Breeding microbes: How genetic diversity shapes gut microbial communities in the critically endangered European mink (Mustela lutreola)

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Abstract

Host's fitness can be affected by its genotype and gut microbiota, defined as the microbes living in the host's intestinal tract. This study explored how the genetic diversity of the host influences its bacterial communities in the context of captive breeding programs, for the critically endangered European mink (Mustela lutreola). As stated by the ecosystem on a leash model, mechanisms such as inbreeding depression may lead to changes in immunomodulation and will therefore induce modifications of the gut microbiota. We investigated variation in the gut bacteria through 16S rRNA metabarcoding, related to the genetic diversity of European mink held in captivity in two breeding centers representing separate breeding stocks originating from the western and eastern populations. The genetic diversity of the host was assessed through diversity analysis of the adaptive MHC class I and II genes as well as neutral microsatellite markers. Results indicate lower diversity in neutral and MHC class I genes for the western population, and the opposite for MHC class II. A lower MHC class II gene variability led to an increase in microbial phylogenetic diversity and in abundance depending on the presence of specific MHC-II motifs. This shows the importance of integrating both neutral and adaptive markers when investigating genetic variation in the context of ex situ conservation, as well as gut microbial community assessment. We advocate for more natural mating systems in captive breeding program to foster genetic diversity as a whole to mitigate the effects of genetic drift on those small, isolated populations.

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Running title: Gut bacteria & genetics of the European mink

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Keywords : Mustela lutreola, microbiota, MHC, immunogenetics, breeding program, genetic diversity, captivity

Introduction

More than 5,800 animal species to date are endangered, as the Earth experiences a mass extinction event (Ceballos et al., 2015). Conservationists have multiple factors to consider in order to arrest population declines. Extrinsic of population declines include habitat loss and degradation, over-exploitation, emerging infectious diseases, invasive species, and climate change (Butchart et al. 2010). Intrinsic drivers of extinction, such as genetic factors, also play a key role for population viability, especially when species are reduced to small, isolated populations that can be negatively affected by genetic load (Hedrick, 2001). In this scenario, finding a suitable mate is challenging and reproduction with related individuals can occur, leading to inbreeding depression. Inbreeding has largely been documented in small populations in the wild (Hedrick, 2001; reviewed in Spurgin & Gage, 2019), impacting individual and population fitness through the fixation of detrimental alleles. An increase of detrimental alleles in endangered species increases their susceptibility to extrinsic ecological drivers of extinction (Frankham, 2005). One *ex situ*conservation tool used to mitigate the decrease of genetic diversity in endangered species are Captive Breeding Programs (CBPs). Captive populations of endangered species have the difficult goal of ensuring the survival of stable, self-sustaining populations for later reintroduction into the native habitat (Mallinson, 1995). A key challenge of CBPs is to maintain genetic diversity and avoid inbreeding depression with a small number of founders (Bouman, 1977; Ralls et al., 1979).

The majority of captive breeding schemes rely on studbooks that document pedigree information within the CBPs. While studbooks can be useful to minimize inbreeding effects (Pelletier et al., 2009; Witzenberger & Hochkirch, 2011), information from pedigrees can be flawed in some captive populations (Bowling et al., 2003; Marshall et al., 1999; Signer et al., 1994). Molecular genetic analyses can provide more insights into the relationships within captive populations and their genetic structure. Recently, genetic studies of endangered species have increased, using highly variable loci non-coding for fitness traits such as microsatellite markers (Witzenberger & Hochkirch, 2011). Microsatellites are known to be highly informative for small populations that have reached bottleneck events and are considered a tool to measure neutral genetic variation, and generally represent the extent and pattern of molecular variation within a population (Selkoe & Toonen, 2006). However, both empirical and simulated data indicate that patterns of variation and divergence in adaptive traits are not always associated with concomitant variation in neutral markers (Hedrick, 2001; Larson, 2012; Reed & Frankham, 2001), and some conservation biologists advocate for genetic diversity

analysis for adaptive variation in CBPs (Hughes, 1991; Sommer, 2005). One targeted adaptive region is the Major Histocompatibility Complex (MHC) because its genes play a crucial role in the adaptive immune system. Historical events such as bottlenecks and founder effects, but also constraints of the mating system, such as limited sexual selection in CBPs (Schulte-Hostedde and Mastromonaco 2015), can be reflected in low numbers of MHC alleles (Schad et al., 2004; Hapke et al., 2004). However, in some free-ranging populations, genetic variation at the MHC might persist due to balancing selection, through heterozygote and/or rare allele fitness advantage, despite low levels of variability shown by neutral markers (Jarvi et al., 2004; Rico et al., 2016). These studies support the importance of balancing selection as a mechanism to maintain variation in small populations, and the difficulty of using neutral markers as surrogates for variation in fitness-related loci. Furthermore, MHC genes, particularly of class I, contribute to participate in pheromone recognition, playing a role in sexual selection as well (Penn, 2002).

MHC genes have a crucial role in adaptive immunity in jawed vertebrates, by encoding proteins that bind peptide antigens and present them at the cell surface to lymphocytes for their activation (Ujvari & Belov, 2011). T and B lymphocytes are known to interact directly with gut microbial communities to prevent invasion, pathogenesis, and detrimental immune response towards commensals (Ost & Round, 2018). T cells react to foreign molecules via cell co-receptors: glycoproteins CD8 and CD4 encoded by MHC class II and class I genes, respectively (Penn & Potts 1999). MHC-I molecules are present on all host nucleated cells and antigen presenting cells, and are known to act at the intracellular level, while MHC-II molecules are strictly found on antigen presenting cells and target extracellular microbes (Ost & Round, 2018). MHC presentation of antigens to T cells is thus the basis for initiating antigen specificity and, immune responses are considered specific to the organisms they target.

