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The ability of ddRAD-Seq to estimate genetic diversity and genetic introgression in endangered native livestock

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Abstract

Unplanned crossbreeding between a native livestock and a specific productive breed was one of the main reasons that caused the loss of valuable genetic resources in native livestock. To avoid further loss and damage of genetic resources in the native livestock, introgressed individuals should be distinguished to eliminate them by preventing any further employment in future mating plans. In general, the genetic diversity of native livestock had already decreased and mass elimination of introgressed individuals from the population endangers their existence. To solve this problem, high-resolution markers are required to discriminate between introgressed variation and native variation. Here, we applied ddRAD-Seq markers for native Japanese horse “Taishu” that has undergone recent genetic introgression. Genome-wide ddRAD-Seq markers can distinguish five breeds of native Japanese horses and Anglo-Arabian

introgressed breeds. We found the signatures of genetic introgression of Anglo-Arabian at only two chromosomes; however, the signatures were separated in their genome suggesting that it might not be the cause of recent introgression. The genetic diversity of Taishu was less than other Japanese breeds and the decreasing genetic diversity is an urgent issue compared to genetic introgression. Although few signatures of recent introgression were detected, a lot of shared SNPs (10% of all SNPs in Taishu) were detected between Taishu and Anglo-Arabian. To avoid misestimation of the presence and degree of introgression in native livestock, information regarding shared SNPs and population genetic approaches need to be assessed by using the large number of genome-wide markers such as ddRAD-Seq.

Keywords

ddRAD-Seq, livestock, genetic introgression, genetic diversity

Introduction

The diversity and populations of native livestock breeds have dramatically decreased due to the dominance of a few breeds that have been selected for greater productivity (Rischkowsky and Pilling, 2007; Scherf and Pilling, 2015). Rapid and unplanned crossbreedings between native and productive breeds have frequently occurred, further depleting the valuable genetic resources of native livestock (Hanotte et al. 2010). To avoid further loss and damage to genetic resources of native livestock, introgressed individuals should be identified and removed. However, most endangered native livestock populations already have depleted genetic diversity, and the easy removal of introgressed individuals endangers their existence. Therefore, we should maintain a careful balance between preserving genetic diversity and removing genetic introgression by focusing at the genomic level (i.e., chromosomes and loci) to identify original genetic variations in native breeds and introgressed variations from other breeds. This could then be used to maintain the genetic diversity of native variants and eliminate the introgressed variants.

Genetic introgression is the movement of genes from one species into the genome of another via crossing. Therefore, genetic introgression can be difficult to detect without molecular data (Fitzpatrick et al. 2010; Ryan et al. 2009). The development of the Sanger-PCR methods has enabled estimates of genetic introgression using partial DNA sequences, such as mtDNA sequences and single sequence repeat (SSR) markers. However, detecting genetic introgression using partial DNA has some problems. For example, mtDNA is maternally inherited and does not recombine, so it can only be used to detect genetic introgression through females and cannot reflect the degree of genetic introgression. Meanwhile, SSR markers are usually no more than several hundred loci and incur the possibility of overestimating or underestimating genetic introgression. As genetic introgression has progressed for several generations, a

larger number of markers is needed to accurately reflect the degree of introgression in individuals. Furthermore, individuals can be identified as hybrids from only a few markers even if it there is little introgressed variation in other regions. This can have a fatal effect on the conservation of native breeds because the few-introgressed individuals who carry native variations could be eliminated from the population, causing further decline in genetic diversity.

Endangered native livestock often have two problems: reduced genetic diversity and genetic introgression from other breeds. Removing genetic variation that has been introgressed from other breeds simultaneously removes the genetic variation of the native breed, further depleting the genetic diversity of native livestock breeds. Therefore, genome-wide, high-resolution markers are needed to achieve a suitable balance between removing the introgressed genetic variations and retaining the genetic diversity of native livestock.

Double-digested restriction site-associated DNA sequencing (ddRAD-Seq) is used to obtain thousands of single nucleotide polymorphisms (SNPs) across the genome (Baird et al. 2008; Peterson et al. 2012). To detect genome-wide SNPs, various molecular methods have been used to generate libraries for use in next-generation sequencing (Futschik and Schlotterer, 2010; Mardis, 2008; Nielsen et al. 2011). One effective method is ddRAD-Seq, a variation of genotyping-by-sequencing (GBS) (Poland and Rife, 2012). In this strategy, genomic DNA is fragmented using restriction enzymes and sequenced using next-generation sequencing technologies to obtain SNPs that are located next to target restriction sites. As there are far fewer sequences, this strategy increases the coverage of fragments and provides reliable data for many samples. There are some favorable aspects for applying ddRAD-seq to endangered native livestock. For example, ddRAD-seq can be applied to all target species and is cost-effective compared to whole-genome sequencing and SNP chips. Although SNP chips designed for some livestock can provide

tens of thousands of accurate SNPs, it is possible that the SNPs do not include those of native livestock. Also, SNP chips are not readily available for some native livestock, whereas ddRAD-seq can be applied to almost all native livestock without additional experiments. Thus, it is clear that ddRAD-Seq is useful for detecting the genetic introgression of native livestock and is a versatile method for genetic introgression problems for many native livestock.

ddRAD-Seq has often been used to detect genetic introgression and hybridization in wild species (Chattopadhyay et al. 2016; Combosch and Vollmer, 2015). Most of the previous studies aimed at finding the geographical hybrid zone and detect signatures of hybridization and backcrossing between target species. In contrast, ddRAD-seq approaches for endangered native livestock aimed to identify introgressed individuals and decide whether the individuals were needed to be included in the conservation plan. As native livestock breeds have lower genetic diversity compared to wild species, introgressed loci and regions should be identified using high-resolution markers to create sustainable conservation plans. Here, we confirmed the ability of ddRAD-Seq to identify introgression variations with high resolution.

In this study, we examined native Japanese horse breeds on Tsushima-island in Nagasaki prefecture, Japan. The breed Taishu is listed by the FAO as “critical maintained” (Rischkowsky and Pilling, 2007; Scherf and Pilling, 2015). Taishu was introgressed with the Anglo-Arabian breed for military use during World war II (Hayashida, 1972). There is documented evidence of this genetic introgression, which describes one main event of crossing between these two breeds (Hayashida, 1972). This single introgression of Taishu presents a good opportunity to estimate genetic diversity and genetic introgression in native livestock using ddRAD-Seq. In addition, mtDNA sequences, SSR markers, and SNP chips for horse (*Equus*) are available. SNP chips for horse can produce 54 000 and 670 000 SNPs (McCue et al.

2012; Schaefer et al. 2017). Some studies used these methods in some native Japanese horses. Therefore, we can compare the results of our ddRAD-Seq with those from previous studies. We obtained about 10 000 SNPs by ddRAD-seq and identified genetic introgression and native loci on each chromosome in Taishu breed. We demonstrate the utility of ddRAD-Seq for the conservation of endangered native livestock breeds.

Material and methods

Sample collection and DNA extraction

We collected fresh blood samples from 57 individuals of 5 different breeds of Japanese native horse (*Equus caballus*), Taishu (N = 38), Kiso (N = 5), Miyako (N = 9), Yonaguni (N = 5), and Hokkaido (N = 6) in Japan. There are records that Taishu, Kiso, and Hokkaido were introgressed with European breeds, whereas Miyako and Yonaguni are not introgressed. Taishu is genetically closer to the Kiso-Hokkaido clade than to the Miyako-Yonaguni clade (Tozaki et al. 2003). In addition, blood samples from the Anglo-Arabian breed (N = 5) that was introgressed into Taishu, were provided by Goryo Bokujo (Imperial Stock Farm). None of these breeds have genetic crossing between them at present. The blood samples were collected in a tube with EDTA and kept at −20 °C until DNA extraction. Total genomic DNA was extracted from the whole blood using the Maxwell 16 Blood DNA Purification Kit (Promega, USA).

