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Conservation Genetics and Gut Microbial Communities' Variability of the Critically Endangered European Mink Mustela Lutreola: Implications for Captive Breeding Programs

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1 **Title:** Conservation genetics and gut microbial communities' variability of the critically
2 endangered European mink *Mustela lutreola*: Implications for captive breeding programs

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13 Running title: Gut bacteria & genetics of the European mink

14 **Abstract**:
15 Host's fitness can be affected by its genotype and gut microbiota, defined as the microbes
16 living in the host's intestinal tract. This study explored how the genetic diversity of the host
17 influences its bacterial communities in the context of captive breeding programs, for the
18 critically endangered European mink (*Mustela lutreola*). As stated by the ecosystem on a
19 leash model, loss of host genetic diversity may lead to changes in immunomodulation and
20 will therefore induce modifications of the gut microbiota. We investigated variation in the
21 gut bacteria through 16S rRNA metabarcoding, related to the genetic diversity of European
22 mink held in captivity in two breeding centers representing separate breeding stocks
23 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. Those results seem to be linked to management practices that differs between the two programs, especially the number of generations in captivity. Long term *Ex situ* conservation practices can thus modulate gut microbial communities, that might potentially have consequences on the survival of reintroduced animals. We suggest strategies to foster genetic diversity in captive breeding program to mitigate the effects of genetic drift on those small, isolated populations.

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). Intrinsic drivers of extinction, such as genetic factors, 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 population fitness through the fixation of detrimental alleles. An increase

47 of detrimental alleles in endangered species increases their susceptibility to extrinsic
48 ecological drivers of extinction (Frankham, 2005). One *ex situ* conservation tool used to
49 mitigate the decrease of genetic diversity in endangered species are Captive Breeding
50 Programs (CBPs). Captive populations of endangered species have the difficult goal of
51 ensuring the survival of stable, self-sustaining populations for later reintroduction into the
52 native habitat (Mallinson, 1995). A key challenge of CBPs is to maintain genetic diversity
53 and avoid inbreeding depression with a small number of founders (Bouman, 1977; Ralls et
54 al., 1979).
55 The majority of captive breeding schemes rely on studbooks that document pedigree
56 information within the CBPs. While studbooks can be useful to minimize inbreeding
57 effects (Pelletier et al., 2009; Witzenberger & Hochkirch, 2011), information from
58 pedigrees can be flawed in some captive populations (Bowling et al., 2003; Marshall et al.,
59 1999; Signer et al., 1994). Molecular genetic analyses can provide more insights into the
60 relationships within captive populations and their genetic structure. Recently, genetic
61 studies of endangered species have increased, using highly variable loci non-coding for
62 fitness traits such as microsatellite markers (Witzenberger & Hochkirch, 2011).
63 Microsatellites are known to be highly informative as a tool to measure neutral genetic
64 variation, and generally represent the extent and pattern of molecular variation within a
65 population (Selkoe & Toonen, 2006). However, both empirical and simulated data indicate
66 that patterns of variation and divergence in adaptive traits are not always associated with
67 concomitant variation in neutral markers (Hedrick, 2001; Larson, 2012; Reed & Frankham,
68 2001), and some conservation biologists advocate for genetic diversity analysis for
69 adaptive variation in CBPs (Hughes, 1991; Sommer, 2005). One targeted adaptive region

70 is the Major Histocompatibility Complex (MHC) because its genes play a crucial role in
71 the adaptive immune system. Historical events such as bottlenecks and founder effects, but
72 also constraints of the mating system, such as limited sexual selection in CBPs (Schulte-
73 Hostedde and Mastromonaco 2015), can be reflected in low numbers of MHC alleles
74 (Schad et al., 2004; Hapke et al., 2004). However, in some free-ranging populations,
75 genetic variation at the MHC might persist due to balancing selection, through rare allele
76 fitness advantage, despite low levels of variability shown by neutral markers (Jarvi et al.,
77 2004; Rico et al., 2016). These studies support the difficulty of using neutral markers as
78 surrogates for variation in fitness-related loci.
79 MHC genes are considered one of the most diverse loci in jawed vertebrates and good
80 candidates for genetic diversity analysis in endangered species (Hughes, 1991). They have
81 a crucial role in adaptive immunity, by encoding proteins that bind peptide antigens and
82 present them at the cell surface to lymphocytes for their activation (Ujvari & Belov, 2011).
83 MHC-I molecules are known to act at the intracellular level, while MHC-II molecules
84 target extracellular non-self-recognition (Ost & Round, 2018). High genetic diversity in
85 these loci could allow targeting numerous combinations of gut microbes (Ost & Round,
86 2018), reflected in variable immunity or tolerance among individuals through rare allele
87 and heterozygous advantage in balancing selection.
88 Within this context, Foster et al. (2017) proposed a theoretical framework known as the
89 ecosystem on a leash model, which posits that the host is under strong selection to evolve
90 mechanisms to keep the microbiota under control. The presence of a genetically diverse
91 microbiota leads to the dominance of the fastest growing microbes instead of the microbes
92 that are most beneficial to the host (Foster et al., 2017). The targeting of microbial taxa to

93 either limit their proliferation could thus be beneficial to the host, through its adaptive
94 immune response. Bolnick et al. (2014) examined the role of MHC-II motifs (amino acid
95 sequences) in gut microbial community variation in sticklebacks (*Gasterosteus aculeatus*)
96 and found that common MHC motifs were linked to increases in microbial abundance and
97 diversity, and rare motifs had the opposite impacts. Similarly, the microbiota was less
98 phylogenetically diverse in individuals with high MHC-II diversity in the plumage of blue
99 petrels (*Halobaena caerulea*, Leclaire et al., 2018), the gut of laboratory mouse strains
100 (BALB/c, Khan et al., 2019), and the fur microbiota of fur seals (*Arctocephalus gazella*,
101 Grosser et al., 2019). However, no study to date has investigated the MHC-gut microbiota
102 relationships in endangered species under CBPs. We therefore hypothesize that less host
103 control, expressed by more genetically diverse gut microbes, should happen in individuals
104 with reduced genetic diversity in both neutral and adaptive markers. To test this hypothesis,
105 we investigated the genetic diversity and gut microbial community assemblages in the
106 critically endangered European mink (*Mustela lutreola*).
107 The European mink (E-mink) is a semi-aquatic carnivore from the *Mustelidae* family. Once
108 widespread throughout Europe, it was evaluated as "critically endangered" in 2011 (Maran
109 et al., 2016). There have been drastic declines in population and range, historically due to
110 overexploitation and nowadays notably driven by habitat loss, degradation and
111 fragmentation, road collisions, and the impacts of the alien American mink (*Mustela vison*).
112 E-mink populations are now restricted to enclaves in western France and northern Spain,
113 referred as the western population, while the eastern population is present in the delta of
114 the Danube in Romania, Ukraine and Russia (Maran et al., 2016), the latter being the focal

