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ORIGINAL ARTICLE

# Evidence of strong population bottleneck in genetics of endangered Brazilian Merganser (*Mergus octosetaceus)*

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## Abstract

The Brazilian Merganser is one of the rarest birds of South America with three known remnant populations in Brazil and only 250 individuals existing in the wild. Thus, evaluating the genetic variability within the remaining population is essential to its conservation. This study assessed genetic diversity of remnant populations by mtDNA (Dloop and cytb) and nDNA (MUSK 13 intron, five microsatellites) markers, giving support to management and conservation efforts. Seventy-one samples were collected during eight years from Chapada dos Veadeiros National Park (PNCV), Serra da Canastra National Park (PNSC), and Alto do Paranaíba region (APR). Cytochrome b, Dloop, and MUSK intron showed only one haplotype, indicating a loss of genetic variability for the Brazilian Merganser. The microsatellites confirmed low genetic variability (1–4 alleles). Despite the low variability observed, it was possible to infer minor differentiation between remnant populations, with PNCV locale being the most differentiated subgroup, due to its being isolated by greater distance from the others. Low genetic diversity observed in Brazilian Merganser, is expected to be found in Critically Endangered species, due to small population size, being result of high inbreeding rates, and gene drift effects. Thus, our genetic data have confirmed the extremely critical status of the Brazilian Merganser, making it necessary to create an immediate recovery program for their populations, and develop a management plan to integrate and preserve all remnant populations to avoid the species extinction.

**Keywords** Genetic conservation · Microsatellite · mtDNA · nDNA · Endangered species

## Zusammenfassung

**Nachweis eines gravierenden populationsgenetischen Engpasses beim Brasilianischen Dunkelsäger (*Mergus octosetaceus*)**

Der Brasilianische Dunkelsäger ist mit nur noch drei bekannten Populationen und 250 wildlebenden Individuen einer der seltensten Vögel Südamerikas. Für seinen Erhalt ist es daher unbedingt notwendig, die genetische Variabilität in den verbliebenen Populationen zu bestimmen. Zur Unterstützung der Bemühungen um den Schutz und Erhalt der Art erfassten wir in dieser Untersuchung die genetische Vielfalt der Populationen anhand der mtDNS- (Dloop und cytb) und nDNS- (MUSK 13

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intron, fünf Mikrosatelliten) –Markern. Über acht Jahre hinweg wurden 71 Proben im Chapada dos Veadeiros-Nationalpark (PNCV), dem Serra da Canastra-Nationalpark (PNSC) und der Umgebung des Alto do Paranaiba erhoben. Bei Cytochrome b, Dloop und MUSK introns gab es nur einen Haplotyp, was auf einen Verlust an genetischer Bandbreite beim Brasilianischen Dunkelsäger hindeutete. Mit 1 – 4 Allelen bestätigten die Mikrosatelliten eine geringe genetische Variabilität. Dennoch war es möglich, auf eine schwache Differenzierung zwischen den Populationen zu schließen, wobei sich die Population im PNCV, die aufgrund ihrer größten Entfernung zu den anderen am isoliertesten ist, als die am stärksten differenzierte Untergruppe erwies. Diese beim Brasilianischen Dunkelsäger festgestellte geringe genetische Diversität wird als Ergebnis einer hohen Inzuchtrate und genetischer Drift-Effekte wegen der geringen Populationsgrößen vermutlich generell bei extrem bedrohten Arten zu finden sein. Unsere zur Genetik erfassten Daten haben den extrem kritischen Status des Brasilianischen Dunkelsägers bestätigt. Sie machen es notwendig, ein sofortiges Schutzprogramm für ihre Populationen aufzustellen und einen Organisations-Plan zur Einbeziehung und zum Erhalt aller übriggebliebenen Populationen auszuarbeiten, um sie vor dem Aussterben zu bewahren.

## Introduction

Endangered species normally have evidence limited population size, leading to suffering from strong effects of inbreeding, and genetic drift (Frankham et al. 2002). Thus, it is expected that these populations lose genetic diversity which affects short- and long-term viability of the wild population. Small population size can be due to habitat fragmentation, hunting pressures, environmental pollution, and disease. Effects of population reduction are proportional to intensity of reduction and the time that population remains small (Luikart et al. 1998; Funk et al. 2010). Genetic bottlenecks can increase the probability of mating among related individuals, consequently increasing frequencies of otherwise rare alleles and causing inbreeding depression, that can decrease individual adaptive potential (fitness) (Funk et al. 2010). Thus, bottlenecks can reduce genetic diversity, leading to concerns about population viability over the long term.