DNA sequencing of mtDNA

16S rRNA sequences of mtDNA were amplified using primers designed by Achilli et al. (2012). All amplifications followed PCR protocols for a reaction volume of 10 µl: 100 ng of the DNA template, 5.0 µl 2× KAPA, and 0.2 µM each primer. The amplification conditions were as follows: 2 min at 98 °C; then 30 cycles of 30 s at 98 °C, 30 s at 66 °C, and 1.5 min at 72 °C; ending with 15 min at 72 °C. The PCR

127 products were purified and cleaned using ExoSAP-IT Express PCR Product Clean-UP (Affymetrix).
128 Sequencing was performed by Macrogen (Seoul, Korea). Sequencing was performed using nested primers
129 as designed by Achilli et al. 2012. The obtained sequences were aligned using Clustal X software (Larkin
130 et al. 2007).
131
132 **Library preparation and sequencing in ddRAD-Seq**
133 Library preparation was composed of 5 steps. First, restriction enzyme digestion and adapter ligation
134 were performed in 10 µL reaction mix: 2 µL sample DNA (20 ng/µL), 0.5 µL EcoRI (10 U/µL, Takara,
135 Osaka, Japan), 0.5 µL BglII (10 U/µL, Takara), 1 µL 10x NEB buffer 2 (New England Biolabs, Ipswich,
136 MA, USA), 0.1 µL 100x BSA (Takara), 0.4 µL EcoRI adaptor (5 µM), 0.4 µL BglII adaptor (5 µM), 0.1
137 µL ATP (100 mM), 0.5 µL T4 DNA Ligase (600 U/µL, Enzymatics, Beverly, MA, USA) and 4.5 µL
138 nuclease-free water. The digestion and ligation were performed at 37 °C for 16 h.
139
140 The two Y-shape adapters were prepared by annealing two partially complementary oligo-DNAs. A
141 mixture of 100 µM adapter F and R was annealed using a thermal cycler with the following program: 95 °C
142 for 2 min, slow-cooled to 25 °C (0.1 °C/s), followed by 30 min at 25 °C. The annealed adapter (50 µM)
143 was stored at −20 °C. It was diluted to the working concentration (0.4 µM) just before use. The
144 oligonucleotide sequences of the Y-shaped adaptors were as follows: BglII_adaptor_F: 5′-A*A*T GAT
145 ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT* C*C-3′;
146 BglII_adaptor_R: 5′-G*A*T CGG AAG AGC TGT GCA GA*C* T-3′; EcoRI_adaptor_F: 5′-/Phos/
147 A*A*TTGAGATCGGAAGAGCACACGTCTGAACTCCAGTC*A*C -3′; and EcoRI_adaptor_R:
148 5′-G*T*C AAG TTT CAC AGC TCT TCC GAT C*T*C-3′, where *signifies a phosphorothioate bond and
149 "/Phos/" signifies a phosphorylation.

The ligation product was purified using AMpure XP beads (Beckman Coulter, Brea, CA, USA) as follows: 10 µL of the AMpure XP and 10 µL ligation product were mixed by pipetting and kept at 25 °C. for 5 min. The purification was performed according to the manufacturer's instructions. Then, the purified adaptor-ligated DNA was subsequently amplified by PCR. Amplification was performed in 10 µL reactions: 2 µL DNA, 2 µL Index primer (5 µM), 1 µL TruSeq_Univ_primer (10 µM), 5 µL 2X KAPA HiFi HS ReadyMix (KAPA Biosystems). The PCR was executed with 94 °C for 2 min and 20 cycles of 98 °C for 10 s, 65 °C for 15 s, and 68 °C for 15 s. After PCR, the product was preserved at 4 °C. The oligonucleotide sequences of the primers were as follows: TruSeq_Univ_primer: 5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GA-3′; and Index primer: 5′-CAA GCA GAA GAC GGC ATA CGA GAT XXX XXX GTG ACT GGA GTT CAG ACG TGT-3′, where "XXXXXX" signifies an index sequence.

The PCR products of all samples were combined and concentrated using AMpure XP beads. The combined PCR product was mixed with equal volume of AMpure XP. Then, the mixture was placed on a magnet, and after 5 min its supernatant was removed. The remained beads were washed by adding 75% EtOH in excess volume than the mixture and removing the supernatant after 30 s; this was repeated twice on the magnet. After addition of 50 µL nuclease-free water, the beads were resuspended by pipetting and kept at 25 °C. for 1 min. The concentrated DNA was obtained by collecting the supernatant. The concentrated DNA was purified by size selection using E-Gel SizeSelect 2% agarose (Life Technologies, Carlsbad, CA, USA). Approximately 350 bp fragments were retrieved; their concentration was measured using a QuantiFluor dsDNA System (Promega, Madison, WI, USA), and the quality was measured with a Bioanalyzer DNA HS kit (Agilent Technologies, Santa Clara, CA, USA). After preparation of the library, 50-bp sequences of the

173 *Bgl*II digested side of the DNA fragments were read using a HiSeq2000 and Hiseq2500 (Illumina, San
174 Diego, CA, USA) by Macrogen. The sequenced reads were demultiplexed by CASAVA 1.8.2 (Illumina).
175 Fastq files were deposited into the DNA Data Bank of Japan Sequence Read Archive as accession no.
176 DRA007047.
177
178 **SNP calling**
179 After removing the reads that contained low-quality bases and adapter sequences from the raw sequence
180 reads using Trimmomatic ver. 0.33 (Bolger et al. 2014), SNPs were called with Stacks ver. 1.37 (Catchen
181 et al. 2013). This process was performed with the default settings of the pipeline *ref_map.pl* in Stacks
182 (population analysis, -m 3 -M 2 -n 1). We then generated an HTML report using a program *Stacks binder*
183 (Yasugi et al. 2018) to visually check the summary of the ddRAD-Seq library and the results of SNP
184 calling (Supplemental Material S1).
185
186 **Genome-wide locus-based phylogeny**
187 We also reconstructed the phylogeny of the genome-wide ddRAD-Seq data set in RAxML (Stamatakis,
188 2014). We used the GAMMA+P-Invar model of sequence evolution and performed a single full maximum
189 likelihood tree search. We applied that the rapid bootstrap algorithm with 1000 replicates to each data set.
190 The resultant tree was plotted using Figtree (http://tree.bio.ed.ac.uk/software/figtree/).
191
192 **Mitochondrial DNA-based phylogeny**
193 We aligned 1828 bp in lengths, including 16S rRNA sequences of 53 samples, and reconstructed the
194 phylogeny of 16S rRNA sequences using maximum likelihood in RAxML, version 8.2.7 (Stamatakis,
195 2014). We used the GAMMA+P-Invar model of sequence evolution and performed a single full maximum

196 likelihood tree search. We applied the rapid bootstrap algorithm with 1000 replicates to each dataset. The
197 resultant tree was plotted using Figtree (http://tree.bio.ed.ac.uk/software/figtree/).
198
199 **Test of genetic introgression using allele frequency data**
200 We analyzed 9 609 SNPs using TreeMix ver. 1.12 software (Pickrell and Pritchard, 2012), which were
201 used to infer population history, including divergence and gene flow, using allele frequency data under
202 genetic drift. TreeMix showed the maximum-likelihood tree with estimated hybridization events, including
203 the direction of gene flow. We ran TreeMix with 0 and 1 migration events from Anglo-Arabian breeds to
204 Taishu breed with various migration rates.
205
206 We used a model-based clustering approach in STRUCTURE (Pritchard et al. 2000) to identify genetic
207 clusters within Japanese native horses and to investigate the degree of genetic introgression at individual
208 level. We ran STRUCTURE from K = 1 to 6, with 10 iterations per K by using all individual data. Then,
209 we ran STRUCTURE from K = 1 to 3, with 10 iterations per K by using data of Anglo-Arabian individuals
210 and Taishu individuals. Each iteration included a burn-in of 50 000 generations, followed by MCMC for
211 100 000 generations. We obtained the optimal K using methods in Evanno et al. (2005) (EVANNO et al.
212 2005) the STRUCTURE harvester (Earl and vonHoldt, 2012). We plotted the results using STRUCTURE
213 PLOT version 2.0 (Ramasamy et al. 2014).
214
215 To test if variations shared SNPs, the Taishu and Anglo-Arabian breeds could be explained by genetic
216 introgression, rather than ancestral variation, we performed ABBA/BABA tests by calculating D-statistics
217 and Z-scores. This measured the signatures of alternative phylogenetic asymmetry and the proportion of the
218 genome that was shared between the two breeds due to genetic introgression. We defined 1 of the 4

Japanese native breeds and Taishu as sister clades. “Anglo-Arabian” and “Thoroughbred” were assumed as the introgression breed and the outgroup breed, respectively. For the test, we used the “CalcD” and “WinCalcD” functions in the “evobiR” R package (Blackmon et al. 2013), with 1000 replicates for each individual test and 100 replicates for each chromosome and regions to estimate variance.