115 origin of a captive breeding effort in Estonia, with successful reintroduced populations on
116 Hiiumaa Island.
117 Two major studies have documented the genetic diversity of the free-ranging E-mink
118 populations (Michaux et al., 2005; Cabria et al., 2015). The western population had a much
119 lower microsatellite genetic diversity and allelic richness compared to the eastern
120 population. The authors concluded that the western free-ranging population reached a
121 recent bottleneck, and potentially inbreeding depression due to geographic isolation.
122 However, no proof of fitness reduction in this population through inbreeding has been
123 reported as of yet (Carbonell et al., 2015). The antigen-binding site, encoded by exon 2 of
124 the DRB MHC class II gene, was also investigated in the eastern captive population by
125 Becker et al. (2009). They detected nine alleles within the 20 individuals investigated,
126 estimating low to moderate variability when comparing to other endangered species in
127 similar situations to the E-mink. However, no comparison is yet available for the captive
128 western population.
129 Both populations are currently in CBP's. The eastern breeding stock is only composed of
130 captive-born individuals for over thirty generations (Maran, pers. comm., 2021) and is
131 managed under an EAZA Ex situ Program (EEP). On the other end, the western breeding
132 stock managed by the Fundación para la Investigación en Etología y Biodiversidad (FIEB)
133 originates from the free-ranging western population captures in Spain within the last seven
134 years (i.e. seven generations), and wild-born individuals from Spain are still being
135 introduced as founders to this date. Those populations are considered as two distinct stocks
136 and are bred separately, although few cross breeds are currently being conducted.

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

146 **Methods**

147 *2.1 Sample collection and DNA extraction*

148 Samples were collected from captive sexually mature mink from both populations in 2020.
149 Fourteen E-mink were sampled in the EEP conservation breeding center at Zoodyssée in
150 France (representing the eastern population), and ten E-mink were sampled in captive
151 settings at the FIEB breeding center in Spain (representing the western population) Table
152 S1]. Two mink sampled in the Spanish breeding center were wild-born individuals but
153 spent at least a year in captivity before sampling. One individual sampled in the Spanish
154 breeding center was the result of a crossbreed between western and eastern mink. For MHC
155 and microsatellite markers analysis, hair samples were collected using sterilized tweezers
156 from each animal during a routine procedure. For the gut microbiota, fresh fecal samples
157 were collected in the enclosure of each animal separately using sterilized tools and kept in
158 96% ethanol tubes at 4°C until further processing. As the E-mink's diet in captivity varies
159 by day, samples were collected at four occasions depending on the item fed to the animal

160 the previous day. The diet of the E-mink from both breeding centers relied on 3 types of
161 food: trout, mice and chicken.
162 DNA from hair samples were extracted using the DNeasy Blood and Tissue Kit from
163 Qiagen using the manufacturer’s protocol. DNA extractions from the fecal samples
164 collected were conducted in duplicates using the QiaAmp Mini Kit with Inhibitex (Qiagen,
165 Germany) following the manufacturer's instructions. Two blank extractions were made to
166 control for contamination during the extraction process. A mock community sample (HM-
167 783D, BEI resources) containing genomic DNA from 20 bacterial strains, at concentrations
168 ranging from 0.6 to 1400 pg/µl, was also added in each library to confirm the reliability of
169 our method.
170 *2.2 Microsatellite analysis*
171 Multilocus genotypes were obtained by PCR amplification of 16 autosomal microsatellites
172 (Fleming et al., 1999; Cabria et al., 2007). The forward primer of each locus was 5’-end
173 labeled with a fluorescent dye. The following three multiplex sets were designed: mix 1
174 (Mvi 114, MLUT 25, MLUT 27, Mvis 099, Mvi 4001), mix 2 (Mvi 087, MLUT 32, MLUT
175 35, Mvis022, Mvi 1341) and mix 3 (MLUT 04, MER009, Mvis075, Mvis072, MER41,
176 MER022). PCR and genotyping steps were carried out following Pigneur et al. (2019).
177 Length variation determination (alleles and genotypes) was performed using Genemapper
178 4.0 (Applied Biosystems). To construct consensus multilocus genotypes, an allele was only
179 accepted if observed at least twice. We thus accepted heterozygous genotypes that were
180 observed twice. A homozygote was accepted after three positive PCRs gave the same
181 single allele.

182 The genetic structure of both sampled captive breeding centers was inferred using Bayesian
183 clustering analysis with Structure 2.3 software (Pritchard et al., 2000). We ran 10 iterations
184 for each K value from 1 to 5 using the admixture model. A total of 10^6 MCMC repetitions
185 were performed after a burn-in period of 20%. The results of the 5 iterations for each K
186 value were summarized and averaged using the Clumpp method (Jakobsson & Rosenberg,
187 2007). The optimal number of clusters was investigated using the ΔK method (Evanno et
188 al., 2005). F-statistics (pairwise F_{ST}, D_{Jost} and F_{is}), allelic richness (Ar), the expected (He)
189 and observed (Ho) heterozygosity, as well as inbreeding coefficient (F) and multilocus
190 heterozygosity (MLH) were calculated for each defined group/individual in R version 3.5.2
191 (R Development Core Team, 2008). A R_{ST} genetic distance estimation matrix between
192 individuals was generated using GenAlex 6.5 (Peakall & Smouse, 2006).

193 *2.3 Amplification, sequencing and analysis of MHC genes*

194 The fragment of DRB gene from exon 2 of MHC class II (Beta 1, 231 bp) was targeted
195 using the primers designed by Becker et al. (2009) for *Mustela lutreola* and a fragment
196 from exon 2 (alpha 1) from MHC class I, using the primers Meme-MHC-Iex2F and
197 PpLAa1L250 designed by Sin et al. (2012) for mustelids. PCRs were carried out in 25 µl
198 volumes containing 0.9 µl of primer mix, 5 µl of GoTaq reaction buffer (Promega), 2 µl of
199 $MgCl_2$,0.04 µl of BSA, 0.8 µl of dNTPs, 0.125 µl of GoTaq G2 DNA polymerase
200 (Promega, France) and 3 µl of DNA. The specific protocol was used for PCR: annealing
201 with touchdown protocol from 65°C to 56°C for 30 s. Amplified DNA in duplicates were
202 pooled after quantification using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo
203 Fisher Scientific Inc., Austria). The library preparation and sequencing were performed by
204 Novogene (UK). Using their designated library protocol, 2×250 bp paired-end sequencing