MHC genes are considered one of the most diverse loci in jawed vertebrates and good candidates for genetic diversity analysis in endangered species (Hughes, 1991). High genetic diversity in these loci could allow targeting more combinations of gut microbes, reflected in variable immunity or tolerance among individuals through rare allele and heterozygous advantage in balancing selection. Bolnick et al. (2014) examined the role of MHC-II motifs (amino acid sequences) in gut microbial community variation in sticklebacks (*Gasterosteus aculeatus*) and found that common MHC motifs were linked to increases in microbial abundance and diversity, and rare motifs had the opposite impacts. Similarly, the microbiota was less phylogenetically diverse in individuals with high MHC-II diversity in the plumage of blue petrels (*Halobaena caerulea* , Leclaire et al., 2018), the gut of laboratory mouse strains (BALB/c, Khan et al., 2019), and the fur microbiota of fur seals (*Arctocephalus gazella* , Grosser et al., 2019). However, no study to date has investigated the MHC-gut microbiota relationships in endangered species under CBPs.

While the host environment has a strong impact on its gut microbial community, the genetics and biology of the host should also be taken into account to fully understand the complex dynamics that occur in this system (Koskella et al., 2017; Spor et al., 2011). This is especially true when considering the gut microbiota of endangered species, where a small number of founders in CBPs are likely to experience overall low genetic variation and diversity. Transition from natural to captive settings for breeding can become more challenging in these conditions when considering host-associated microbes (Trevelline et al., 2019; West et al., 2019). Within this context, Foster et al. (2017) proposed a theoretical framework known as the leash model, which posits that the host is under strong selection to evolve mechanisms to keep the microbiota under control, or "on a leash". The presence of a genetically diverse microbiota leads to the dominance of the fastest growing microbes instead of the microbes that are most beneficial to the host (Foster et al., 2017). The targeting of microbial taxa to either promote or limit their proliferation could thus be beneficial to the host, through its adaptive immune response. We therefore hypothesize that less host control, expressed by more genetically diverse gut microbes, should happen in individuals with reduced genetic diversity in both neutral and adaptive markers. To test this hypothesis, we investigated the genetic diversity and gut microbial community assemblages in the critically endangered European mink (*Mustela lutreola*).

The European mink (E-mink) is a semi-aquatic carnivore from the*Mustelidae* family. Once widespread throughout Europe, it was evaluated as "critically endangered" in 2011 (Maran et al., 2016). There have

been drastic declines in population and range, historically due to overexploitation and still today driven by habitat loss, degradation and fragmentation, road collisions, and the impacts of the alien American mink (*Mustela vison*). E-mink populations are now restricted to enclaves in western France and northern Spain (referred as the western population), the delta of the Danube in Romania, and Ukraine and Russia (referred as the eastern population, Maran et al., 2016), the latter being the focal origin of a captive breeding effort in Estonia with successful reintroduced populations on Hiiumaa Island.

Two major studies have documented the genetic diversity of the free-ranging E-mink populations (Michaux et al., 2005; Cabria et al., 2015). The western population was characterized by a single mitochondrial DNA haplotype against 17 haplotypes in the overall eastern population. Western E-mink had also a much lower microsatellite genetic diversity and allelic richness compared to the eastern population. The authors concluded that the western free-ranging population reached a recent bottleneck, and potentially inbreeding depression due to geographic isolation. However, no proof of fitness reduction in this population through inbreeding has been reported as of yet (Carbonell et al., 2015). The antigen-binding site, encoded by exon 2 of the DRB MHC class II gene, was also investigated in the eastern captive population by Becker et al. (2009). They detected nine alleles within the 20 individuals investigated, estimating low to moderate variability when comparing to other endangered species in similar situations to the E-mink. However, no comparison is yet available for the captive western population.

Both populations are currently in a CBP. The majority of the eastern breeding stock is held at the Tallinn Zoo and extending to other zoos in Europe such as Zoodyssée in France and is only composed of captive-born individuals for over thirty of generations (Maran, pers. comm., 2021). This stock is managed under an EAZA Ex situ Program (EEP). On the other hand, the western breeding stock is held in facilities in Spain. Due to the recent implementation of a CBP in Spain, most of the breeding stock originates from the free-ranging western population captures in Spain within the last seven years (i.e. seven generations), and wild-born individuals from Spain are still being introduced as founders to this date. Those populations are considered as two distinct stocks and are bred separately, although few cross breeds are currently being conducted in Spanish facilities and at the Tallinn Zoo.

Both captive E-mink populations therefore offer a range of variation in neutral and adaptive genetic diversity. Due to extreme population variation over time and the emergence of small and isolated populations, the E-mink provides a unique framework to study the relationship between host genetics and gut microbial communities. Following the ecosystem on a leash model, the aims of this study were to (i) characterize the genetic diversity in the two captive E-mink populations with neutral and adaptive genetic markers as well as their gut microbial communities, (ii) examine the relationship between gut microbial diversity and genetic diversity, and (iii) investigate if gut microbial community structure and composition is linked to specific MHC motifs.

Methods

2.1 Sample collection and DNA extraction

Samples were collected from captive live animals from two populations in 2020. Ten E-mink were sampled in captive settings at the Fundación para la Investigación en Etología y Biodiversidad (FIEB) breeding center in Spain [originated from Spanish free-ranging population (western population)] and fourteen E-mink from the conservation breeding center at Zoodyssée in France [originated from the captive EEP population (representing the eastern population), Table S1]. Two mink sampled in the Spanish breeding center were wild-born individuals but spent at least a year in captivity. One individual sampled in the Spanish breeding center was the result of a crossbreed between western and eastern mink. For MHC and microsatellite markers analysis, hair samples were collected using sterilized tweezers from each animal during a routine procedure. For the microbiota, fresh fecal samples were collected in the enclosure of each animal separately using sterilized tools and kept in 96% ethanol tubes at 4°C until further processing. As the E-mink's diet in captivity varies by day, samples were collected at four occasions depending on the item fed to the animal the previous day. The diet of the E-mink from both breeding centers relied on 3 types of food: trout, mice

and chicken.