Linkage disequilibrium in Taishu populations

Before detecting regions of high linkage disequilibrium (LD) in the Taishu breed, we conducted phasing of genotype data and imputation of missing data using Beagle ver. 5 (B. L. Browning and S. R. Browning, 2016). We used the phased data with Haploview software ver 4.2 (Barrett, 2009) to calculate pairwise measures of LD among SNPs on the same chromosomes. Using the default method, we divided the region into blocks of strong LD using a standard block definition based on confidence intervals for strong LD (Gabriel et al. 2002) and minor allele frequencies > 0.05. If the haploblock size was > 10 kb, we defined the regions with variations shared between Taishu and Anglo-Arabian as potentially introgressed variation.

Results

SNPs detection in the native Japanese horse

We obtained 312 035 915 sequence reads after removing undesirable reads. The median read number per sample was 4 588 763 (interquartile range: 2 747 437–6 587 774). The median average quality value per sample was 37.465 (interquartile range: 36.77–37.63). RAD-seq library summary is reported in Supplemental Material S1. From these reads, Stacks was used to build 1 363 411 loci that contained 0 or more than 1 SNP. Supplemental Material S1a and A.1b show the number of loci shared by individuals. The number of loci decreased as the number of matching samples increased, with or without SNPs. All information regarding the reads used in Stacks, including that described above, is given in Supplemental Material S1a. We used 9 609 SNPs for analysis, filtered using the criteria over 75% matching samples, 1 SNP, 2 alleles, cut off 0.05% minor alleles, and mapped them to the chromosomes.

Genetic introgression at the population level - Maximum Likelihood phylogenies of 5 native Japanese breeds and Anglo-Arabian -

To confirm admixture between Anglo-Arabian population and Taishu population, we reconstructed the ML phylogeny using 9 609 SNPs. The phylogeny showed that each breed consisted of a single clade (Fig. 1). All individuals of Taishu were grouped in a single clade and separated from all individuals of Anglo-Arabian. Some population structure was detected in the Taishu clade and the genetic distances between Taishu subpopulations were relatively large, but the genetic distances in both subpopulations of Taishu and Anglo-Arabian were large. We sequenced 1829 mtDNA sequences, including 16S rRNA that have slowest evolutionary rate of the present *Equidae* (Achilli et al. 2012). We detected 8 SNPs in 16S rRNA of mtDNA. The phylogeny constructed using mtDNA did not show the pattern of breeds (Supplemental Material S2.).

256

257 **Genetic introgression at the population level - TreeMix analysis -**

258 To estimate a migration rate of the genetic introgression from Anglo-Arabian into Taishu, the TreeMix
259 approach was applied to all 6 breeds. A tree with 0 edges (i.e., no introgression event) explained 98.55% of
260 the variance in relatedness between breeds (Fig. 2a). When a 4% migration edge was allowed from
261 Anglo-Arabian into Taishu, the variance in relatedness between breeds was explained by the model that
262 reached 98.80 %, which was the best-explained migration weight at an introgression from Anglo-Arabian
263 to Taishu (Fig. 2b), based on the assumption that migration did not dramatically improve the variance in
264 relatedness of the tree.

265

266 **Genetic introgression at the individual level - STRUCTURE analysis -**

267 To estimate the degree of genetic introgression from Anglo-Arabian individuals to Taishu at individual
268 level, we used 9609 loci with 1 SNP and 2 alleles in the STRUCTURE analysis. When we used genomic
269 data from all individuals, based on *Ln P*(K) and delta K (EVANNO et al. 2005), we determined that the
270 optimal value of K was 2 (Fig. 3a). One cluster included Taishu, another included Miyako and Yonaguni.
271 Kiso, Hokkaido, and Anglo-Arabian consisted of 1/3 Taishu cluster and 2/3 Miyako-Yonaguni cluster.
272 When K = 5 and 6, results showed an Anglo-Arabian cluster, and that all breeds were grouped individually;
273 however, some Taishu were partially included in the same cluster as Anglo-Arabian. Then, we conducted
274 additional structure analysis using genomic data from only Taishu and Anglo-Arabian. Based on *Ln P*(K)
275 and delta K (EVANNO et al. 2005), we determined that the optimal value of K was 2 (Fig. 3b). When K =
276 3, results showed an Anglo-Arabian cluster. In both analyses, we observed 10 individuals who comprised
277 0.1–10 % of the Anglo-Arabian cluster. However, this degree comprising Anglo-Arabian cluster was
278 detected in all 5 Japanese native breeds (Fig. 3a and Fig. 3b).

279

280 **Genetic introgression at the individual level - ABBA/BABA tests for each Taishu individuals**

281 **-**

282 To confirm that the genetic introgression from Anglo-Arabian to Taishu is more often than in other

283 native Japanese breeds, we conducted four ABBA/BABA tests for all Taishu individuals (Fig. 4 and

284 Supplemental Material S6). The ABBA/BABA test calculates the proportion of ABBA and BABA patterns.

285 An excess of any of these patterns indicates the genetic introgression that can be detected using Patterson's

286 D statistic (Green et al. 2010). If D is significantly different from 0, then the null hypothesis of no genetic

287 introgression is rejected. When using the SNPs of Taishu and 3 of the native Japanese breeds, without

288 Yonaguni as a sister species, almost all individuals had negative Patterson's D. Thus, the genetic

289 introgression from Anglo-Arabian to Taishu did not occur as often as it did in the other 3 breeds. On the

290 other hand, genetic migration from Anglo-Arabian into Taishu occurred more often than that into Yonaguni.

291 If genetic introgression from Anglo-Arabian into Taishu during WWII has left a signature in Taishu

292 individuals, then Patterson's D should be positive when using SNPs of both Miyako and Yonaguni as

293 non-introgressed breeds.

294

295 **Genetic introgression at chromosomal and regional levels - ABBA/BABA tests for each**

296 **chromosome -**

297 After a genetic introgression event, it is possible that the genetic variations from introgression partially

298 remain in the genome of successive generations of Taishu. Thus, the signature of genetic introgression was

299 not detected using individual data of whole genome SNPs. According to the preceding analysis, it is

300 possible that genetic introgression from Anglo-Arabian into Taishu remained in small regions of Taishu

301 genomes. Therefore, we calculated Patterson's D for each chromosome per sample to detect the signatures

of genetic introgression from Anglo-Arabian to Taishu at each chromosome (Fig. 5 and Supplemental Material S7). The results of the ABBA/BABA tests at the chromosomal level showed negative Patterson's D for almost all chromosomes when using the SNPs of Taishu and 3 of the native Japanese breeds, without Yonaguni, as a sister species. However, Patterson's D for chromosomes 21 and 24 were positive in all four patterns of the ABBA/BABA tests (Fig. 5). This strongly indicated that chromosomes 21 and 24 had retained the genetic introgression.

Genetic introgression at chromosomal and regional levels - ABBA/BABA tests for regions at Chr 21 and Chr 24 -

It is assumed that because of incomplete genetic recombination after genetic introgression, the signatures of recent genetic introgression (i.e., during WWII) combined on the each of the chromosomes. To confirm that the signatures on the each of Chr 21 and Chr 24 combined with each other, we calculated Patterson's D for Chr 21 and Chr 24 using 10 non-overlapping SNPs (roughly 10 kb regions) sliding window analysis (Supplemental Material S3, S8 and S9). The regions with positive D were calculated in approximately half of the chromosomes and were on separate chromosomes.

Genetic introgression at the locus level - Shared SNPs among 5 Japanese native breeds and Anglo-Arabian breed -

We counted shared SNPs between Anglo-Arabian to Taishu as the potential introgressed SNPs. The number of breed-specific SNPs is shown in Fig. 6. In current Taishu population, 8 504 loci showed variations. There were 554 loci with Taishu-specific SNPs, which should be retained in conservation plans for this breed. For SNPs that were shared between Taishu and Anglo-Arabian, there were 961 loci that had "potential" introgressed SNPs, which could have reduced the frequency of the SNPs in Taishu. Another 6

988 loci were considered as ancestral variations, which is a common ancestor of all six breeds in this study and should be retained as much as possible.

