Genetic monitoring of the captive population of the critically endangered Brazilian Merganser (*Mergus octosetaceus*)

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Supplementary Materials

Figures

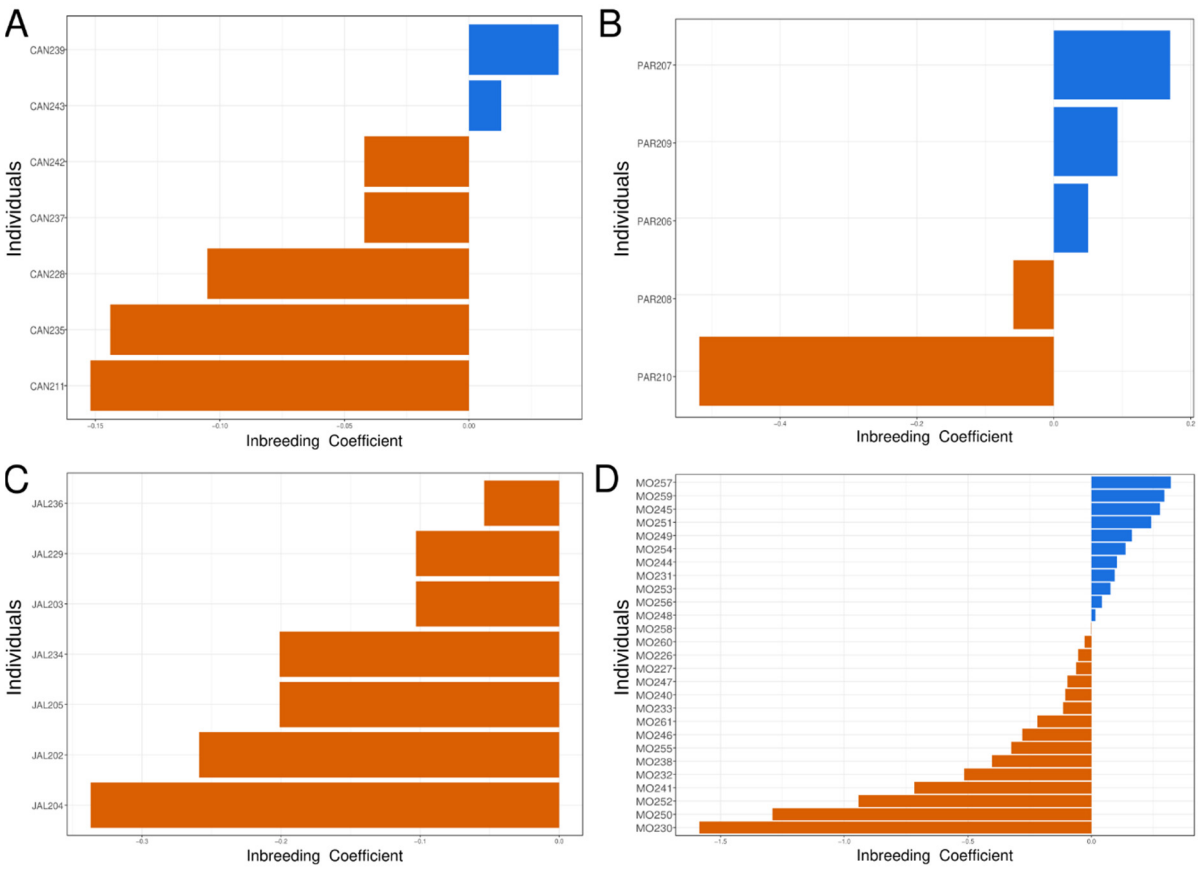

Figure S1. Inbreeding coefficients for the four subdivisions of the captive population of Brazilian Merganser: **A –** Serra da Canastra National Park (Canastra - CAN) individuals; **B –** Alto Paranaíba region (Paranaíba - PAR) individuals; **C –** Jalapão State Park (Jalapão - JAL) individuals; **D –** Captive-bred individuals (MO) individuals.