205 with a depth of 50,000 reads/sample for MHC genotyping and was completed using an
206 Illumina NovaSeq platform (Illumina Biotechnology Co., Novogene, UK).
207 To analyze MHC-I and MHC-II amplicon sequences, we used the three-step pipeline
208 AmpliSAS (Sebastian et al., 2015). Low-quality sequences with Phred scores lower than
209 20 were removed and clustering was conducted using the default parameters for Illumina
210 sequences. Already identified alleles of MHC-II DRB for E-mink were extracted from
211 NCBI (Becker et al., 2009), as well as sequences from related species (*Mustela putorius*
212 and *Mustela itatsi*) for MHC-I exon 2. If NCBI blast reveled 100% of sequence identify
213 between the discovered alleles in this study and already identified one, their name was
214 replaced by the accession number of these sequences. For the subsequent analysis, we
215 focused on the amino acid translated sequences (referred as MHC motifs) as they are in
216 direct contact with bacteria. We measured motif richness as the number of sequences per
217 individual for each locus. We calculated functional distances between individuals
218 following the approach described in Strandh et al. (2012). A maximum-likelihood tree was
219 constructed based on the chemical binding properties of the amino acids, as described by
220 five physico-chemical descriptor variables (z-descriptors) for each amino acid, using
221 sequences of *Meles meles*, *Meles leucurus*, *Meles anakuma* and *Martes zibelina* as out-
222 group retrieved through NCBI blast (Figure S1). The trees were used as reference from
223 which the functional distances between individuals were calculated using unweighted
224 UniFrac for both genes (Lozupone & Knight, 2005). Following Bolnick et al. (2014), the
225 genetic distance between each amino acid sequences within each individual (Faith's PD)
226 were calculated, and further defined as motif divergence.
227 *2.5 Statistical analysis for MHC and microsatellites markers between populations*

228 For subsequent analyses, individuals were sorted according to their population origin
229 (western and eastern) that corresponded to structure results (admixed individuals ($q < 0.9$)
230 were excluded = 0 individuals). Non-parametric Kruskal-Wallis rank sum tests were used
231 to investigate neutral genetic variation with F and MLH calculated from microsatellite
232 markers analysis, between the two E-mink populations and host sex. Adaptive genetic
233 diversity and variation between E-mink populations and individual sex was also observed
234 using the same approach for motif richness for both MHC genes and divergence.
235 Permutational multivariate analyses of variance (PERMANOA) models *adonis* from the
236 vegan package were constructed with 9,999 permutations with reported F, R2, and p-
237 values, to determine whether there were significant differences in genetic distance matrices
238 for neutral and adaptive markers between E-mink population, individual sex and birth
239 location. Pearson's pairwise correlation tests between the presence of MHC motifs, present
240 in at least three individuals, were conducted to potentially represent haplotype blocks for
241 the two genes investigated. The significance cutoff was set to p-value < .05 for each test.
242 Mantel tests were also conducted between each neutral, MHC-I and MHC-II genetic
243 distance matrices with 1,000 permutations to investigate correlation between each marker.
244 *2.6 Microbiota data generation and processing*
245 After DNA extraction, the targeted gene for bacterial taxonomic affiliation using broad
246 bacterial primers of the region V4 of the 16S rRNA gene (515F-806R, 390 bp) was
247 amplified through PCRs. Amplification, library preparation and sequencing were carried
248 out in a similar manner to MHC genotyping, with a depth of 100,000 reads/samples of two
249 libraries composed of 48-52 samples.

250 The quality controls of the demultiplexed paired-end reads were performed through the
251 software FastQC (Andrews, 2010). Demultiplexed sequence reads denoising and amplicon
252 sequence variants (ASVs) picking steps were done with the QIIME2 tool (Bolyen et al.,
253 2018; v. 2019.1), using the DADA2 pipeline (Callahan et al., 2016; Callahan et al., 2017).
254 Samples were pooled by individuals to limit bias from diet foods prior to rarefaction.
255 Rarefaction was conducted at 27,000 reads/samples in sampling depth. ASVs—or also
256 referred to as bacterial phylotypes—were then screened to the 97% 16S rRNA gene full-
257 length reference sequences from the Silva v.132 database (Pruesse et al., 2007) for
258 taxonomical association using the VSEARCH classifier implemented in QIIME2
259 (Bokulich et al., 2018). Sequence alignment and phylogeny building were conducted in
260 QIIME2 for the construction of UniFrac distance matrices.

261 *2.7 Statistical analysis for α-diversity of gut bacteria according to host information*

262 All statistical analyses were conducted using the phyloseq and microbiome packages for
263 manipulation of data. Total observed number of bacterial taxa, Chao1, Shannon's diversity
264 index and Faith's PD in each sample were used as metrics to measure the α-diversity of gut
265 bacteria between samples. Chao1 characterizes the overall phylotype richness within a
266 host, Shannon's diversity index considers richness and abundance, and Faith's PD is an
267 indicator of genetic diversity within a sample. Differences in the indices according to E-
268 mink populations, sex and birth location of the host were analyzed using Kruskal-Wallis
269 rank sum tests. Linear regression models were also conducted with the different measures
270 of the microsatellites and MHC analysis (MLH, F for microsatellite data, MHC motif
271 richness and divergence) as predictors, and microbial richness indexes as response variable.

272 Homogeneity of variance assumptions were tested using Levene tests and normality of the
273 residuals with Shapiro-Wilk tests and visual representations.

274 *2.8 Statistical analysis for β-diversity of gut bacteria between population and differential*
275 *abundance*

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

286 A differential abundance analysis was conducted on the raw ASVs count (after filtering,
287 prior to rarefaction) that were present in more than 10% of all the samples and that had a
288 relative abundance of more than 5% among all taxa. It corresponds to the core microbiota
289 of the dataset, represented by 1203 phylotypes. The abundance analysis was made at the
290 ASV level with the DESeq2 package, using a negative binomial Wald test to test
291 significance in contrast between each E-mink population and each common MHC motifs
292 that were present in at least two individuals. Only microbial ASV with a significance level
293 (α) below .001 after false discovery rate (FDR) corrections were considered using the
294 Benjamini–Hochberg method (Love et al., 2014). From the same core microbiota, we also

295 tested for Pearson correlations between microbial genera' relative abundance per
296 individual and continuous genetic variables (MLH, motif richness and PD for both MHC
297 class I and II) with FDR corrections, for genera that were encountered at least in three
298 individuals.