The number of Taishu-specific SNPs was almost correlated with the size of the chromosome (All SNPs = 31.6 + 2.9 x genome size of each chromosome (Mb), $R^2 = 0.88$, SE = 38.6, $P < 0.001$), but the positive correlation between the potential introgressed SNPs and chromosome size was lower than that for all SNPs (introgressed SNPs = 0.37 + 2.5 x genome size of each chromosome (Mb), $R^2 = 0.68$, SE = 9.12, $P < 0.001$). For example, there were a low number of potential introgressed SNPs on chromosome X, which is the second longest chromosome in the horse genome (Supplemental Material S4). We counted the number of "potential" introgression SNPs at 7 loci on chromosome 21 (9.9% of all shared SNPs on Chr 21) and 19 loci on chromosome 24 (38.8% of all shared SNPs on Chr 24). Chromosomes 21 and 24 which were estimated as introgressed chromosomes by the preceding analysis did not show a substantial number of shared SNPs more than other chromosomes (Supplemental Material S4).

Genetic introgression at the locus level - Shared SNPs between Taishu and Anglo-Arabian on high linkage disequilibrium -

Patterson's D differed depending on the sister breed of the four Japanese native breeds (Fig. 4 and 5); therefore, we conducted another test for recent genetic introgression without using the data from the four Japanese breeds. The genetic introgression from Anglo-Arabian into Taishu occurred relatively recently; therefore, we expected that recent-introgressed SNPs should be in the genomic regions of high linkage disequilibrium (high LD). We counted the shared SNPs in the high LD regions. Of the potential introgressed SNPs, 36 were in high LD regions (10% of all potential introgressed SNPs in >10 kb high LD regions) in 38 Taishu individuals. The frequency of the potential introgressed SNPs in the high LD regions

did not significantly affect the frequency of all SNPs in the high LD regions (2-sample test for equality of proportions with continuity correction, $\chi^2 = 2.03$, df =1, $p = 0.154$).

Genetic introgression at the locus level - Genetic diversity of Japanese native horses -

We calculated the nucleotide diversity (π) of 5 Japanese native horse breeds as the index of genetic diversity. The nucleotide diversity of Taishu was significantly lower than that of the other breeds (Fig. 7, Bonferroni-adjusted Welch's *t*-tests, p <0.001 each). However, the inbreeding coefficient (F_{IS}) for Taishu was not lower than that of other breeds (Supplemental Material S5). Taishu had many unique SNPs (Fig. 6), but most of them have low frequency in the current population.

Discussion

Genetic introgression from Anglo-Arabian to Taishu

We conducted the main 8 analyses to confirm genetic introgression from Anglo-Arabian to Taishu and to estimate genetic diversity of Taishu (Table 1). First, we reconstructed the ML phylogeny by using ddRAD-seq markers to confirm admixture between Anglo-Arabian population and Taishu population (Fig. 1 and Table 1). All individuals grouped based on the breeds and this clear pattern of phylogeny corresponded with previous studies (Tozaki et al. 2003). Tozaki et al. (2003) showed that Kiso and Hokkaido were grouped in a sister clade and that Kiso-Hokkaido clade and Taishu were grouped in a sister clade. It is reasonable to group Yonaguni and Miyako in a sister clade because their habitats are geographically very close. Both Yonaguni and Miyako have smaller body sizes compared with other breeds of Japanese native horses. Thus, we considered that the ML phylogeny by using ddRAD-seq markers is reliable. The phylogeny showed that Taishu population has sub-populations, and the genetic distances between Taishu subpopulations were relatively large. One possible cause could be the rapid decrease in

population size. Another possible cause could be because of the inconvenience caused by the blockage of movement between the north and south islands, which may have resulted in the differences in the breeding populations on Tsushima island . The genetic distances of both subpopulations of Taishu and Anglo-Arabian were large; therefore, we concluded that this structure was not derived from introgression of the Anglo-Arabian breed with Taishu. Then, we reconstructed using the phylogeny of 16S rRNA. The 16S rRNA phylogeny did not reflect the pattern of breeds (Supplemental Material S2) and this concurred with previous studies that show mtDNA in the horse is highly diverse and does not show the phylogenetic pattern of breeds in European and Asian horses (Achilli et al. 2012). This indicated that using partial DNA sequences was not suitable for detecting introgression because they show biased variation in the genomes. Specific regions of horse genomes have undergone rapid and strong artificial selection while others have maintained the ancestral variations from before domestication (Achilli et al. 2012).

Second, we estimated the migration rate of Anglo-Arabian population to Taishu population by TreeMix analysis. The results showed that the migration rate was 4 % in the best-explained tree and the assumption that migration from Anglo-Arabian to Taishu did not dramatically improve the variance in relatedness of the tree, indicated that almost no introgression from Anglo-Arabian into Taishu remained in the current population.

Then, we conducted STRUCTURE analysis to assess the degree of genetic introgression at individual level (Fig. 3 and Table 1). The results of STRUCTURE Analysis showed that 10 individuals of all Taishu individuals comprised 0.1–10 % of the Anglo-Arabian cluster. As this degree of Anglo-Arabian clusters were detected in individuals in other Japanese native breeds (Fig. 3a and 3b), it is not enough to conclude that Taishu individuals who comprised the Anglo-Arabian clusters are introgressed individuals.

Subsequently, we conducted another analysis, ABBA/BABA test, to assess the degree of genetic introgression in comparison with 4 other Japanese breeds (Fig. 4 and Table 1). When using the SNPs of

393 Taishu and 3 of the native Japanese breeds, without Yonaguni as a sister species, almost all individuals had
394 negative Patterson's D, which indicated that the introgression from Anglo-Arabian did not occur in more
395 than 3 breeds. When Yonaguni and Taishu were included, the results indicated that genetic migration from
396 Anglo-Arabian into Taishu occurred more often than into Yonaguni. If recent genetic introgression (i.e.,
397 during WWII) from Anglo-Arabian into Taishu has left a signature in Taishu individuals, then Patterson's
398 D should be positive when using SNPs of non-introgressed breeds (Miyako and Yonaguni). It is difficult to
399 conclude the results of ABBA/BABA test. ABBA/BABA test showed positive D when using Yonaguni as
400 a sister species of Taishu, meaning that genetic introgression from Anglo-Arabian to Taishu was often
401 more than to Yonaguni; however, ABBA/BABA test showed negative D when using Miyako , meaning
402 that genetic introgression from Anglo-Arabian to Taishu was not often more than Miyako, even though
403 both Yonaguni and Miyako were non-introgressed breeds. There are some possible causes of the negative
404 D when using 3 of the native Japanese breeds, without Yonaguni as a sister species. One possibility could
405 be that introgression partially remained in the genome of Taishu individuals and another is that the
406 signatures of introgression was derived from genetic admixture long before genetic introgression during
407 WWII. Therefore, we calculated Patterson’s D for each chromosome per sample (Fig. 5 and Table 1).
408 Patterson’s D for chromosomes 21 and 24 were positive in all four patterns of the ABBA/BABA tests.
409 These results indicated strongly that chromosomes 21 and 24 had retained the genetic introgression. If the
410 signatures of genetic introgression on Chr 21 and Chr 24 were derived from the recent genetic introgression,
411 they must have combined on the each of the chromosomes. To confirm that the signatures on Chr 21 and 24
412 combined each other, we calculated Patterson’s D at region level. Regions with positive Patterson’s D were
413 on separate chromosomes (Supplemental Material S3). The results suggested that the signatures of “recent”
414 genetic introgression were not found in the current Taishu population.
415

"Recent" genetic introgression from Anglo-Arabian to Taishu

We counted the shared SNPs to assess the genetic introgression at loci level and to confirm the signatures of genetic introgression were derived from recent genetic introgression during WWII (Fig. 6). There was a low number of potential introgressed SNPs on chromosome X (Supplemental Material S4). Although mean values of Patterson's D at chromosome X were negative, chromosomes 21 and 24 that were estimated as introgressed chromosomes by the preceding analysis did not show a substantial amount of shared SNP when compared to other chromosomes (Supplemental Material S4). This suggested that the estimation of genetic introgression using only the number of shared variations is not completely accurate. Then, we counted the shared SNPs in the high LD regions because if the genetic introgression from Anglo-Arabian into Taishu occurred recently, the introgressed SNPs were present in the high LD regions (Table 1). 36 SNPs were in high LD regions (10% of all shared SNPs) in 38 Taishu individuals. The frequency of the shared SNPs in the high LD regions did not significantly affect the frequency of all SNPs in the high LD regions.