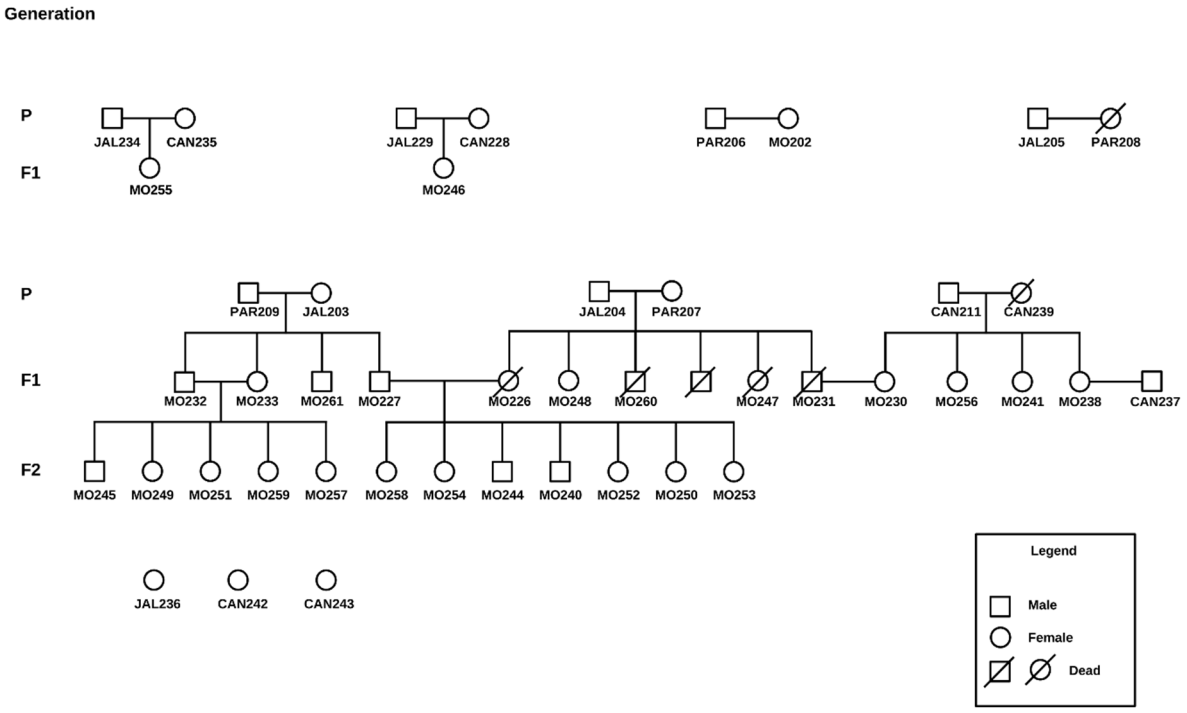

Figure S2. Pedigree of individuals from the *ex situ* population of Brazilian Merganser. Individuals are labeled with sample numbers: MO (captive-bred individuals derived from *ex situ* crossings), CAN (from Canastra region), PAR (from Alto Paranaíba region), and JAL (from Jalapão State Park). CAN, PAR and JAL individuals are the founders of this *ex situ* population.

Tables

Table S1. List of samples used in this work.

