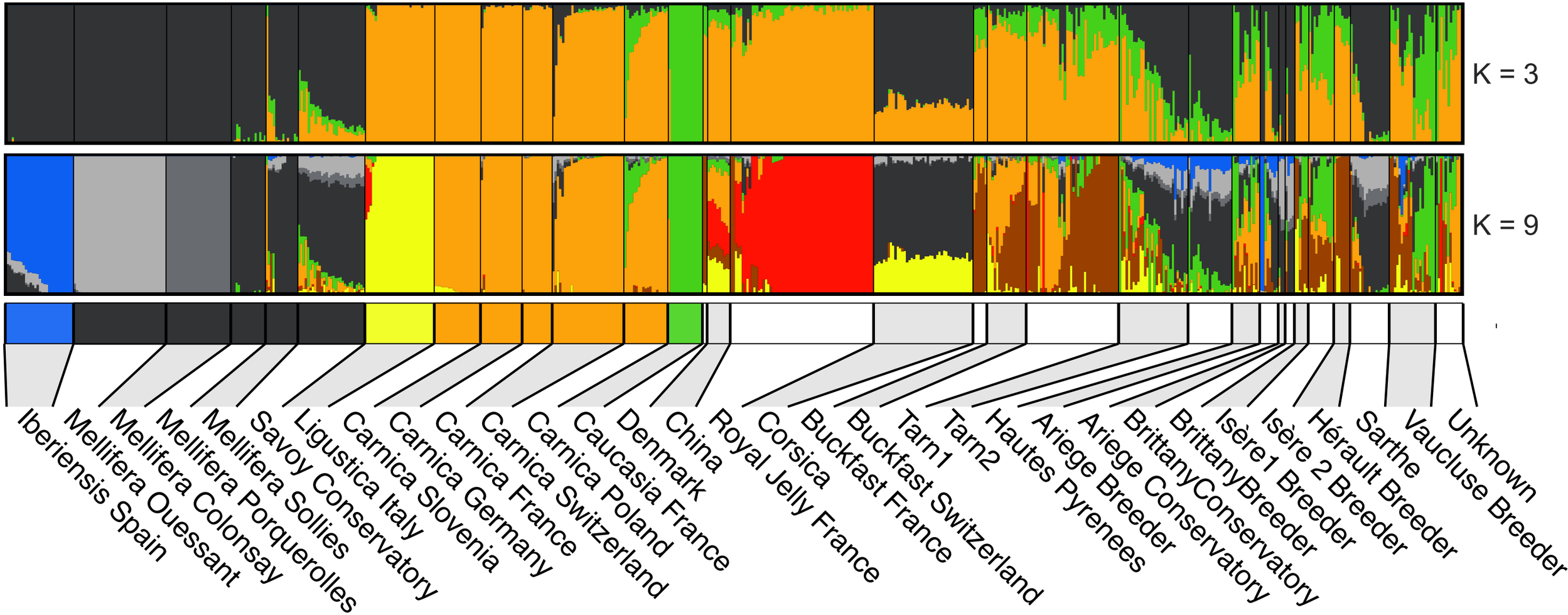
A



B



C



Mellifera Colonsay

Denmark

Carnica

Carnica

Mellifera Ouessant

Brittany

Sarthe

Buckfast

Carnica

Caucasia

Carnica

Royal Jelly

Tarn

Mellifera Savoy

Isère

Vaucluse

Ligustica

Hautes Pyrénées

Mellifera Sollies

Ariège

Hérault

Mellifera Porquerolles

Corsica

Iberiensis

A

Mean L(m) +/- SD

Variance Explained

likelihoods +/- SD

% variance

99.8% threshold

Δm

m (migration edges)

B

RoyalJelly

Ligustica

Carnica

Buckfast

Colonsay

Ouessant

Iberiensis

Mellifera

Corsica

Caucasia

Migration weight

0.5

0

H

10 s.e.