299 **Results**

300 *3.1 Genetic diversity of the European mink captive populations*

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

314 Raw MHC amplicon sequencing data consisted of 3,084,478 raw reads with an average
315 length of 230 base pairs for MCH-I and 130 base pairs for MHC-II. After processing, we
316 characterized 13 MHC-I motifs (amino acid sequences) and 6 MHC-II motifs. The average
317 number of motifs per individual was 5.3 and 3.08 (range: 2-9; 2-4) for MHC-I and MHC-

318 II genes respectively, indicating the presence of at least five and two copies for the two
319 regions. For the MHC-I gene, three motifs were strictly present in the eastern E-mink, and
320 one motif in the western E-mink. Comparatively, no motif were unique to eastern E-mink
321 for MHC-IIex2 gene, and three were strictly found in western E-mink (Figure 1). Spearman
322 correlation tests allowed us to detect haplotype blocks for both genes, mostly attributed to
323 the eastern population (with the motifs Mulu:MHC-I*0003, Mulu:MHC-I*0008,
324 Mulu:MHC-I*0012, Mulu:MHC-I*0013 and Mulu:MHC-I*0015 for MHC-I and
325 Mulu:DRB*90701, EU263553 for MHC-II) and western E-mink (Mulu:MHC-I*0007,
326 Mulu:MHC-I*0009 and Mulu:MHC-I*0011 for MHC-I, KM371114_EU263551,
327 EU263558_LC055119, EU263550_EU263557 and EU263554_EU263552_EU263556 for
328 MHC-II, Figure S2 & Table S2). Most of the variation encountered in both genes was
329 expressed in amino acid residues that influence the binding of CD4 and CD8 glycoproteins
330 involved in antigen presentation for adaptive immunity (Figure S3).
331 Motif richness and divergence (Faith's PD) were significantly higher in the western E-
332 mink compared to eastern E-mink for MHC-II gene (Kruskal-Wallis: χ^2=13.456, p-
333 value=0.0002; χ^2=8.0614, p-value=0.0045; respectively). However, for MHC-I,
334 divergence was higher in eastern E-mink compared to western E-mink, but not motif
335 richness (Kruskal-Wallis: χ^2=5.0097, p-value=0.0252; χ^2=1.5456, p-value=0.2138,
336 respectively). No changes in motif richness nor divergence were observed according to sex
337 for the two genes. However, we did observe significant variation in MHC-II richness
338 according to birth location (Kruskal-Wallis: χ^2=10.854, p-value=0.0125), and a Dunn test
339 with Benjamini-Hochberg correction only detected higher motif richness for the MHC-II
340 gene in captive-born E-mink in Spain compared to the EEP (Dunn: Z=-2.748, adjusted p-

341 value=0.0358). PERMANOVA detected a significant influence of E-mink sex for MHC-I
342 genetic distance, as well an influence of mink population close to the significance threshold
343 (Figure S5), whereas E-mink population was the only variable influenced MHC-II
344 composition variation (Table 2). Finally, Mantel tests showed a positive correlation
345 between MHC - I and neutral markers distances (Mantel: r=0.2761, p-value=0.001).

346 *3.2 α-diversity of gut bacteria according to host information*

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

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

360 Despite an overall observation of lower microbial richness in the western compared to the
361 eastern populations, no significant results were observed in multiple microbial richness
362 indices (Shannon index, Chao1; Figure 3). However, we did observe slightly lower Faith's
363 PD in western compared to eastern individuals (χ^2= 2.8834, p-value = 0.0895). Western

364 females had significantly lower microbial phylogenetic diversity compared to males
365 (Figure 3). Despite not reaching statistical significance (R^2=0.1626, F=0.5502, p-
366 value=0.7633), linear regression models with alpha diversity measures as response
367 variables showed negative correlations with adaptive genetic richness measures, and
368 particularly strong estimates for MHC-I richness (Figure S4).

369 *3.3 β-diversity of gut bacteria between E-mink and differential abundance*

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

381 We recovered several bacterial genera and families whose relative abundances were
382 significantly correlated with MHC-I and MHC-II richness and divergence, while some
383 marginally correlated with multilocus heterozygosity of neutral markers (Table 3). A
384 majority (65%) of the Pearson correlations appeared to be negative between bacterial
385 genera and genetic indexes. Differential abundance analysis in microbial families
386 according to presence of MHC motifs for both genes between E-mink populations detected

387 a significant increase in abundance for 13 families in the eastern population, for 8 in
388 western E-mink (Figure 5).
389 From the differential abundance analysis, 22 phylotypes were found to be significantly
390 different in abundance according to MHC motifs presence. MHC-I motifs explained the
391 variation of 3 phylotypes, and only impacted the *Clostridiaceae* family (Clostridium sensu
392 stricto 1). MHC-II motifs presence was attributed to the altered the abundance of 14
393 microbial phylotypes and those were mostly more abundant in the eastern E-mink as 13
394 phylotypes were more abundant in eastern E-mink against one in the western E-mink.
395 MHC-II motifs impacted over twelve genera compared to one for MHC-I motifs. It is also
396 worth noting that both MHC-I and MHC-II motifs presence were observed to alter taxa
397 abundance for 5 phylotypes, mostly from the Proteobacteria phylum (Table 4). The MHC-
398 II motifs KM371114_EU263551, EU263554_EU263552_EU263556, and
399 Mulu:DRB*90701 respectively took part in the variation in abundance for 17, 15 and 12
400 phylotypes respectively. MHC-I motif Mulu:MHC-I*00008 was significantly involved in
401 the variation for 4 phylotypes, whereas the other motifs had relatively low impact, as they
402 took part in abundance variation of 1-2 families.
403
404 **Discussion**
405 *Genetic variation in the two E-mink captive breeding programs*
406 Microsatellite markers for both populations exhibited low allelic richness and
407 heterozygosity indices, with the western population having the lowest values, in line with
408 previously published results (Michaux et al., 2005; Cabria et al., 2007; Cabria et al. 2015).
409 However, the eastern E-mink sampled in this study might not reflect the full genetic

410 variation within the entire population, as collected E-mink originated from a subset of the
411 EEP since its start 25 years ago (Becker et al., 2009). Conversely, western E-mink sampled
412 came from wild-born and captive-born individuals from a recent breeding program. Our
413 Bayesian clustering analysis suggests the existence of at least two main genetic units of E-
414 mink defined by their origin with the captive programs, validating our use of the two E-
415 mink groups for studying their genetic and gut microbial variation.

416 Nonetheless, the two MHC genes investigated revealed differential variation between the
417 two E-mink populations, the MHC-I gene being more divergent in eastern E-mink and the
418 MHC-II gene exhibiting more richness and divergence in western E-mink. Interestingly,
419 the adaptive genetic diversity followed the neutral markers trend only for one gene and not
420 the other, making the assessment of genetic diversity in captive breeding complex. The
421 maintenance of genetic variation in neutral markers through non-selective evolutionary
422 forces (genetic drift, inbreeding) depend on the number of founders in a population, as well
423 as the breeding system of the species. However, balancing selection is believed to
424 counteract those non-selective evolutionary forces in functional genes (Hedrick, 1999),
425 resulting in an excess of heterozygotes in small, isolated populations for MHC-II loci. This
426 pattern has been observed in several isolated populations (Aguilar et al., 2004; Jarvi et al.,
427 2004; Schad et al., 2004), but all species investigated were free ranging, implying less
428 restrictions in the mating system compared to CBPs and therefore stronger sexual selection.
429 In line with previous evidence of the role of sexual selection for MHC pattern distribution
430 in vertebrates (Edwards & Hedrick, 1998), we observed that sex had an influence on MHC-
431 I gene composition. It has been shown that MHC class I genes may be involved in
432 pheromone recognition, and that mate preferences can be reflected in dissimilarity of MHC

433 patterns (Penn, 2002). In the case of the E-mink, captive-bred males are less successful
434 breeders compared to wild-born males (Kiik et al., 2013). Therefore, mate pairing based
435 only on pedigree might not provide enough information and might be hindered by MHC-I
436 similarities between potential mates. Variation at neutral markers may thus not accurately
437 reflect variation at potentially relevant genes, particularly those under selection like the
438 MHC (Ujvari & Belov, 2011), and a global genetic assessment should be taken in
439 consideration in conservation genetics for management decisions (Mardsen et al., 2013).