These results suggested that although the genetic introgression event between Anglo-Arabian and Taishu was recorded during WWII, the incontestable signatures of the recent genetic introgression on their genome were not detected in the current Taishu population. Therefore, it is likely that the Taishu population has undergone a drastic decrease in size with a hard bottleneck, and the introgressed offspring were not preferred by residents on the Tsushima islands. It is possible that many Anglo-Arabian offspring could not tolerate the environment on the islands. The number of Taishu individuals on Tsushima island was 2405 in 1952 (Hayashida, 1972); thus, the population has declined to about 1/60 of its former size. Moreover, the residents of Tsushima island prefer individuals with a smaller body size that are more suitable for agricultural purposes (Hayashida, 1972). Also, Taishu has been maintained on the island without forage,

438 indicating that Taishu has a higher tolerance to low nutrition (Hayashida, 1972), whereas the offspring of
439 Anglo-Arabian and Taishu might have weaker resistance.
440
441 **Genetic diversity of Taishu**
442 The nucleotide diversity of Taishu was significantly lower than that of the other breeds (Fig. 7 and Table
443 1), despite the genetic introgression from Anglo-Arabian, which increased the nucleotide diversity of this
444 breed. This is consistent with the results of other tests of introgression (Table 1). The inbreeding coefficient
445 (F_{IS}) for Taishu was not higher than that of other breeds (Supplemental Material S5), indicating that the low
446 nucleotide diversity of Taishu was not due to a failure of recent artificial breeding. There are many unique
447 SNPs in Taishu (Fig. 6), but they are less frequent in the current population and thus, will be lost. In
448 general, low genetic diversity affects the long-term potential for survival of populations (Bouzat, 2010),
449 and individual fitness because of decreased sperm quality (Hedrick and Fredrickson, 2010), reduced litter
450 size (Hedrick and Fredrickson, 2010), increased mortality of juveniles (RALLS et al. 1988), and increased
451 susceptibility to diseases and parasites (Coltman et al. 1999). Although Taishu has undergone genetic
452 introgression from Anglo-Arabian, there were no, or very few, signatures of recent genetic introgression in
453 the current Taishu population. This suggests that the decrease in genetic diversity is a more urgent issue
454 than the removal of genetic introgression.
455
456 **The utility of ddRAD-Seq for native livestock**
457 Population genetic approaches using ddRAD-Seq can distinguish the breeds of 5 native Japanese horses
458 that have few genetic differences and can evaluate the genetic introgression status and genetic diversity of
459 breeds. In this study, the phylogeny by using ddRAD-seq markers was consistent with the results from

Tozaki et al. (2013), suggesting that ddRAD-seq provides reliable data. ddRAD-seq can provide variable genome wide markers for native livestock that do not have SNP chips or genomic information.

By applying ddRAD-Seq to native livestock, we could have conducted further downstream analysis. Genome-wide markers can provide two methods of analysis: all markers together and markers divided into regions. After several generations, it is possible that the ability to detect introgression is weaker using all genome-wide markers together, because the introgressed regions represent only parts of the genome. In fact, the signatures of introgression in Taishu were found at the chromosomal level and not at the individual level. However, "recent" genetic introgression was not supported by other analyses, and we concluded that the signatures of introgression reflected events older than the introgression event during WWII. Although the signatures of recent genetic introgression events were not detected in many of the analyses, many of the shared SNPs between Taishu and Anglo-Arabian were detected. This indicated that defining genetic introgression using only shared SNPs might lead to overestimation of genetic introgression, while using an insufficient number of, and unevenly distributed, markers also carries a risk of misestimation of introgression. Thus, to detect both the presence and the degree of introgression in native livestock, we need to use both shared SNPs and population genetic approaches using large numbers of genome-wide markers.

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590 **Tables**

591 **Table 1. The summary of the analysis and the results in this study.**

Method	Software	Purpose in this study	Result
Reconstructing phylogenies with ML	RAxML (Stamatakis, 2014)	To confirm admixture between Anglo-Arabian and Taishu populations	Taishu and Anglo-Arabian did not construct the same cluster
The inference of patterns of population splitting and mixing	TreeMix (Pickrell and Pritchard, 2012)	To estimate migration rate from Anglo-Arabian population to Taishu population	4 %, but the assumption of migration does not improve the variance in relatedness of the tree
Model-based clustering of individuals	STRUCTURE (Pritchard et al. 2000)	To estimate the degree of genetic introgression from Anglo-Arabian to Taishu in each individual	Some individuals comprised 0.1-10% of the Anglo-Arabian cluster
ABBA/BABA test for individuals	evobiR (Blackmon et al. 2013)	To confirm genetic introgression from Anglo-Arabian to Taishu in each individual compared with other breeds	Almost all individuals showed negative D
ABBA/BABA test for chromosomes	evobiR (Blackmon et al. 2013)	To confirm introgression from Anglo-Arabian to Taishu at each chromosome	Chr 21 and 24 showed positive D
ABBA/BABA test for regions in Chr 21 and Chr 24	evobiR (Blackmon et al. 2013)	To confirm the “recent “genetic introgression from Anglo-Arabian to Taishu	Positive D regions were separated on the chromosomes
Shared SNPs on High Linkage disequilibrium	Haploview (Barrett, 2009)	To detect shared SNPs between Anglo-Arabian and Taishu on High LD	36 SNPs were detected and did not significantly different a prediction by random distribution of SNPs.

		regions	
Nucleotide diversity	Tassel (Bradbury et al. 2007)	To estimate genetic diversity of Taishu population	Lower than all Japanese native breeds

592 **Figure legends**

593 **Fig. 1. Phylogeny of 5 Japanese horse breeds and Anglo-Arabian.** Maximum Likelihood phylogeny of
594 genome-wide SNPs. The color boxes represent different breeds. Green, purple, yellow, orange, blue, and
595 gray represent Kiso, Hokkaido, Yonaguni, Miyako, Taishu, and Anglo-Arabian, respectively.

596

597 **Fig. 2. Results of TreeMix analysis of 6 Japanese native horse breeds.** Maximum-likelihood trees and
598 the matrices of pairwise residuals for a model allowing (a) 0 migration events and (b) 1 migration event
599 from Anglo-Arabian to Taishu. We estimated that the current Taishu population would have 4% of their
600 ancestry from Anglo-Arabian. Large positive values in the residual matrix indicate a poor fit for the
601 respective pair of populations. Edges representing mixture events are colored according to the weight of the
602 inferred edge.

603

604 **Fig. 3. Genetic clustering using STRUCTURE with an admixture model.** (a) Structure results for 5
605 Japanese horse breeds and Anglo-Arabian from K = 2 to 6. (b) Structure results for Taishu and
606 Anglo-Arabian from K = 2 and K = 3.