Individuals	Origin	Reads	Present_loci	Average depth of coverage	Frequency_ missing loci*
JAL202	Jalapão	1195968	21806	53.32	0.39
JAL203	Jalapão	1699213	21390	75.43	0.4
JAL204	Jalapão	1134441	19495	52.58	0.45
JAL205	Jalapão	625129	15808	34.89	0.55
PAR206	Paranaíba	1713969	20109	79.33	0.43
PAR207	Paranaíba	1499821	21441	66.93	0.4
PAR208	Paranaíba	466157	15378	26.97	0.57
PAR209	Paranaíba	2005642	20594	90.65	0.42
PAR210	Paranaíba	1069332	19055	51.65	0.46
CAN211	Canastra	2133359	21189	95.32	0.4
MO226	Captivity	1364027	18966	65.5	0.47
MO227	Captivity	1275949	17837	65.52	0.5
CAN228	Canastra	846324	17027	44.05	0.52
JAL229	Jalapão	630868	16186	33.69	0.54
MO230	Captivity	4405580	25212	182.92	0.29
MO231	Captivity	1966384	20495	91.51	0.42
MO232	Captivity	706231	19161	33.36	0.46
MO233	Captivity	693680	17267	35.51	0.51
JAL234	Jalapão	1970681	20752	90.48	0.42
CAN235	Canastra	980387	17550	49.24	0.51
JAL236	Jalapão	1266070	18381	64.02	0.48
CAN237	Canastra	2641404	21972	122.91	0.38
MO238	Captivity	1919106	20385	90.79	0.43
CAN239	Canastra	1327781	19295	65.67	0.46
MO240	Captivity	512261	15069	28.97	0.58
MO241	Captivity	1579876	19992	75.89	0.44
CAN242	Canastra	1358351	18337	68.79	0.48
CAN243	Canastra	2693056	22035	125.35	0.38
MO244	Captivity	2729312	21679	129.83	0.39
MO245	Captivity	19044767	19742	94.16	0.45
MO246	Captivity	1283030	16729	69.14	0.53
MO247	Captivity	1750366	19627	85.62	0.45
MO248	Captivity	841464	17650	49.36	0.51
MO249	Captivity	2127977	21167	98.91	0.41

MO250	Captivity	4424525	23392	198.17	0.33
MO251	Captivity	2886368	20831	140.95	0.41
MO252	Captivity	668985	18724	32.4	0.47
MO253	Captivity	1374798	19388	67.45	0.45
MO254	Captivity	1822056	19926	87.31	0.44
MO255	Captivity	1820240	19401	91.03	0.45
MO256	Captivity	952460	21827	42.97	0.39
MO257	Captivity	2552464	24502	109.95	0.31
MO258	Captivity	2295233	21572	104.47	0.39
MO259	Captivity	547169	17095	29.27	0.52
MO260	Captivity	487416	16694	24.93	0.52
MO261	Captivity	808756	19083	38.19	0.46

*The frequency of missing loci shown in this table refers to the result after Stacks filtering. The final data, after vcftools filtering does not show missing data

Table S2. Number of SNPs in heterozygosity. INDV = individual; O(HOM) = observed number of SNPs in homozygosity; O(HET) = observed number of SNPs in heterozygosity; F = inbreeding coefficient. The expected homozygosity was 305.5, the expected heterozygosity was 119.5 and the number of analyzed SNPs was 425.

INDV	O (HOM)	O (HET)	F
JAL202	296	129	-0.07958
JAL203	312	113	0.05432
JAL204	288	137	-0.14653
JAL205	302	123	-0.02936
PAR206	338	87	0.27191
PAR207	349	76	0.36397
PAR208	328	97	0.18822
PAR209	342	83	0.30539
PAR210	278	147	-0.23022
CAN211	286	139	-0.16327
MO226	304	121	-0.01263
MO227	303	122	-0.02100
CAN228	284	141	-0.18000
JAL229	312	113	0.05432
MO230	128	297	-1.48554
MO231	321	104	0.12964
MO232	251	174	-0.45617
MO233	297	128	-0.07121
JAL234	302	123	-0.02936
CAN235	279	146	-0.22185
JAL236	317	108	0.09617
CAN237	292	133	-0.11305