DNA from hair samples were extracted using the DNeasy Blood and Tissue Kit from Qiagen using the manufacturer's protocol. DNA extractions from the fecal samples collected were conducted in duplicates using the QiaAmp Mini Kit with Inhibitex (Qiagen, Germany) following the manufacturer's instructions. Two blank extractions were made to control for contamination during the extraction process. A mock community sample (HM-783D, BEI resources) containing genomic DNA from 20 bacterial strains, at concentrations ranging from 0.6 to 1400 pg/µl, was also added in each library to confirm the reliability of our method.

2.2 Microsatellite analysis

Multilocus genotypes were obtained by PCR amplification of 16 autosomal microsatellites (Fleming et al., 1999; Cabria et al., 2007). The forward primer of each locus was 5'-end labeled with a fluorescent dye. The following three multiplex sets were designed: mix 1 (Mvi 114, MLUT 25, MLUT 27, Mvis 099, Mvi 4001), mix 2 (Mvi 087, MLUT 32, MLUT 35, Mvis022, Mvi 1341) and mix 3 (MLUT 04, MER009, Mvis075, Mvis072, MER41, MER022). PCR and genotyping steps were carried out following Pigneur et al. (2019). Length variation determination (alleles and genotypes) was performed using Genemapper 4.0 (Applied Biosystems). To construct consensus multilocus genotypes, an allele was only accepted if observed at least twice. We thus accepted heterozygous genotypes that were observed twice. A homozygote was accepted after three positive PCRs gave the same single allele.

The genetic structure of both sampled captive breeding centers was inferred using Bayesian clustering analysis with Structure 2.3 software (Pritchard et al., 2000). We ran 10 iterations for each K value from 1 to 5 using the admixture model. A total of 10^6 MCMC repetitions were performed after a burn-in period of 20%. The results of the 5 iterations for each K value were summarized and averaged using the Clumpp method (Jakobsson & Rosenberg, 2007). The optimal number of clusters was investigated using the ΔK method (Evanno et al., 2005). F-statistics (pairwise F_{ST}, D_{Jost} and F_{is}), allelic richness (Ar), the expected (He) and observed (Ho) heterozygosity, as well as inbreeding coefficient (F) and multilocus heterozygosity (MLH) were calculated for each defined group/individual in R version 3.5.2 (R Development Core Team, 2008). A R_{ST} genetic distance estimation matrix between individuals was generated using GenAlex 6.5 (Peakall & Smouse, 2006).

2.3 Amplification, sequencing and analysis of MHC genes

The fragment of DRB gene from exon 2 of MHC class II (Beta 1, 231 bp) was targeted using the primers designed by Becker et al. (2009) for*Mustela lutreola* and a fragment from exon 2 (alpha 1) from MHC class I, using the primers Meme-MHC-Iex2F and PpLAa1L250 designed by Sin et al. (2012) for mustelids. PCRs were carried out in 25 µl volumes containing 0.9 µl of primer mix, 5 µl of GoTaq reaction buffer (Promega), 2 µl of $MgCl_2$,0.04 µl of BSA, 0.8 µl of dNTPs, 0.125 µl of GoTaq G2 DNA polymerase (Promega, France) and 3 µl of DNA. The specific protocol was used for PCR: annealing with touchdown protocol from 65°C to 56°C for 30 s. Amplified DNA in duplicates were pooled after quantification using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific Inc., Austria). The library preparation and sequencing were performed by Novogene (UK). Using their designated library protocol, 2×250 bp paired-end sequencing with a depth of 50,000 reads/sample for MHC genotyping and was completed using an Illumina NovaSeq platform (Illumina Biotechnology Co., Novogene, UK).

To analyze MHC-I and MHC-II amplicon sequences, we used the three-step pipeline AmpliSAS (Sebastian et al., 2015). Low-quality sequences with Phred scores lower than 20 were removed and clustering was conducted using the default parameters for Illumina sequences. Already identified alleles of MHC-II DRB for E-mink were extracted from NCBI (Becker et al., 2009), as well as sequences from closely related species (*Mustela putorius* and *Mustela itatsi*) for MHC-I exon 2. If NCBI blast reveled 100% of sequence identify between the discovered alleles in this study and already identified one, their name was replaced by the accession number of these sequences. For the subsequent analysis, we focused on the amino acid translated sequences (referred as MHC motifs) as they are in direct contact with bacteria. We measured motif richness as the number of sequences per individual for each locus. We calculated functional distances between individuals following

the approach described in Strandh et al. (2012). A maximum-likelihood tree was constructed based on the chemical binding properties of the amino acids, as described by five physico-chemical descriptor variables (z-descriptors) for each amino acid, using sequences of *Meles meles* , *Meles leucurus* , *Meles anakuma* and *Martes zibelina* as out-group retrieved through NCBI blast (Figure S1). The trees were used as reference from which the functional distances between individuals were calculated using unweighted UniFrac for both genes (Lozupone & Knight, 2005). Following Bolnick et al. (2014), the genetic distance between each amino acid sequences within each individual (Faith's PD) were calculated, and further defined as motif divergence.

2.5 Statistical analysis for MHC and microsatellites markers between populations

For subsequent analyses, individuals were sorted according to their population origin (western and eastern) that corresponded to structure results (admixed individuals (q < 0.9) were excluded = 0 individuals). Non-parametric Kruskal-Wallis rank sum tests were used to investigate neutral genetic variation with F and MLH calculated from microsatellite markers analysis, between the two E-mink populations and host sex. Adaptive genetic diversity and variation between E-mink populations and individual sex was also observed using the same approach for motif richness for both MHC genes and divergence.