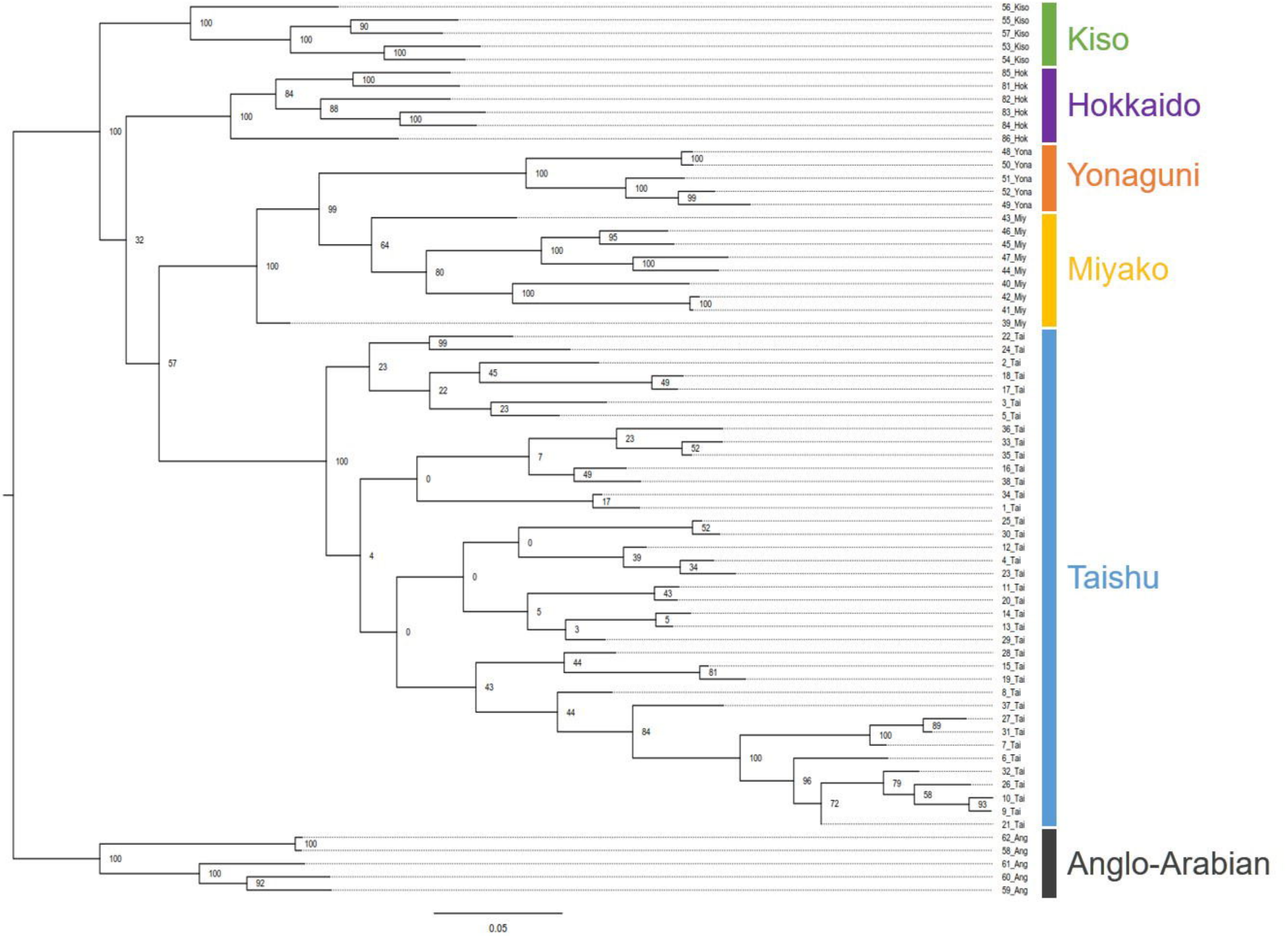
607

608 **Fig. 4. Patterson's D statistic to test for genetic introgression at the individual level.** Positive values
609 indicate gene flow from Anglo-Arabian into Taishu, while negative values indicate gene flow from
610 Anglo-Arabian to other Japanese native breeds. Exact values are shown in Supplemental Material S6.

611

612 **Fig. 5. Patterson's D statistic to test for genetic introgression at the chromosome and region levels.**