440 *The ecosystem on a leash model in mammalian gut microbiota*

441 The hypothetical framework presented by the ecosystem on a leash model (Foster et al.,
442 2017) suggests that more host control in distantly related microbes, illustrated by the
443 Faith's PD index and Unifrac distances, should be found in individuals with high MHC
444 diversity. This pattern was observed, although weakly, in alpha diversity analysis for the
445 western population, highlighting the importance of the MHC class II gene above MHC
446 class I and microsatellite markers. It is also worth noting that this result only involves
447 distantly related microbes, emphasizing the fact that a phylogenetically diverse microbiota
448 could lead to the dominance of the fastest growing microbes instead of the microbes that
449 are most beneficial to the host, lending support to the ecosystem on a leash model.

450 Similar more robust trends were found in beta-diversity analysis, where gut microbial
451 composition was different according to the number of MHC-II motifs a E-mink possessed
452 and the more distant two E-mink are in MHC-II haplotype, the more different in rare gut
453 microbiota composition as well. Mostly negative correlations were observed between
454 microbial abundance and MHC genes richness and divergence, likewise suggesting more
455 host control in individuals with high adaptive genetic variation. This also supports an

456 advantage in balancing selection despite strong genetic drift. The differential abundance
457 analysis also revealed stronger host control in the western population, mostly explained by
458 the presence of specific MHC-II motifs. Moreover, the MHC-II gene was more likely to
459 impact a wide range of microbial taxa. These results are in line with previous studies
460 conducted on fish, mice and birds (Bolnick et al., 2014; Khan et al., 2019; Leclaire et al.,
461 2018). However, we did observe that one motif of MHC-II present in the eastern population
462 also impacted the abundance of several bacterial families, indicating that the captive
463 eastern population still possess interesting motifs for host control.

464 Because the MHC-I gene targets intracellular non-self-molecules recognition, it would
465 impact a smaller number of bacteria compared to MHC-II (Ost & Round, 2018). Other
466 taxonomic groups such as viruses and protists would need to be targeted for further
467 investigation, and eastern population might be more equipped to recognize them, given the
468 increased diversity for this MHC-I gene (Kubinak et al., 2012). This is of particular
469 importance knowing the circulation of several viruses in free-ranging western E-mink
470 (Fournier-Chambrillon et al., 2004; Philippa et al., 2008; Mañas et al., 2016). The canine
471 distemper virus, which results in a high mortality rate in E-mink, is currently re-emerging
472 in many wild carnivore populations in Europe (Origgi et al., 2012) and has had a major
473 impact on population of E-mink in Navarra, Spain (Fournier-Chambrillon et al., 2022).

474 Overall, our prediction that less host control will be observed in mink with lower genetic
475 diversity is supported by both alpha and beta diversity for the E-mink. However, both
476 populations have low genetic diversity, and the MHC class II DRB gene seemed to have a
477 stronger influence in gut microbes than other markers. To further validate our results,
478 replicating the study to see if those differences are observable when individuals from the

479 two populations are kept in the same facility to control for the influence of the external
480 environment should be conducted. Given that we only had access to samples from a small
481 fraction of the captive eastern population, our results might also not be representative of
482 the entire captive breeding stock. Despite the gut microbiota variation being a complex
483 puzzle, our study gives more importance to host immunogenetics in the context of species
484 conservation.

485 *Adaptation to captivity and management practices*

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

499 Inadvertent genetic adaptation to captivity for endangered species has been documented
500 over recent years (reviewed in Frankham, 2008). This has been related to a fitness reduction
501 when animals are released in the wild environment, increasing with the numbers of captive-

502 bred generations, including changes in reproductive success, morphology and behavior
503 (Williams & Hoffman, 2009; Willoughby & Christie, 2019). Becker et al. (2009)
504 previously investigated the MHC-II DRB gene in the captive eastern E-mink population,
505 and detected nine alleles, representing 6 motifs. However, ten years later, we observed 3
506 motifs in the eastern group. The EEP in Estonia started in 1992 and has not been
507 supplemented by wild individuals for at least 25 generations (T. Maran, pers. comm.,
508 2021). Moreover, given the moderate success of the breeding program due to captive-born
509 male behavior (Kiik et al., 2013), this suggests that high number of generations in captivity
510 led to loss of genetic diversity and deleterious genetic fixation took place for this
511 population (Woodworth et al., 2002; Frankham, 2008; Witzenberger & Hochkirch, 2011;
512 Parmar et al., 2017). Even though 90% of the initial gene diversity has been maintained
513 through studbook calculations (T. Maran, pers. comm., 2021), it is likely that studbook
514 measurements might not reflect this trend for all E-mink genes. However, different
515 management strategies have been proposed to mitigate fitness reduction for future
516 reintroduction (reviewed in Williams & Hoffman, 2009) that could be implemented for the
517 E-mink.
518 One strategy is to translocate animals between breeding centers for reproduction to prevent
519 loss of genetic diversity. Similar to the western captive population of E-mink, these
520 translocations could be composed of wild-born individuals, free of captive selection
521 pressure (Schulte-Hostedde & Mastromonaco, 2015). Occasional translocations from
522 western to eastern captive populations could also be conducted and would potentially
523 mitigate the modest reproductive success within the program. It is worth noting that wild-
524 born animals have been out of reach from the EEP breeding stock so far. However,