Permutational multivariate analyses of variance (PERMANOA) models*adonis* from the vegan package were constructed with 9,999 permutations with reported F, R2, and p- values, to determine whether there were significant differences in genetic distance matrices for neutral and adaptive markers between E-mink population, individual sex and birth location. Pearson's pairwise correlation tests between the presence of MHC motifs, present in at least three individuals, were conducted to potentially represent haplotype blocks for the two genes investigated. The significance cutoff was set to p-value < .05 for each test. Mantel tests were also conducted between each neutral, MHC-I and MHC-II genetic distance matrices with 1,000 permutations to investigate correlation between each marker.

2.6 Microbiota data generation and processing

After DNA extraction, the targeted gene for bacterial taxonomic affiliation using broad bacterial primers of the region V4 of the 16S rRNA gene (515F-806R, 390 bp) was amplified through PCRs. Amplification, library preparation and sequencing were carried out in a similar manner to MHC genotyping, with a depth of 100,000 reads/samples of two libraries composed of 48-52 samples.

The quality controls of the demultiplexed paired-end reads were performed through the software FastQC (Andrews, 2010). Demultiplexed sequence reads denoising and amplicon sequence variants (ASVs) picking steps were done with the QIIME2 tool (Bolyen et al., 2018; v. 2019.1), using the DADA2 pipeline (Callahan et al., 2016; Callahan et al., 2017). Samples were pooled by individuals to limit bias from diet foods prior to rarefaction. Rarefaction was conducted at 27,000 reads/samples in sampling depth. ASVs—or also referred to as bacterial phylotypes—were then screened to the 97% 16S rRNA gene full-length reference sequences from the Silva v.132 database (Pruesse et al., 2007) for taxonomical association using the VSEARCH classifier implemented in QIIME2 (Bokulich et al., 2018). Sequence alignment and phylogeny building were conducted in QIIME2 for the construction of UniFrac distance matrices.

2.7 Στατιστιχαλ αναλψσις φορ α-διερσιτψ οφ γυτ βαχτερια ασχορδινγ το ηοστ ινφορματιον

All statistical analyses were conducted using the phyloseq and microbiome packages for manipulation of data. Total observed number of bacterial taxa, Chao1, Shannon's diversity index and Faith's PD in each sample were used as metrics to measure the α-diversity of gut bacteria between samples. Chao1 characterizes the overall phylotype richness within a host, Shannon's diversity index takes into account richness and abundance, and Faith's PD is an indicator of genetic diversity within a sample. Differences in the indices according to E-mink populations, sex and birth location of the host were analyzed using Kruskal-Wallis rank sum tests. Linear regression models were also conducted with the different measures of the microsatellites and MHC analysis (MLH, F for microsatellite data, MHC motif richness and divergence) as predictors, and microbial richness indexes as response variable. Homogeneity of variance assumptions were tested using Levene tests and normality of the residuals with Shapiro-Wilk tests and visual representations.

2.8 Στατιστιχαλ αναλψσις φορ β-διερσιτψ οφ γυτ βαχτερια βετωεεν ποπυλατιον ανδ διφφερεντιαλ αβυνδανχε

Weighted and unweighted UniFrac distance matrix between samples were used to investigate differences in gut microbial communities between E-mink population and host sex. These metrics take into account the differences in phylogenetic distance and abundance of each bacterial community between samples, pairwise, emphasizing on rare (unweighted) or abundant microbial taxa (weighted). PERMANOVA tests were conducted in similar conditions than PERMANOVA tests conducted on MHC matrixes. Additionally, models were constructed with adaptive genetic variables: motif richness and divergence from both MHC genes. To test the hypothesis that both neutral and adaptive distances are correlated with gut microbial composition, we employed Mantel tests between each genetic distance and Unifrac distance.

A differential abundance analysis was conducted on the raw ASVs count (after filtering, prior to rarefaction) that were present in more than 10% of all the samples and that had a relative abundance of more than 5% among all taxa. It corresponds to the core microbiota of the dataset, represented by 1203 phylotypes. The abundance analysis was made at the ASV level with the DESeq2 package, using a negative binomial Wald test to test significance in contrast between each E-mink population and each common MHC motifs that were present in at least two individuals. Only microbial ASV with a significance level (α) below .001 after false discovery rate (FDR) corrections were considered using the Benjamini–Hochberg method (Love et al., 2014). From the same core microbiota, we also tested for Pearson correlations between microbial genera' relative abundance per individual and continuous genetic variables (MLH, motif richness and PD for both MHC class I and II) with FDR corrections, for genera that were encountered at least in three individuals.

Results

3.1 Genetic diversity of the European mink captive populations

The microsatellite markers analysis demonstrated an overall population allelic richness per locus of 2.69 with an average of 2.49 in the western population, and 2.82 in the eastern population. Heterozygosity values were lower in the western population compared to eastern population (Table 1). Bayesian assignment recovered two genetic clusters within our population, and no admixture pattern were detected. All individuals clustered according to populations, corresponding to the two different breeding facilities. The offspring with parents of each population was assigned to the western population according to clustering ($p(K_{western})$=0.975). Multilocus heterozygosity was slightly higher in eastern than western populations (Kruskal-Wallis: χ^2=3.4761; p-value=0.0623), but the inbreeding coefficient (F) was not (Kruskal-Wallis: χ^2=0.085714; p-value=0.7697). Overall, sex and birth location had no significant effect on neutral markers' diversity and richness. PERMANOVA on genetic distance based on the microsatellite markers detected no variation according to E-mink population, sex and birth location (Table 2).