Drift parameter

C

support

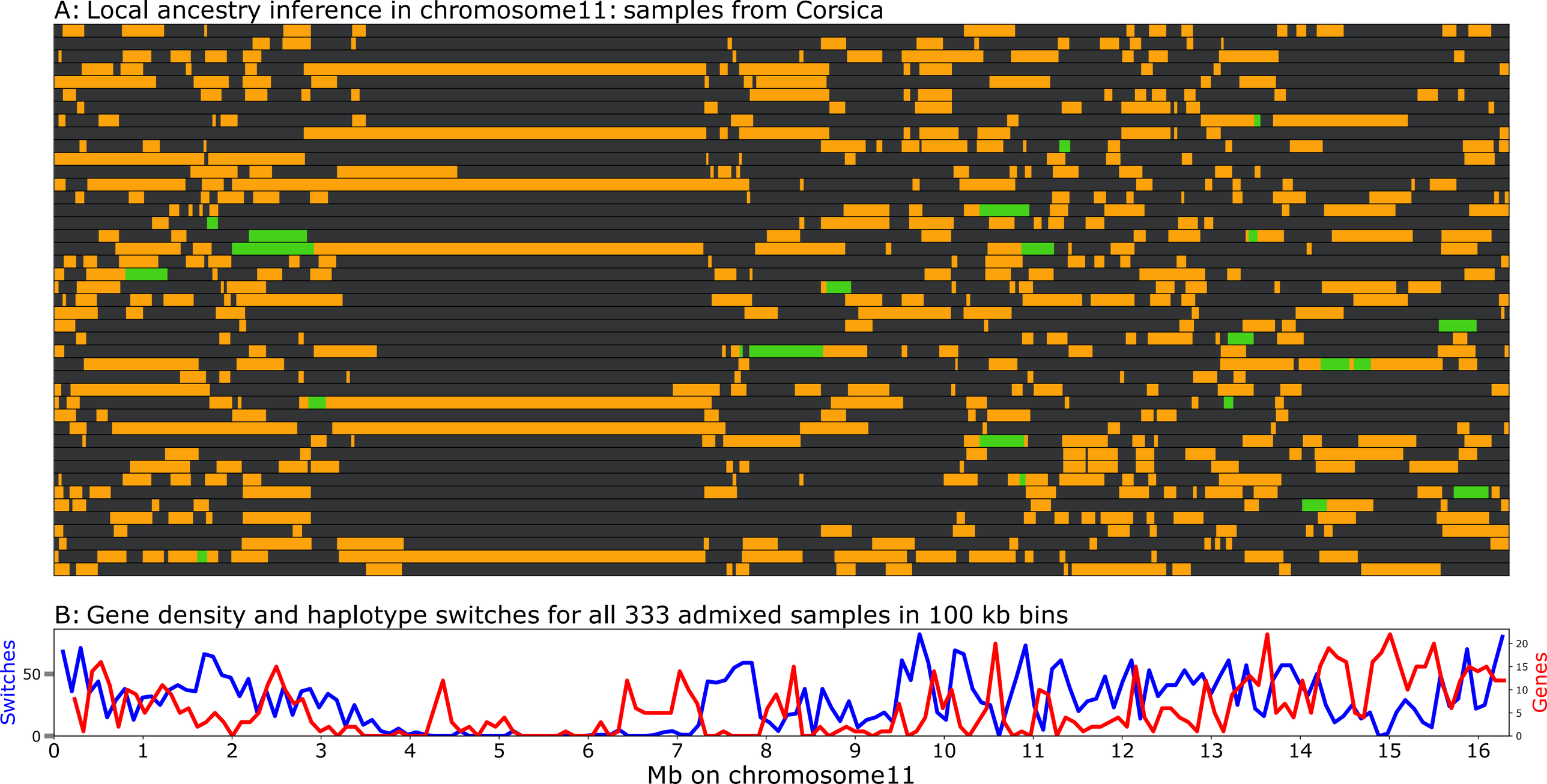
1

0.72

No migration

One migration

Two migrations


A: Local ancestry inference in chromosome11: samples from Corsica
B: Gene density and haplotype switches for all 333 admixed samples in 100 kb bins
Switches
0
50
Genes
0
5
10
15
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Mb on chromosome11