525 conducting preliminary MHC variation assessment on reintroduced animals from the
526 eastern stock present in Hiiumaa island, as they no longer face captivity for a number of
527 generations, could be used to identify potential assets to the current breeding stock.
528 Captivity has been shown to alter gut microbial communities (McKenzie et al., 2017).
529 Combined with this traditional conservation efforts, microbial rescue could also help
530 improve success of managing at-risk populations. For example, the most common cause of
531 mortality in captive cheetahs (*Acinonyx jubatus*) is bacterial infection, possibly because of
532 an increase in pathogenic taxa compared to wild conspecifics (Wasimuddin et al., 2017).
533 Microbial rescue, using probiotics, can stabilize the composition of the gut microbiota of
534 dolphin in captivity (*Lagenorhynchus obliquidens*, Cardona et al., 2018). Implementing
535 wild-like diet-based enrichment could also mitigate captivity effects on gut microbial
536 communities in the same way as captive selection (Mueller et al., 2019; Trevelline et al.,
537 2019; van Leeuwen et al., 2020), but further research is needed in the E-mink case.
538 These types of strategies could increase adaptive genetic diversity related to
539 immunomodulation and therefore a fitness advantage to the mink once reintroduced.
540 Coupled with a more in-depth investigation on the gut microbiota of the E-mink according
541 to diet and environment manipulation, these technics can have synergetic effects and foster
542 the success of the CBPs (Gould et al., 2018; West et al., 2019). This first look into the
543 connection between management strategies, genetic diversity and gut bacteria within the
544 CBPs of the E-mink allowed preliminary assessment of the current situation. It also offers
545 many axes of further research and potential strategies with the on-going challenges that
546 many *ex situ* conservation programs face to mitigate species extinction.
547

548 **Declarations**

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554 Data availability: Supporting information has been made available online. Pooled 16S

555 rRNA sequences available on NCBI accession number: SUB9956843. ASV table,

556 taxonomy table and mapping file have been uploaded, as well as MHC-1, MHC-2 and

557 microsatellite data: Dryad link XXXX, see reference list.

558 Authors' contributions: J.M., A.S.H. and P.V.L. planned and designed the study. P.V.L.

559 with the help of C.F.-C., P.F., C.A. and L.B. performed the sample preparation for

560 sequencing. J.M. provided sequencing services and together with A.S.H. advised on

561 laboratory and sampling procedures. P.V.L. performed bioinformatics, statistical analyses

562 and the interpretation of results with feedbacks provided by A.S.H. and J.M.. P.V.L. wrote

563 the manuscript with input from all authors.

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568 (Spain).

569 **Tables and figures**

570

	Eastern	Western	Distance between populations	
N	14	10	Gst	0.0615
A	50	43	Djost	0.0284
Ar	2.82	2.49	Fst	0.1165
Ho	0.54	0.44		
He	0.48	0.43		
Fis	-0.1241	-0.0403		
Fis_Low	-0.252	-0.2265		
Fis_High	-0.0177	0.1047		

571 Table 1. Measures of neutral genetic diversity through microsatellite marker analysis by E-
572 mink captive population (Eastern = EEP breeding center in Zoodysée, France; Western =
573 FIEB breeding center in Spain). N: number of individuals, A: total number of alleles, Ar:
574 mean allelic richness per locus, Ho: observed heterozygosity, He: expected heterozygosity,
575 Fis: mean inbreeding coefficient. Overall mean value for each population across the 16
576 markers.
577

578

Model	Distance matrix	Variables	F statistic	R squared	P-value
1	Microsatellite	Mink population	1.45837	0.06286	0.2318
		Birth location	1.42653	0.06149	0.2390
		Sex	0.77162	0.09978	0.7236
		Residuals		0.77587	
2	MHC-I	Mink population	2.8442	0.09820	0.0797
		Birth location	0.9910	0.10264	0.4476
		Sex	**5.1459**	**0.17767**	**0.0200 (0.387)**
		Residuals		0.62148	
3	MHC-II	**Mink population**	**5.4984**	**0.21090**	**0.0284(0.418)**
		Birth location	0.7741	0.08908	0.5412
		Sex	0.2503	0.00960	0.7140
		Residuals		0.69042	
4	Microbiota (UWU)	**Mink population**	**1.44739**	**0.06203**	**0.0003 (0.229)**
		Birth location	0.96248	0.12375	0.7692
		Sex	0.99747	0.04275	0.4722
		Residuals		0.77146	
5	Microbiota (UWU)	MLH	0.97674	0.04194	0.5673
		MHC-II divergence	1.06218	0.04561	0.2064
		MHC-II richness	**1.21292**	**0.05195**	**0.0219 (0.821)**
		MHC-I divergence	0.99014	0.04252	0.5019
		MHC-I richness	1.10666	0.04752	0.1151
		Residuals		0.77292	
6	Microbiota (WU)	Mink population	1.26796	0.06009	0.2749
		Birth location	0.40095	0.05701	0.8163
		Sex	0.62848	0.02979	0.6250
		Residuals		0.85311	
7	Microbiota (WU)	MLH	1.24147	0.05512	0.2745
		MHC-II divergence	0.27059	0.01201	0.7573
		MHC-II richness	1.37532	0.06106	0.2411
		MHC-I divergence	0.57734	0.02563	0.6026
		MHC-I richness	1.05875	0.04701	0.3326
		Residuals		0.79917	

579
580
581 Table 2. Model results of PERMANOVAs for 9,999 permutations for each distance matrix
582 according to variables of interest. Values in bracket are p-values from Marti Anderson's

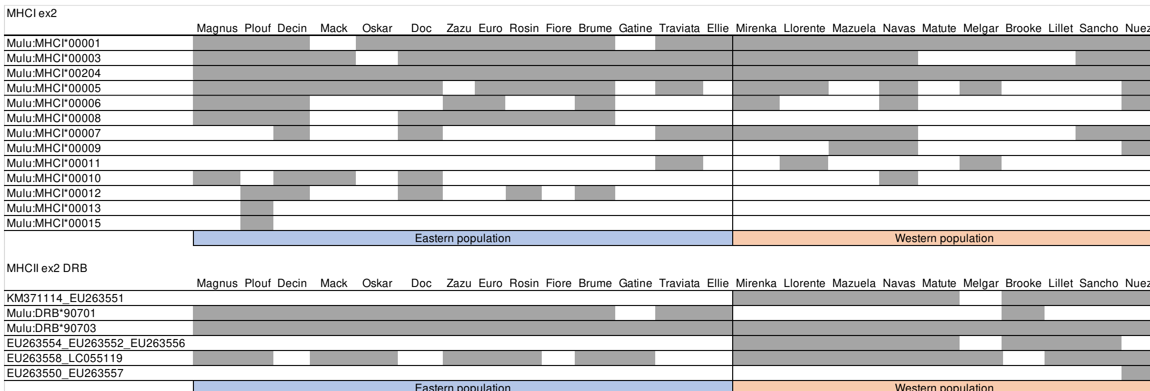
PERMDISP2 procedure for the analysis of multivariate homogeneity of group dispersions (variances). UWU: Unweighted Unifrac, WU: Weighted Unifrac distances.