Raw MHC amplicon sequencing data consisted of 3,084,478 raw reads with an average length of 230 base pairs for MCH-I and 130 base pairs for MHC-II. After processing, we characterized 13 MHC-I motifs (amino acid sequences) and 6 MHC-II motifs. The average number of motifs per individual was 5.3 and 3.08 (range: 2-9; 2-4) for MHC-I and MHC-II genes respectively, indicating the presence of at least five and two copies for the two regions. For the MHC-I gene, three motifs were strictly present in the eastern E-mink, and one motif in the western population. Comparatively, no motifs were unique to eastern E-mink for MHC-IIex2 gene, and three were strictly found in western E-mink (Figure 1). Spearman correlation tests allowed us to detect haplotype blocks for both genes, mostly attributed to the eastern population (with the motifs Mulu:MHC-I*0003, Mulu:MHC-I*0008, Mulu:MHC-I*0012, Mulu:MHC-I*0013 and Mulu:MHC-I*0015 for MHC-I and Mulu:DRB*90701, EU263553 for MHC-II) and western E-mink (Mulu:MHC-I*0007, Mulu:MHC-I*0009 and Mulu:MHC-I*0011 for MHC-I, KM371114_EU263551, EU263558_LC055119, EU263550_EU263557 and EU263554_EU263552_EU263556 for MHC-II, Figure S2 & Table S2). Most of the variation encountered in both genes was expressed in amino acid residues that influence the binding of CD4 and CD8 glycoproteins involved in antigen presentation for adaptive immunity (Figure S3).

Motif richness and divergence (Faith's PD) were significantly higher in the western population compared to eastern E-mink for MHC-II gene (Kruskal-Wallis: χ^2=13.456, p-value=0.0002; χ^2=8.0614, p-value=0.0045;

respectively). However, for MHC-I, divergence was higher in eastern E-mink compared to the western population, but not motif richness (Kruskal-Wallis: χ^2=5.0097, p-value=0.0252; χ^2=1.5456, p-value=0.2138, respectively). No changes in motif richness nor divergence were observed according to sex for the two genes. However, we did observe significant variation in MHC-II richness according to birth location (Kruskal-Wallis: χ^2=10.854, p-value=0.0125), and a Dunn test with Benjamini-Hochberg correction only detected higher motif richness for the MHC-II gene in captive-born E-mink in Spain compared to the EEP (Dunn: Z=-2.748, adjusted p-value=0.0358). PERMANOVA detected a significant influence of E-mink sex for MHC-I genetic distance, as well an influence of mink population close to the significance threshold (Figure S5), whereas E-mink population was the only variable influenced MHC-II composition variation (Table 2). Finally, Mantel tests showed a positive correlation between MHC class I and neutral markers distances (Mantel: r=0.2761, p-value=0.001).

3.2 α-διερσιτψ οφ γυτ βαστερια ασσορδινγ το ηοστ ινφορματιον

A sample of mock community containing known concentrations of genomic DNA from 20 bacterial strains was sequenced. 19 of the 20 different strains originally included in the sample were detected. The undetected strain was present at the lowest concentration. Therefore, our protocol allowed bacterial DNA detection and identification to the genus level as long as its concentration in the DNA extract was at least 2.8 pg/µl, and provided that the sequence was included in the reference database.

After reads processing, a total read count of 624,796 was obtained for gut microbial communities in captive E-mink, with an average counts per sample of 26,033 after rarefaction to limit sequencing depth artifacts. A total number of 5703 ASVs – or phylotypes – were distinguished in the samples. The gut microbiota of the E-mink was mostly composed of the*Firmicute* phylum (74%), which was dominated by the*Clostridiaceae* and *Peptostreptococcaceae* families, followed by *Proteobacteria* (14%) with *Enterobacteriaceae* ,*Moraxellaceae* and *Pseudomonadaceae* families (Figure 2).

Despite an overall observation of lower microbial richness in the western compared to the eastern populations, no significant results were observed in multiple microbial richness indices (Shannon index, Chao1; Figure 3). However, we did observe slightly lower Faith's PD in western compared to eastern individuals (χ^2= 2.8834, p-value = 0.0895). Western females had significantly lower microbial phylogenetic diversity compared to males (Figure 3). Despite not reaching statistical significance (R^2=0.1626, F=0.5502, p-value=0.7633), linear regression models with alpha diversity measures as response variables showed negative correlations with adaptive genetic richness measures, and particularly strong estimates for MHC-I richness (Figure S4).

3.3 β-διερσιτψ οφ γυτ βαστερια βετωεεν Ε-μινκ ανδ διφφερεντιαλ αβυνδανσε

Beta diversity analyses revealed that bacterial communities were significantly different in composition according to E-mink population only when considering unweighted Unifrac distances, whereas no significant differences in microbial community composition were found between host sexes nor birth locations (Models 4 and 6, Table 2). Moreover, MHC-II gene richness had a small significant influence on gut microbial composition (Model 5, Table 2). This is reflected in the results of the PCoA, which clustered individuals with differences in number of MHC-II motifs (Figure 4). Mantel tests also shown a significant positive correlation between unweighted Unifrac distance and MHC-II genetic distance (Mantel: r=0.4811, p-value=0.019), and despite not reaching statistical significance, a negative correlation with MHC-I genetic distance (Mantel: r=-0.0823, p-value=0.862) and close to zero for neutral markers' distance (Mantel: r=0.0065, p-value=0.229).

We recovered several bacterial genera and families whose relative abundances were significantly correlated with MHC-I and MHC-II richness and divergence, while some marginally correlated with multilocus heterozygosity of neutral markers (Table 3). A majority (65%) of the Pearson correlations appeared to be negative between bacterial genera and genetic indexes. Differential abundance analysis in microbial families according to presence of MHC motifs for both genes between E-mink populations detected a significant increase in abundance for 13 families in the eastern population, for 8 in western E-mink (Figure 5).