613 Patterson's D statistics for each chromosome. Red triangles indicated mean D >0 among four patterns of
614 ABBA/BABA tests. Black circles, black bars, and gray circles indicated mean, median, and outliers of D,
615 respectively.
616
617 **Fig. 6. SNP configurations.** SNPs of Taishu, non-introgressed breeds (including Miyako and Yonaguni),
618 introgressed breeds (including Kiso and Hokkaido), and Anglo-Arabian are represented by blue, yellow,
619 green, and grey, respectively.
620
621 **Fig. 7. Boxplot of genetic diversity of 5 Japanese native horse breeds.** Nucleotide diversity (π) of 5
622 Japanese native horse breeds. Nucleotide diversity of Taishu (n = 38, mean = 0.3075) was significantly
623 lower than that of the other breeds (Miyako: n = 9, mean = 0.3598; Yonaguni: n = 5, mean = 0.4133; Kiso:
624 n = 5, mean = 0.4134; Hokkaido: n = 6, mean = 0.3733).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55_Kiso
57_Kiso
53_Kiso
54_Kiso
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81_Hok
82_Hok
83_Hok
84_Hok
86_Hok
48_Yona
50_Yona
51_Yona
52_Yona
49_Yona
43_Miy
46_Miy
45_Miy
47_Miy
44_Miy
40_Miy
42_Miy
41_Miy
39_Miy
22_Tai
24_Tai
2_Tai
18_Tai
17_Tai
3_Tai
5_Tai
36_Tai
33_Tai
35_Tai
16_Tai
38_Tai
34_Tai
1_Tai
25_Tai
30_Tai
12_Tai
4_Tai
23_Tai
11_Tai
20_Tai
14_Tai
13_Tai
29_Tai
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9_Tai
21_Tai
62_Ang
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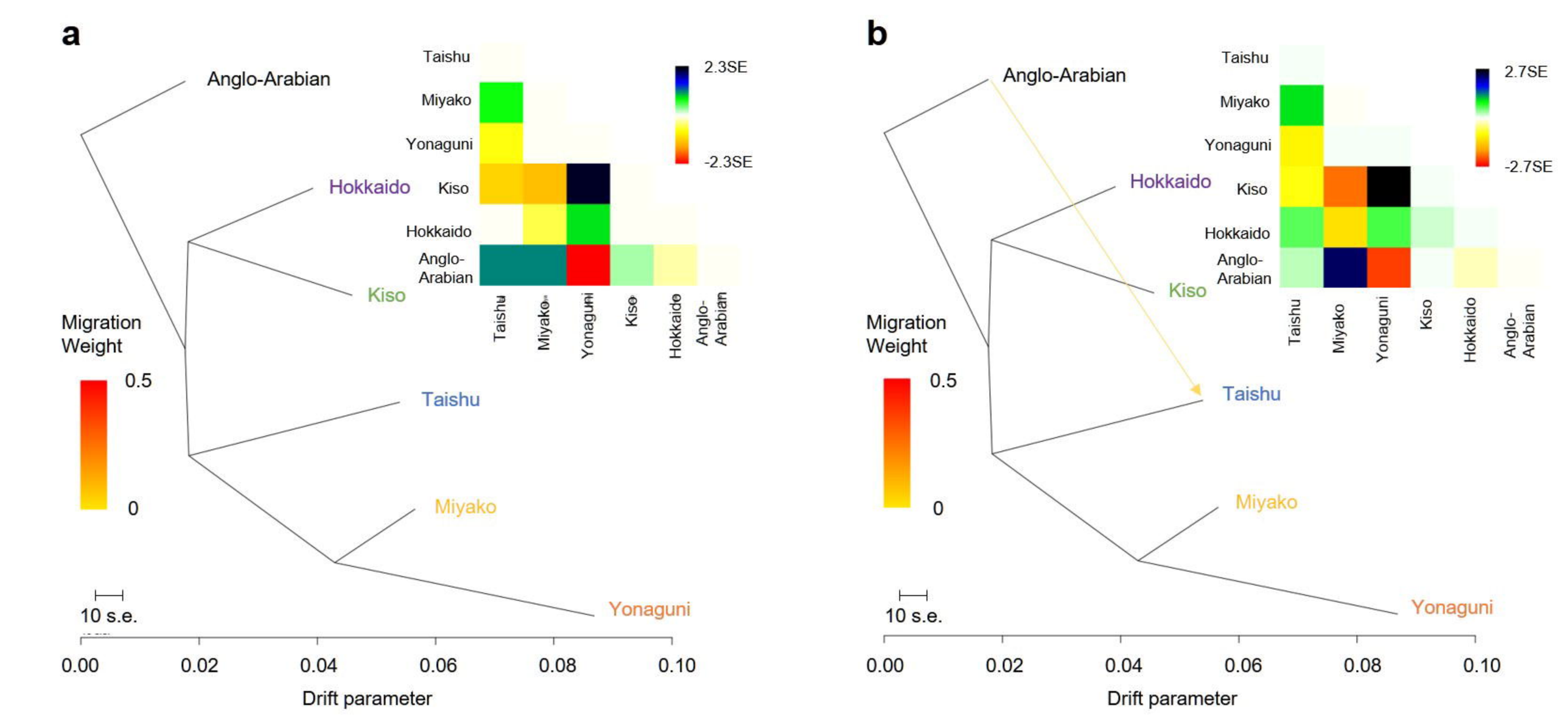
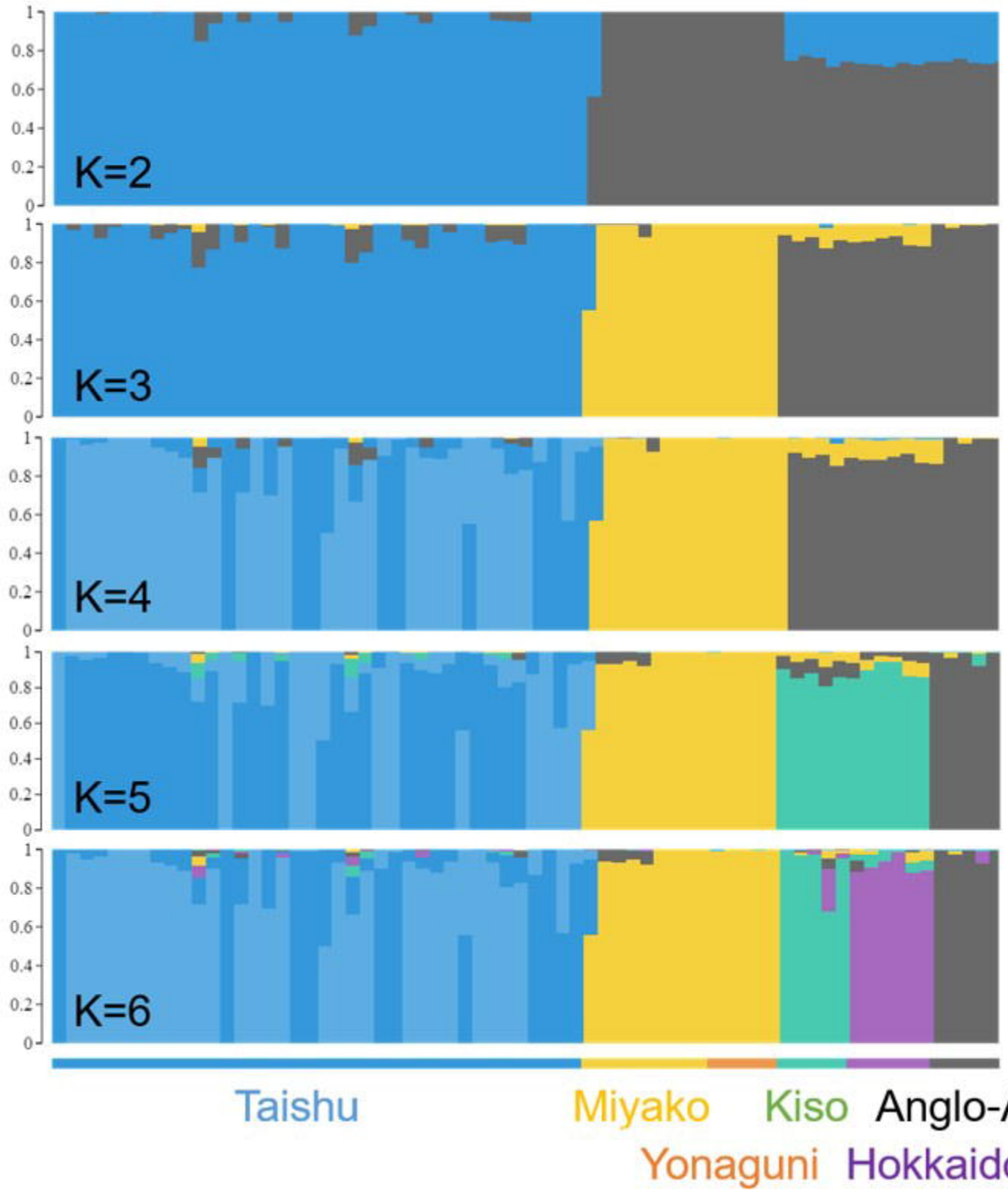
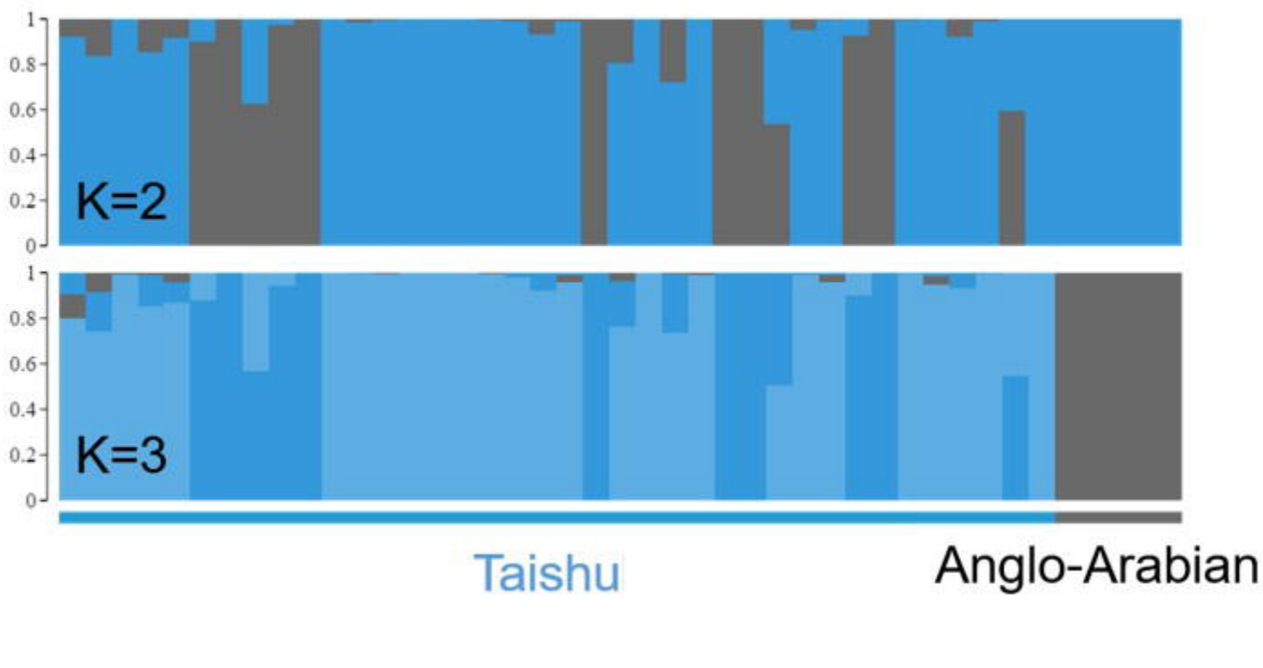
59_Ang
Kiso
Hokkaido
Yonaguni
Miyako
Taishu
Anglo-Arabian
0.05


a
Anglo-Arabian
Taishu
Miyako
Yonaguni
Hokkaido
Kiso
Hokkaido
Anglo-Arabian
2.3SE
-2.3SE
Migration Weight
0.5
0
10 s.e.
0.00
0.02
0.04
0.06
0.08
0.10
Drift parameter
b
Anglo-Arabian
Taishu
Miyako
Yonaguni
Hokkaido
Kiso
Hokkaido
Anglo-Arabian
2.7SE
-2.7SE
Migration Weight
0.5
0
10 s.e.
0.00
0.02
0.04
0.06
0.08
0.10
Drift parameter

a


1
0.8
0.6
0.4
0.2
0
K=2
K=3
K=4
K=5
K=6
Taishu
Miyako
Kiso
Anglo-Arabian
Yonaguni
Hokkaido