Genus (family)	Pearson's rho	FDR p-value
Multilocus heterozygosity (neutral markers)		
Clostridium sensus stricto 13 (Clostridiaceae)	0.365	*0.065*
Epulopiscium (Lachnospiraceae)	0.410	0.034
Gemmela (Gemellaceae)	-0.352	*0.077*
Providencia (Morganellaceae)	0.367	*0.063*
MHCI ex2 richness		
Helicobacter (Helicobacteraceae)	-0.400	0.040
Staphylococcus (Staphylococcaceae)	-0.386	0.049
MHCI ex2 divergence (PD)		
Aeromonas (Aeromonadaceae)	0.382	*0.065*
Helicobacter (Helicobacteraceae)	-0.436	0.033
Paeniclostridium (Peptostreptococcaceae)	-0.384	*0.064*
Pseudomonas (Pseudomonadaceae)	0.473	0.019
Staphylococcus (Staphylococcaceae)	-0.467	0.021
MHCII ex2 richness		
Actinomyces (Actinomycetaceae)	-0.438	0.022
Luteolibacter (Rubritaleaceae)	0.366	*0.064*
Sporosarcina (Planococcaceae)	0.398	0.048
Streptococcus (Streptococcaceae)	-0.395	0.043
MHCII ex2 divergence (PD)		
Helicobacter (Helicobacteraceae)	-0.456	0.025
Lysinibacillus (Planococcaceae)	-0.632	0.0035
Staphylococcus (Staphylococcaceae)	-0.540	0.046
Streptococcus (Streptococcaceae)	-0.711	0.0001
Terrisporobacter (Peptostreptococcaceae)	-0.516	*0.063*

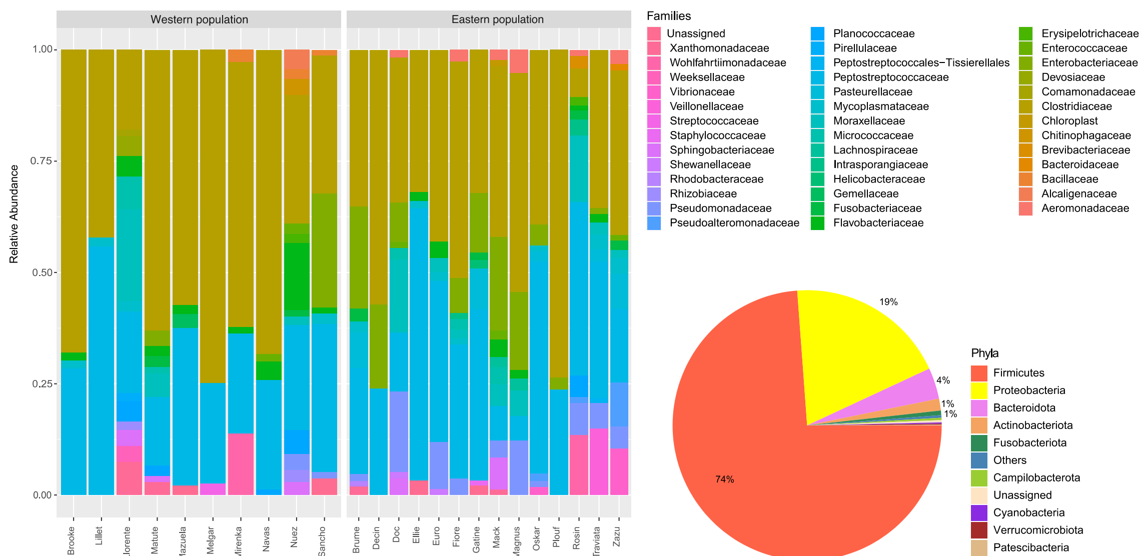
Table 3. Genera that were significantly correlated with or differed across variables associated with gut microbial communities. Italicized P-values were marginally significant after False Discovery Rate (FDR) correction of p-values.

Bacteria Taxonomy				Increased abundance in:	Eastern mink			Western mink		
				Explained by presence of:	MHC-I motifs	MHC-II motifs	Both	MHC-I motifs	MHC-II motifs	Both
Phylum	Class	Order	Family	Genus						
Campilobacterota										
	Campylobacteria	Campylobacterales	Helicobacteraceae	Helicobacter		1				
Firmicutes										
	Bacilli	Bacillales	Planococcaceae	Sporosarcina					1	
		Mycoplasmatales	Mycoplasmataceae	Mycoplasma						1
	Clostridia	Clostridiales	Clostridiaceae	Clostridium_sensu_stricto_1	2			1		
				Clostridium_sensu_stricto_13		1				
		Lachnospirales	Lachnospiraceae	Epulopiscium		1				
		Peptostreptococcales-Tissierellales	Peptostreptococcaceae	Romboutsia		1				
Proteobacteria										
	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Oceanisphaera		1				
		Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas			1			
			Shewanellaceae	Shewanella		2				
		Enterobacterales	Enterobacteriaceae	Escherichia-Shigella		1				
		Oceanospirillales	Unassigned	Unassigned		1				
		Pseudomonadales	Moraxellaceae	Acinetobacter		2				
			Pseudomonadaceae	Pseudomonas		2	1			
		Vibrionales	Vibrionaceae	Vibrio			1			
		Xanthomonadales	Xanthomonadaceae	Luteimonas						1
				Total by gene and population	2	13	3	1	1	2