MO238	264	161	-0.34738
CAN239	302	123	-0.02936
MO240	298	127	-0.06284
MO241	228	197	-0.64866
CAN242	292	133	-0.11305
CAN243	299	126	-0.05447
MO244	322	103	0.13801
MO245	342	83	0.30539
MO246	278	147	-0.23022
MO247	299	126	-0.05447
MO248	312	113	0.05432
MO249	329	96	0.19659
MO250	162	263	-1.20100
MO251	338	87	0.27191
MO252	202	223	-0.86625
MO253	319	106	0.11291
MO254	326	99	0.17149
MO255	273	152	-0.27206
MO256	315	110	0.07943
MO257	347	78	0.34723
MO258	310	115	0.03759
MO259	344	81	0.32213
MO260	307	118	0.01248
MO261	285	140	-0.17163

ddRAD Library Assembly Protocol

This protocol is adapted from Thrasher et al. (2017) and Peterson et al. (2012). The digestion enzymes used were *Sbf*I and *Msp*I. The adapter annealing process was performed according to the protocol by Thrasher et al (2017).

DNA extraction

The first step in the library assembly process is DNA extraction. The extraction protocols can be varied (Kits, Phenol-Chloroform, etc.) as long as the DNA obtained is of high quality.

The verification of the DNA quality must be done in the Nanodrop where the absorbance curves 260/280 must have a value greater than or equal to 1.75 and 260/230 must have a value greater than or equal to 1.80. The quantification must be done in Qubit™ (Thermo Fisher), DNA concentration values greater than or equal to 30 ng/ul are expected. The verification of the integrity of the DNA must be done in an agarose gel electrophoresis, where samples that present smearing (a signal of degraded DNA) must be discarded. After all these processes, the amount of genomic DNA must be normalized to the same concentration for all samples (30 ng/ul).

Double digestion

To calculate the DNA amount of each sample that we will be used in the library construction, we will consider the quantification found in the previous step, if your sample was stored and quantified for a long time, it is recommended that you redo the quantification.

We should use approximately 500 ng of genomic DNA per reaction. The DNA volume must be a maximum of 17 ul, if a lower volume is placed, we must add H_2O until reaching the volume of 17 ul.

The restriction enzyme digestion reaction can be done in 96-well plates or in individual Eppendorf tubes (0.8 ml) and should be done according to the table below. A mix can be made with the reagents, except for the genomic DNA, which can then be aliquoted in each well, according to the necessary volumes.

Reagent	**Volume for 1 sample (uL)**
Restriction enzyme *Sbf*I	0.5 (10 units)
Restriction enzyme *Msp*I	0.5 (10 units)
Cutsmart buffer (10x)	2.0
Genomic DNA	17
Total Volume	**20**

Steps

• Working on ice, add 3 ul of the mix (buffer + restriction enzymes) to each well of the 96-well plate (the number of wells you will use depends on the number of samples you will place in your library assembly).

• Load the appropriate DNA volume according to the quantification of each sample

• Seal the plate with the specific sealant (make sure it is well sealed to avoid evaporation).

• Vortex the plate for 6 to 10 seconds and centrifuge briefly at approximately 800 rpm.

• Place the plate in the thermal cycler for 5h at 37°C, then refrigerate at 4°C overnight.

• Run an agarose gel electrophoresis to check digestion with 1 ul of reaction. At this stage, we expect to observe a smearing on the gel showing restriction digested DNA products of smaller sizes.

Cleanup of digested DNA with magnetic beads

• Remove the beads from the refrigerator and leave them at room temperature and vortex them well before use.

• Add 1.5 x volume of the beads to the digested DNA sample.

• Mix the beads and the sample, gently with the pipette (about 10x) to make the solution homogeneous.

• Let the beads act for 10 minutes.

• Place the plate on the magnetic rack and leave until the liquid becomes clear.

• Without removing the plate from the magnetic rack, remove the liquid with the pipette (about 40 ul) and discard.

• Without removing the plate from the magnetic rack, wash twice with 200 ul of 70% ethanol (use fresh ethanol, do not use old preparations). In the second wash, completely remove the liquid, always remembering to use the opposite side of pipette tip to the side where the beads stick.