b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0.2
0
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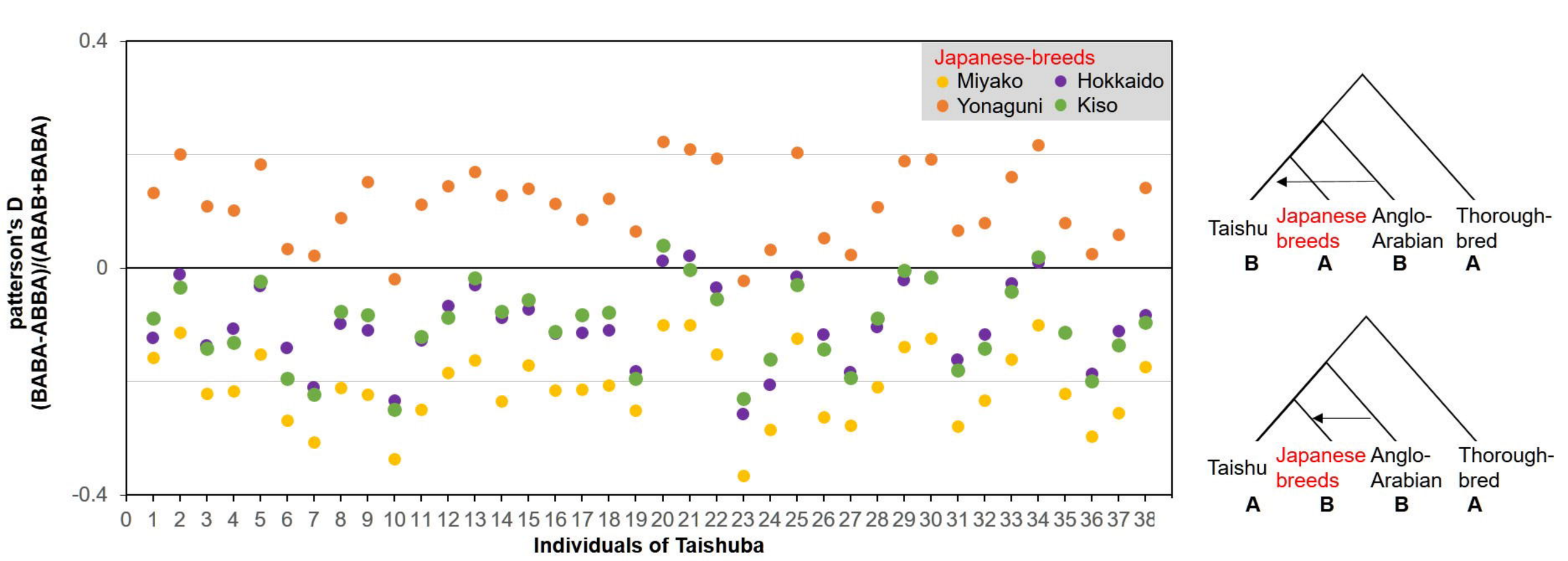
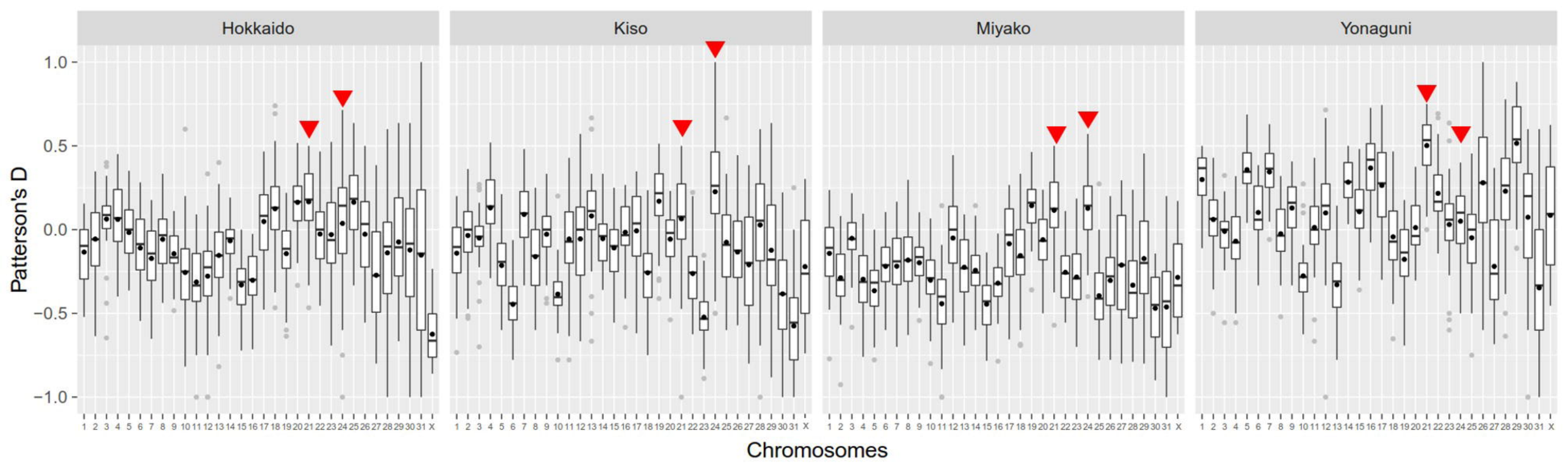
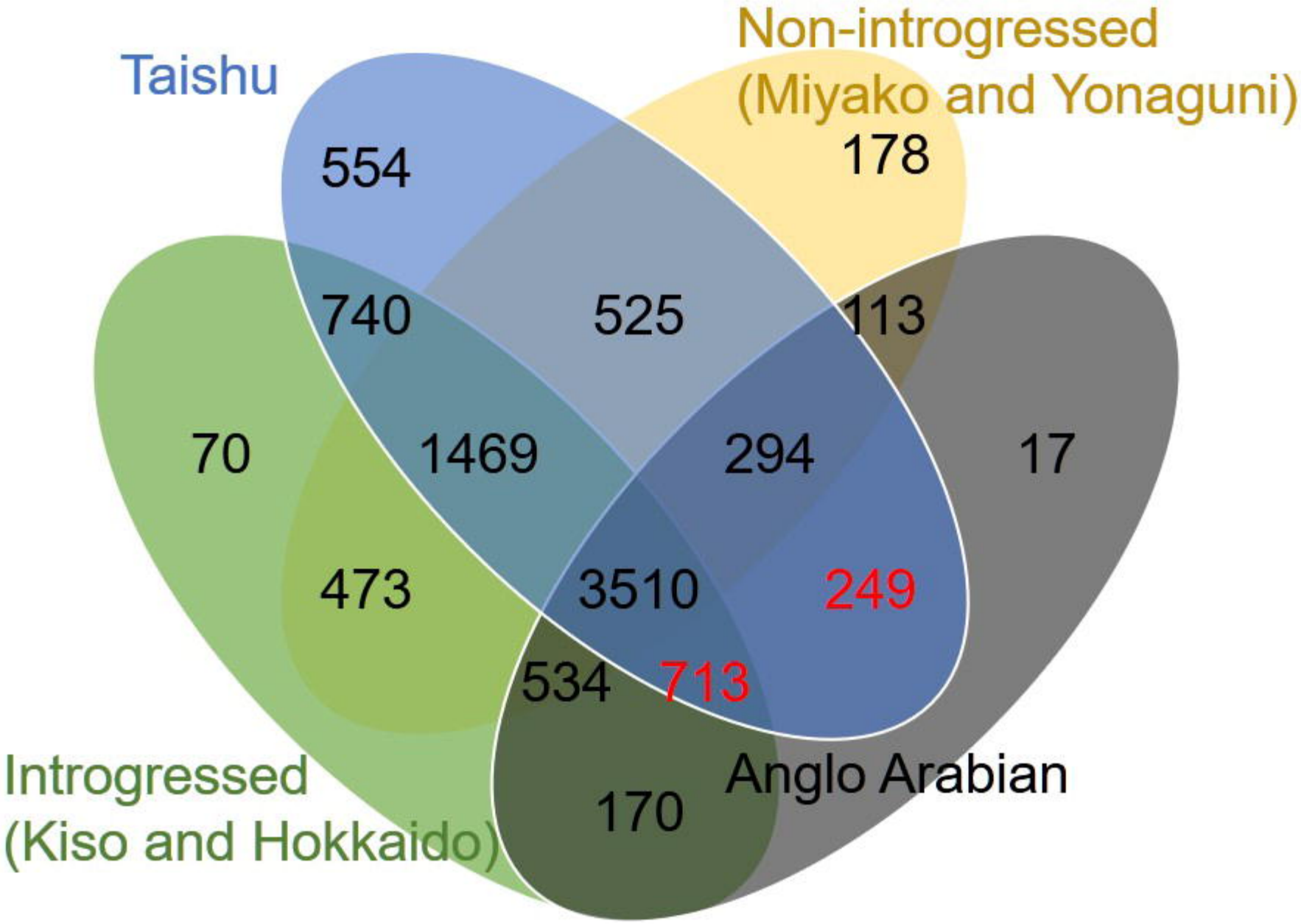
K=3
Taishu
Anglo-Arabian


0.4
0
-0.4
patterson's D
(BABA-ABBA)/(ABAB+BABA)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
Individuals of Taishuba
Japanese-breeds
Miyako
Hokkaido
Yonaguni
Kiso
Taishu
Japanese breeds
Anglo-Arabian
Thorough-bred
B A B A
Taishu
Japanese breeds
Anglo-Arabian
Thorough-bred
A B B A


Hokkaido
Kiso
Miyako
Yonaguni
Patterson's D
1.0
0.5
0.0
−0.5
−1.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 X
Chromosomes


Taishu
Non-introgressed
(Miyako and Yonaguni)
554
178
740
525
113
70
1469
294
17
473
3510
249
534
713
Introgressed
(Kiso and Hokkaido)
Anglo Arabian
170