From the differential abundance analysis, 22 phylotypes were found to be significantly different in abundance

according to MHC motifs presence. MHC-I motifs explained the variation of 3 phylotypes, and only impacted the *Clostridiaceae* family (Clostridium sensu stricto 1). MHC-II motifs presence was attributed to the altered the abundance of 14 microbial phylotypes and those were mostly more abundant in the eastern E-mink as 13 phylotypes were more abundant in eastern E-mink against one in the western E-mink. MHC-II motifs impacted over twelve genera compared to one for MHC-I motifs. It is also worth noting that both MHC-I and MHC-II motifs presence were observed to alter taxa abundance for 5 phylotypes, mostly from the Proteobacteria phylum (Table 4). The MHC-II motifs KM371114_EU263551, EU263554_EU263552_-EU263556, and Mulu:DRB*90701 respectively took part in the variation in abundance for 17, 15 and 12 phylotypes respectively. MHC-I motif Mulu:MHC-I*00008 was significantly involved in the variation for 4 phylotypes, whereas the other motifs had relatively low impact, as they took part in abundance variation of 1-2 families.

Discussion

Genetic variation in the two E-mink captive breeding programs

Our results show low genetic polymorphism in both captive E-mink populations. Microsatellite markers for both populations exhibited low allelic richness and heterozygosity indices, with the western population having the lowest values, in line with previously published results (Michaux et al., 2005; Cabria et al., 2007; Cabria et al. 2015). However, the eastern E-mink sampled in this study might not reflect the full genetic variation within the entire population, as collected E-mink originated from a subset of the EEP since its start 25 years ago (Becker et al., 2009). Conversely, western E-mink sampled came from wild-born and captive-born individuals from a recent breeding program. Our Bayesian clustering analysis suggests the existence of at least two main genetic units of E-mink defined by their origin with the captive programs, validating our use of the two E-mink groups for studying their genetic and gut microbial variation.

Nonetheless, the two MHC genes investigated revealed differential variation between the two E-mink populations, the MHC-I gene being more divergent in eastern E-mink and the MHC-II gene exhibiting more richness and divergence in western E-mink. Interestingly, the adaptive genetic diversity followed the neutral markers trend only for one gene and not the other, making the assessment of genetic diversity in captive breeding complex. The maintenance of genetic variation in neutral markers through non-selective evolutionary forces (genetic drift, inbreeding) depend on the number of founders in a population, as well as the breeding system of the species. However, balancing selection is believed to counteract those non-selective evolutionary forces in functional genes (Hedrick, 1999), resulting in an excess of heterozygotes in small, isolated populations for MHC-II loci. This pattern has been observed in several isolated populations (Aguilar et al., 2004; Jarvi et al., 2004; Schad et al., 2004), but all species investigated were free ranging, implying less restrictions in the mating system compared to CBPs and therefore stronger sexual selection.

In line with previous evidence of the role of sexual selection for MHC pattern distribution in vertebrates (Edwards & Hedrick, 1998), we observed that sex had an influence on MHC-I gene composition. It has been shown that MHC class I genes may be involved in pheromone recognition, and that mate preferences can be reflected in dissimilarity of MHC patterns (Penn, 2002). In the case of the E-mink, captive-bred males are less successful breeders compared to wild-born males (Kiik et al., 2013). Therefore, mate pairing based only on pedigree might not provide enough information and might be hindered by MHC-I similarities between potential mates. It is particularly striking knowing that the only successful breeding of an eastern pair in 2019 at the French facility was composed of mates having dissimilar MHC-I gene composition (Zazu-Rosin, Figure S5; L. Berthomieu pers. comm., 2020). Variation at neutral markers may thus not accurately reflect variation at potentially relevant genes, particularly those under selection like the MHC (Ujvari & Belov, 2011), and a global genetic assessment should be taken in consideration in conservation genetics for management decisions (Mardsen et al., 2013).

The ecosystem on a leash model in mammalian gut microbiota

The hypothetical framework presented by the ecosystem on a leash model (Foster et al., 2017) suggests that more host control in distantly related microbes, illustrated by the Faith's PD index and Unifrac distances,

should be found in individuals with high MHC diversity. This pattern was observed, although weakly, in alpha diversity analysis for the western population, highlighting the importance of the MHC class II gene above MHC class I and microsatellite markers. It is also worth noting that this result only involves distantly related microbes, emphasizing the fact that a phylogenetically diverse microbiota could lead to the dominance of the fastest growing microbes instead of the microbes that are most beneficial to the host, lending support to the ecosystem on a leash model.

Similar more robust trends were found in beta-diversity analysis, where gut microbial composition was different according to the number of MHC-II motifs a E-mink possessed and the more distant two E-mink are in MHC-II haplotype, the more different in rare gut microbiota composition as well. Mostly negative correlations were observed between microbial abundance and MHC genes richness and divergence, likewise suggesting more host control in individuals with high adaptive genetic variation. This also supports an advantage in balancing selection despite strong genetic drift. The differential abundance analysis also revealed stronger host control in the western population, mostly explained by the presence of specific MHC-II motifs. Moreover, the MHC-II gene was more likely to impact a wide range of microbial taxa. These results are in line with previous studies conducted on fish, mice and birds (Bolnick et al., 2014; Khan et al., 2019; Leclaire et al., 2018). However, we did observe that one motif of MHC-II present in the eastern population also impacted the abundance of several bacterial families, indicating that the captive eastern population still possess interesting motifs for host control.

Because the MHC-I gene targets intracellular non-self-molecules recognition, it would impact a smaller number of bacteria compared to MHC-II (Ost & Round, 2018). Other taxonomic groups such as viruses and protists would need to be targeted for further investigation, and eastern population might be more equipped to recognize them, given the increased diversity for this MHC-I gene (Kubinak et al., 2012). This is of particular importance knowing the circulation of several viruses in free-ranging western E-mink (Fournier-Chambrillon et al., 2004; Philippa et al., 2008; Mañas et al., 2016). In particular the canine distemper virus, which results in a high mortality rate in E-mink, is currently re-emerging in many wild carnivore populations in Europe (Origgi et al., 2012) and has had a major impact on population of E-mink in Navarre, Spain (Fournier-Chambrillon et al., 2021).