• Allow the plate to dry for 5 to 10 minutes without removing the plate from the magnetic rack.

• Remove the plate from the magnetic rack.

• Resuspend the beads in 50 ul of ultrapure H_2O, mixing well.

• Let the beads acting for 5 min.

• Return the plate to the magnetic rack, wait for the beads to stick together again and the liquid to become clear.

• Transfer 40 ul of the liquid without the beads to another plate.

• Discard the plate with the beads.

• Quantify the concentrations of the clean product using 2 ul of digested DNA in the Qubit (dsDNA BR kit). These concentrations will be used to calculate volumes to perform the binding of adapters.

Ligation of Adapters

In this step, we will connect the adapters (oligonucleotides), which were previously “annulled” according to the protocol by Thrasher et al. (2017), and the samples already digested. So that there is no imbalance between the amount of DNA of each individual sample, the same concentration of DNA must be used for each of the samples in the library. Put 70 ng of DNA from each sample/individual in the ligation reactions, which must be done according to the table below.

Reagent	Volume for 1 sample (uL)
Barcode P1 (unique tag for each sample)	2.0 (Trasher et al. 2017)
T4 DNA ligase	1.25 (500 units)
Buffer T4	4.0
P2 adapter	2.5 (Trasher et al. 2017)
H_2O	Add for a total 40 ul volume
DNA	Depends on digested DNA concentration

Total volume	40

• To make it easier, a mix can be made with T4 DNA ligase, buffer and P2 adapter.

• Always work on ice.

• Put the H_2O in each well/tube first, then the DNA, the P1 barcode and lastly the mix, which must be kept in the freezer until it is aliquoted in the well.

• Seal the plate with the specific sealant (make sure it is well sealed to avoid evaporation).

• Vortex the plate for 6 to 10 seconds and centrifuge briefly at approximately 800 rpm.

• Place the plate in the thermal cycler to run the ligation reaction for 2h at 23°C, then inactivate the enzyme by keeping the temperature at 65°C for 10 min.

After heat inactivation cool at 2°C for 90 seconds, keep at room temperature.

Cleaning ligated DNA with magnetic beads

• Remove the beads from the refrigerator and leave them at room temperature.

• Combine the ligation products for each index group that will be used in a 2 ml tube. For example, if you digested 40 samples with only 20 unique barcodes, each set of 20 samples linked with their respective barcodes must be associated with 1 different index (1 tube of 2 ml for each index). In this step you will have 800 ul (40 ul x 20) in each tube.

• Vortex beads well before use.

• Add 1.5x the ligation reaction sample volume of beads (In the case of the above example, you would add 1.2 ml of beads to each tube containing 800 ul of ligation product.

• Mix the beads and the sample, gently with the pipette (about 10x) to make the solution very homogeneous.

• Let the beads act for 10 minutes at room temperature.

• Place the tubes on the magnetic rack and leave until the liquid becomes clear.

• Without removing the tubes from the magnetic rack, remove the liquid with the pipette and discard.

• Without removing the tubes from the magnetic rack, wash twice with 2 ml of 70% ethanol (use fresh ethanol, do not use old preparations). In the second wash, completely remove the liquid, always remembering to use the pipette tip in the opposite side where the beads stick.

• Leave the tubes to dry for 5 to 10 minutes without removing them from the magnetic rack.

• Remove the tubes from the magnetic rack.

• Resuspend beads in 36 ul of Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0 - Qiagen). Repeatedly pipette over the side of the tube to resuspend all beads and wash them in the suspension.

• Incubate at room temperature for 5 min.

• Return the tubes to the magnetic rack and wait for the beads to stick together again and the liquid to become clear.

• Transfer 34 ul of the supernatant to a new tube.

• Discard the tubes that were left with the beads.

• Determine DNA concentrations using 2 ul of clean ligated DNA in Qubit (dsDNA BR kit). The concentrations must be above 50 ng/ul.