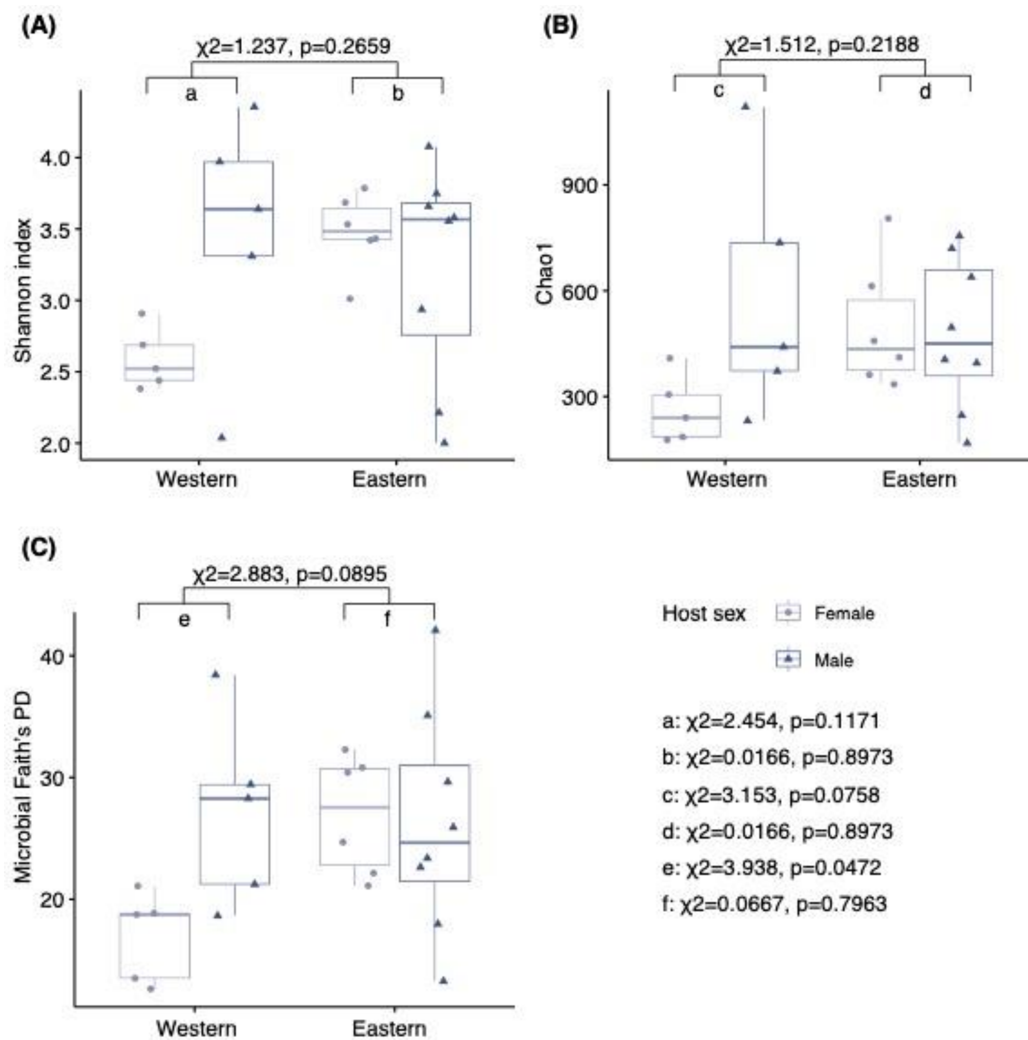
Table 4. Summary of significantly enriched phylotypes among the two mink group according to presence of each MHC motif in each class I and class II genes from the DESEq2 analysis. If multiple motifs explained the variation of one ASV, there were accounted for once if it varied only according to motifs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



598
599
600 Figure 1. Distribution of MHC motifs in both mink population. Grey shading shows the
601 presence of each motif for each individual. Newly discovered motifs were named following
602 the nomenclature, and already identified motifs were named according to the accession
603 number found through NCBI blast.
604

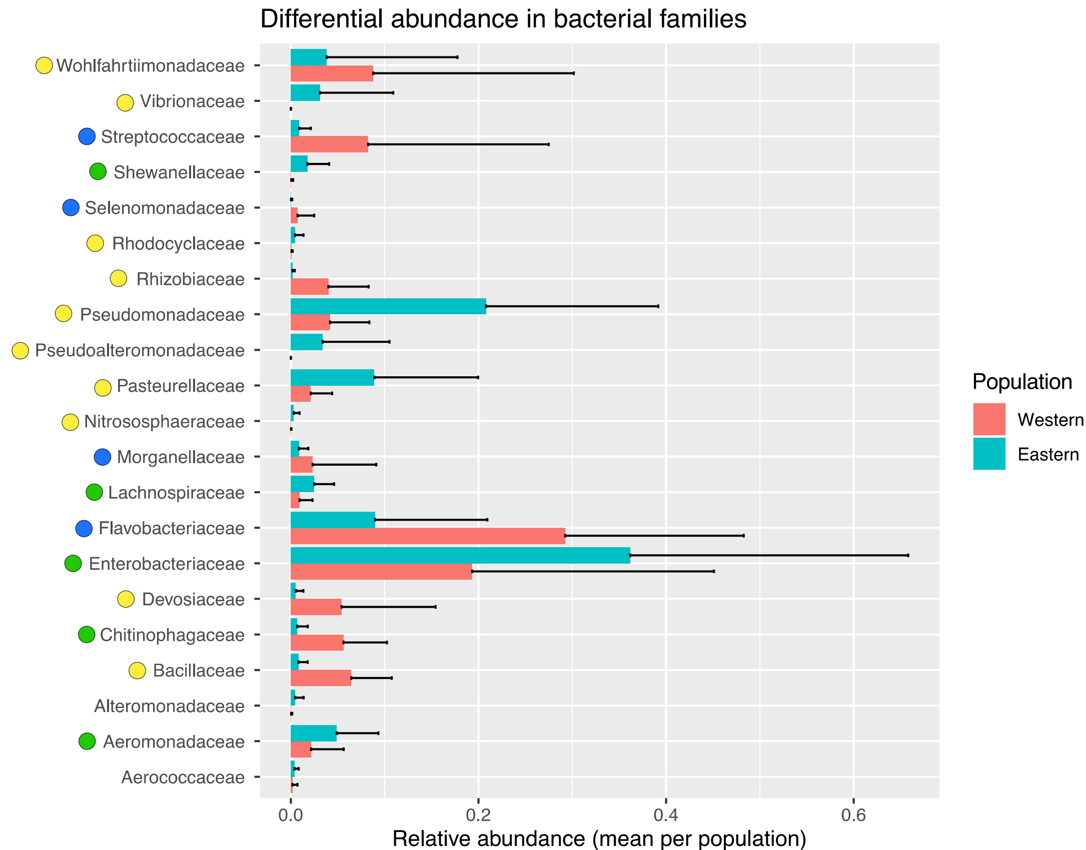


605
606 Figure 2. The relative abundance of common bacterial families across 24 E-mink
607 individuals from the two breeding populations. The pie chart represents the mean
608 proportion of relative abundance for each microbial phylum for all individuals.
609



610
611 Figure 3. Alpha diversity indexes for microbial taxa richness are shown as boxplots (with
612 median, interquartile range (IQR) and whiskers extending to the last data points).
613 Individual values appear as light blue dots for the female mink and dark blue triangle for
614 the males, for each mink populations. Shannon index (A) expresses changes in taxa
615 richness and abundance, Chao1 (B) variation in strict richness, and Faith's PD (C) for
616 phylogenetic diversity. Results from respective Kruskal-Wallis tests are documented.
617

618
619 Figure 4. Differences in E-mink microbial community composition visualized by principal
620 coordinates analysis of unweighted UniFrac distances based on rarefied OTU counts.
621 Individuals are labelled according to their name their population or origin (animal names
622 from the eastern population are underlined, not underlined for western). Clusters
623 correspond to number of MHC-II motifs for each individual where the value is indicated
624 at the centroid.



625
626 Figure 5. Mean relative abundance of each family that experienced significant differential
627 abundance between the two mink populations (orange: Western; blue: Eastern, mean
628 relative abundance per population + standard error). Colored circles next to the family
629 name correspond to which MHC motifs absence/presence the variation in abundance was
630 significant for (yellow: MHCII, blue: MHCI, green: both MHCI and MHCII).
631

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636
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