Overall, our prediction that less host control will be observed in mink with lower genetic diversity is supported by both alpha and beta diversity for the E-mink. However, both populations have low genetic diversity, and the MHC class II DRB gene seemed to have a stronger influence in gut microbes than other markers. To further validate our results, replicating the study to see if those differences are observable when individuals from the two populations are kept in the same facility to control for the influence of the external environment should be conducted. Given that we only had access to samples from a small fraction of the captive eastern population, our results might also not be representative of the entire captive breeding stock. Despite the gut microbiota variation being a complex puzzle, our study gives more importance to host immunogenetics in the context of species conservation.

Adaptation to captivity and management practices

For MHC genes, rare allele and heterozygous advantage are two types of balancing selection that have been suggested to be important in maintaining high levels of adaptive genetic diversity (Sommer, 2005). Assuming that rare and divergent MHC genotypes are more likely to induce host control on gut microbes, giving a fitness advantage to the host, the co-evolutionary arm race with gut microbes will foster adaptation from microorganisms to not be targeted by common MHC alleles (Kubinak et al., 2012). However, the evolutionary time lag of these antagonistic responses can lead to variation in fitness in a cyclic manner and microbe-driven selection could vary over time and space and between E-mink populations. This mechanism could be of influence in the western E-mink population, given that the breeding program started in 2013 and individuals from the wild are still being captured to increase founder size in the program from the natural habitat. Moreover, low MHC class II gene diversity in the eastern population might indicate that non-evolutionary forces overshadow balancing selection for this locus, which could be mainly explained by extensive constrains in the mating system for a long period of time.

Inadvertent genetic adaptation to captivity for endangered species has been documented over recent years (reviewed in Frankham, 2008). This has been related to a fitness reduction when animals are released in the wild environment, increasing with the numbers of captive-bred generations, including changes in reproductive success, morphology and behavior (Williams & Hoffman, 2009; Willoughby & Christie, 2019). Becker et al. (2009) previously investigated the MHC-II DRB gene in the captive eastern E-mink population, and detected nine alleles, representing 6 motifs. However, ten years later, we observed 3 motifs in the eastern group. The EEP in Estonia started in 1992 and has not been supplemented by wild individuals for at least 25 generations (T. Maran, pers. comm., 2021). Moreover, given the moderate success of the breeding program due to captive-born male behavior (Kiik et al., 2013), this suggests that high number of generations in captivity led to loss of genetic diversity and deleterious genetic fixation took place for this population (Woodworth et al., 2002; Frankham, 2008; Witzenberger & Hochkirch, 2011; Parmar et al., 2017). Even though 90% of the initial gene diversity has been maintained through studbook calculations (T. Maran, pers. comm., 2021), it is likely that studbook measurements might not reflect this trend for all E-mink genes. However, different management strategies have been proposed to mitigate fitness reduction for future reintroduction (reviewed in Williams & Hoffman, 2009) that could be implemented for the E-mink.

One strategy is to limit the number of generations captivity through the use of artificial insemination (AI) and gametes cryopreservation. AI allows to balance the genetic contribution between males, even with individuals showing abnormal breeding behavior. Gametes cryopreservation could extend the generation intervals, only requiring mature females kept in captivity. These tools have been conducted in the CBP of the black footed ferret (*Mustela nigripes*) and benefited the program, notably by using sperm that were stored as long as 20 years (Howard et al., 2015). However, these technics present serious limitations as they are expensive to put in place, demand expertise and investigation for success in every single species, and it is therefore unlikely that they become tools on a regular basis for the E-mink programs at this time.

Another strategy is to translocate animals between breeding centers for reproduction to prevent loss of genetic diversity. Similar to the western captive population of E-mink, these translocations could be composed of wild-born individuals, free of captive selection pressure (Schulte-Hostedde & Mastromonaco, 2015). Occasional translocations from western to eastern captive populations could also be conducted and would potentially mitigate the modest reproductive success within the program. It is worth noting that wild-born animals have been out of reach from the EEP breeding stock so far. However, conducting preliminary MHC variation assessment on reintroduced animals from the eastern stock present in Hiiumaa island, as they no longer face captivity for a number of generations, could be used to identify potential assets to the current breeding stock.

Captivity has been shown to alter gut microbial communities (McKenzie et al., 2017). Combined with this traditional conservation efforts, microbial rescue could also help improve success of managing at-risk populations. For example, the most common cause of mortality in captive cheetahs (*Acinonyx jubatus*) is bacterial infection, possibly because of an increase in pathogenic taxa compared to wild conspecifics (Wasimuddin et al., 2017). Microbial rescue, using probiotics, can stabilize the composition of the gut microbiota of dolphin in captivity (*Lagenorhynchus obliquidens* , Cardona et al., 2018). Implementing wild-like diet-based enrichment could also mitigate captivity effects on gut microbial communities in the same way as captive selection (Mueller et al., 2019; Trevelline et al., 2019; van Leeuwen et al., 2020).

These types of strategies could increase adaptive genetic diversity related to immunomodulation and therefore a fitness advantage to the mink once reintroduced. Coupled with a more in-depth investigation on the gut microbiota of the E-mink according to diet and environment manipulation, these technics can have synergetic effects and foster the success of the CBPs (Gould et al., 2018; West et al., 2019). This first look into the connection between management strategies, genetic diversity and gut bacteria within the CBPs of the E-mink allowed preliminary assessment of the current situation. It also offers many axes of further research and potential strategies with the on-going challenges that many *ex situ* conservation programs face to mitigate species extinction.

Declarations

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Conflict of interest: All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data availability: Supporting information has been made available online. Pooled 16S rRNA sequences available on NCBI accession number: SUB9956843. ASV table, taxonomy table and mapping file have been uploaded, as well as MHC-1, MHC-2 and microsatellite data: Dryad link XXXX, see reference list.