Fragment Selection

The selection of fragments should be done in Pippin Prep (Sage Science) in the range of 450-600 bp. For a more accurate selection, the 2% agarose cassettes are recommended. The fragment selection process is done according to the Pippin Prep user manual.

Low cycle PCR to add the indices.

To calculate the concentrations and volume of ligated DNA in the next reaction we must quantify the DNA after the size selection performed in Pippin Prep. The quantification must be performed in the Qubit with the dsDNA HS kit and concentration must be around 1 ng/ul.

We will use 10 ng of size selected DNA per PCR reaction. Considering that, after Pippin Prep, we will have 30 ul of final volume and that the quantification was 1 ng/ul, we will prepare three PCR reactions, as shown in the table below.

Reagent	Volume (ul) for 1 reaction	Volume (ul) for 3 reactions
Ligated and size-selected DNA	10	30
Phusion Master Mix (2X)	12.5	37.5
Primer P1_PCR_for index (5 uM)	1.25	3.75
Index_primer (*P_Index	1.25	3.75
Total volume	**25 ul**	**75 ul**

A different index_primer must be used for each of the groups of samples formed during the cleaning step, after connecting the adapters. Once the reactions have been done place the tubes in the thermocycler according to the program below.

- 98ºC for 30 sec.

- 12 cycles of:
    - 98ºC for 5 sec.
    - 60ºC for 25 sec.
    - 72ºC for 10 sec.
- 72ºC for 5 min.
- 10º for 10min

Note: Even if the concentration before the PCR is below the recommended level, the number of cycles should not be increased, as this could lead to a substantial increase (>1%) of incorrect incorporation of bases.

Verification on the agarose gel electrophoresis

• Place the PCR reactions from the same group in the same 1.5 ml tube.

• Take 5 ul and run it on a 1 or 2% agarose gel electrophoresis.

• At this stage a band between 500 and 600 bp should be visible, but it may be a very weak band.

Post-PCR DNA Cleanup with Magnetic Beads

• Remove the beads from the refrigerator and leave them at room temperature.

• Vortex beads well before use.

• Add 0.8x the bead sample volume.

• Mix the beads and the sample, gently with the pipette (about 10x) to make the solution homogeneous.

• Let the beads act for 10 minutes.

• Place the tubes on the magnetic rack and leave until the liquid becomes clear.

• Without removing the tubes from the magnetic rack, remove the liquid with the pipette and discard.

• Without removing the tubes from the magnetic rack, wash with 500 ul of 70% ethanol (use fresh ethanol, do not use old preparations).

• Wait 2 minutes and remove all ethanol.

• Repeat the last three steps and in the last step remove as much ethanol as possible.

• Leave the tubes to dry for 5 to 10 minutes without removing them from the magnetic rack.

• Remove the tubes from the magnetic rack.

• Resuspend beads in 36 ul of Buffer AE. Repeatedly pipette over the side of the tube to resuspend all beads and wash them in the suspension.

• Incubate at room temperature for 5 min.

• Return the tubes to the magnetic rack and wait for the beads to stick together again and the liquid to become clear.

• Transfer 34 ul of the supernatant to a new tube.

• Discard the tubes that were left with the beads.

• Determine DNA concentration, post-PCR, using 2 ul of cleaned DNA (dsDNA HS kit Qubit). Concentrations may be low but should have increased from pre-PCR quantitation post Pippin Prep size selection.

Post-PCR samples are now ready for pool assembly and submitted to Illumina short read sequencing. If the post-PCR quantifications are too low, the DNA can be concentrated using a vacuum centrifuge. The running time in the centrifuge depends on the final volume of the library and on what is the minimum volume required to undergo sequencing.

The library should be quantified using the KAPA Library Quantification Kit (Roche), following the recommendations in the kit's guide for use.

Once all previous steps have been successfully performed, the library can now be submitted to sequencing.