Authors' contributions: J.M., A.S.H. and P.V.L. planned and designed the study. P.V.L. with the help of C.F.-C., P.F., C.A. and L.B. performed the sample preparation for sequencing. J.M. provided sequencing services and together with A.S.H. advised on laboratory and sampling procedures. P.V.L. performed bioinformatics, statistical analyses and the interpretation of results with feedbacks provided by A.S.H. and J.M.. P.V.L. wrote the manuscript with input from all authors.

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Tables and figures

Hosted file

`image1.emf` available at https://authorea.com/users/733008/articles/710989-breeding-microbes-how-genetic-diversity-shapes-gut-microbial-communities-in-the-critically-endangered-european-mink-mustela-lutreola

Table 1. Measures of neutral genetic diversity through microsatellite marker analysis by mink population. N: number of individuals, A: total number of alleles, Ar: mean allelic richness per locus, Ho: observed heterozygosity, He: expected heterozygosity, Fis: mean inbreeding coefficient. Overall mean value for each population across the 16 markers.

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Table 2. Model results of PERMANOVAs for 9,999 permutations for each distance matrix according to variables of interest. Values in bracket are p-values from Marti Anderson's PERMDISP2 procedure for the analysis of multivariate homogeneity of group dispersions (variances). UWU: Unweighted Unifrac, WU: Weighted Unifrac distances.

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Table 3. Genera that were significantly correlated with or differed across variables associated with gut microbial communities. Italicized P-values were marginally significant after False Discovery Rate (FDR) correction of p-values.

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Table 4. Summary of significantly enriched phylotypes among the two mink group according to presence of each MHC motif in each class I and class II genes from the DESEq2 analysis. If multiple motifs explained the variation of one ASV, there were accounted for once if it varied only according to motifs from the same gene.

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Figure 1. Distribution of MHC motifs in both mink population. Grey shading shows the presence of each motif for each individual. Newly discovered motifs were named following the nomenclature, and already identified motifs were named according to the accession number found through NCBI blast.

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Figure 2. The relative abundance of common bacterial families across 24 E-mink individuals from the two breeding populations. The pie chart represents the mean proportion of relative abundance for each microbial phylum for all individuals.

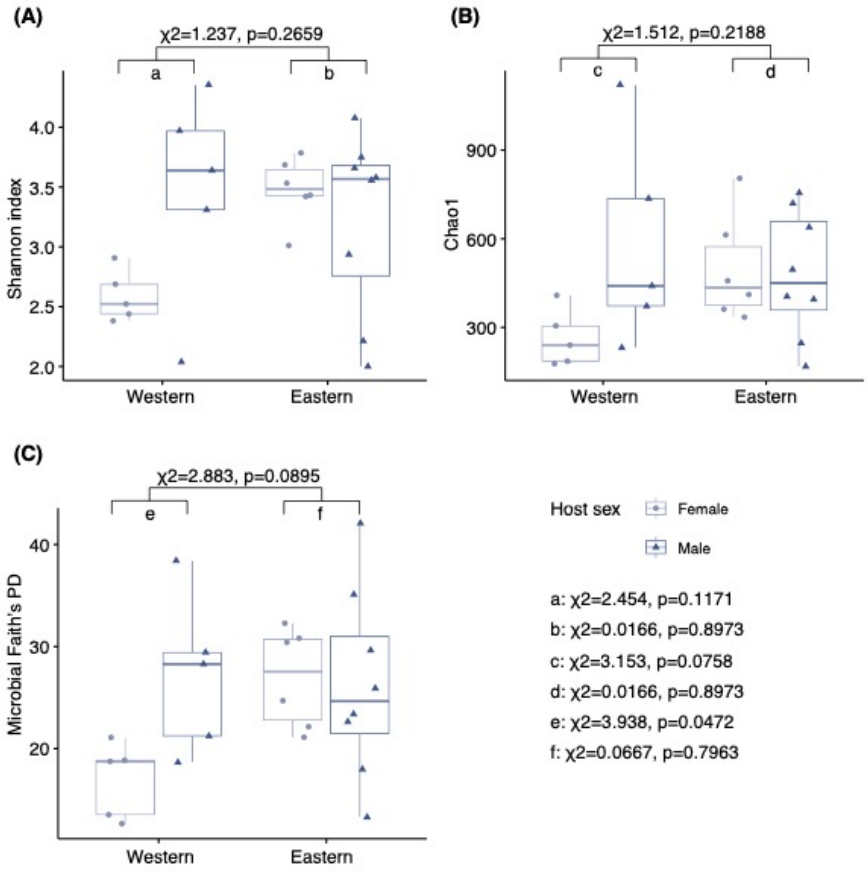

Figure 3. Alpha diversity indexes for microbial taxa richness are shown as boxplots (with median, interquartile range (IQR) and whiskers extending to the last data points). Individual values appear as light blue dots for the female mink and dark blue triangle for the males, for each mink populations. Shannon index (A) expresses changes in taxa richness and abundance, Chao1 (B) variation in strict richness, and Faith's PD (C) for phylogenetic diversity. Results from respective Kruskal-Wallis tests are documented.

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Figure 4. Differences in E-mink microbial community composition visualized by principal coordinates analysis of unweighted UniFrac distances based on rarefied OTU counts. Individuals are labelled according to their name their population or origin (animal names from the eastern population are underlined, not underlined for western). Clusters correspond to number of MHC-II motifs for each individual where the value is indicated at the centroid.

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Figure 5. Mean relative abundance of each family that experienced significant differential abundance between

the two mink populations (orange: Western; blue: Eastern, mean relative abundance per population + standard error). Colored circles next to the family name correspond to which MHC motifs absence/presence the variation in abundance was significant for (yellow: MHCII, blue: MHCI, green: both MHCI and MHCII).

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[dataset] Van Leeuwen, P., Schulte-Hostedde, A., Fournier-Chambrillon, C., Aranda, M.C., Berthomieu, L., Fournier, P., Michaux, J.; 2021; ASV table, taxonomy table and mapping file have been uploaded, MHC-1, MHC-2 and microsatellite data; Dryad; DOI to be determined

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