Modern genetic investigation methods allow researchers to evaluate population bottlenecks, detecting changes in $N_e$ (effective number of alleles), and loss of diversity (Funk et al. 2010). Several markers have been used to estimate demographic events in natural populations; these include microsatellites, nuclear introns, and mitochondrial regions. Nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have distinct inheritance, ploidy, and mutation rates, mtDNA being derived via maternal inheritance, while nDNA is of biparental origin; and effective population size of nDNA is four times higher than mtDNA (Zink and Barrowclough 2008). Thus, it is expected that demographic events that mark the nuclear genome occur more slowly than those of mitochondrial (Solovyeva et al. 2011). However, nuclear loci tend to retain ancestral polymorphism, when they are not subject to selection or drastic population reductions (Warn 2009). On the other hand, microsatellites that also are nuclear markers are characterized by high mutational rates and thus are used in various population studies to recover evidence of recent genetic events and kinship analysis (Grativol et al. 2001; Bristol 2013). For these reasons, inferences based on multiloci approaches demonstrate robust results for recovery of evolutionary history of populations/species (Zink and Barrowclough 2008).

The Brazilian Merganser (*Mergus octosetaceus* Vieillot, 1817) is considered critically endangered on the Red List of Threatened Species of IUCN (International Union for Conservation of Nature, Birdlife 2016) and the National Official Species of Endangered Fauna (IBAMA 2014), due to having a world population estimated as around 250 individuals and being subjected to continued habitat loss. Historically, Brazilian Mergansers had been recorded in Argentina, Paraguay, and Brazil. However, currently this species occurs only in Brazil, as four remaining relict populations (Silveira and Bartmann 2001; Hughes et al. 2006; Lins et al., 2011): existing in Chapada dos Veadeiros at Goiás State, (Yamashita and Valle 1990; Bianchi et al. 2005); in Jalapão State Park at Tocantins State (Braz et al. 2003; Barbosa and Almeida, 2010), and in the Alto do Paranaíba region and the Serra da Canastra National Park at Minas Gerais State (Bartmann 1988; Lamas 2006).

Only one prior study of Brazilian Mergansers characterized genetic diversity and used exclusively mitochondrial genetic markers (Vilaça et al. 2012). These authors found two distinct lineages with high polymorphism between them at Serra da Canastra population, suggesting that current Brazilian Merganser lineages could be the outcome of an ancient geographic isolation structure which was later erased by second contact. However, there are few cases of high genetic diversity being observed in small endangered populations (Kuro-o et al. 2010). One alternative hypothesis to explain observed Brazilian Merganser genetics is that past bottleneck events could have erased genetic diversity in natural populations, leaving only two common ancestral haplotypes remaining.

In this context, the present study sought to discern demographic history of Brazilian Mergansers covering a great part of the whole species actual distribution, three out of four remnants populations, searching for estimated genetic diversity, gene flow, population structure, and testing the

bottleneck hypothesis on the relict populations, through a multilocus study approach.

## Methods

For this study, we used a total of 71 samples from blood, muscles, eggshell, feathers, and skulls of dead animals, that were collected in the period from 2006 to 2014 in three isolated remaining populations of Brazilian Merganser: National Park Chapada dos Veadeiros (PNCV) ($n = 08$); Alto do Paranaíba region (APR) ($n = 08$); and National Park Serra da Canastra (PNSC) ($n = 55$) (Fig. 1). All samples were submitted to DNA genomic extraction by digestion with Proteinase K (20 mg/ml) and posterior purification Phenol:Clorophormio:isoamilic alcohol (25:24:1), and ethanol precipitation protocol (Sambrook et al. 2001). Genomic DNA was quantified using the NanoDrop 2000 (Spectrophotometer Thermo Scientific Uniscience) and diluted to 25 ng/µl.

Control region from mitochondrial DNA was amplified by primers DUCK L81 (5′-TATTTGGYTATGYAYRTC GTGCAT-3′) and DUCK H768 (5′-TATACGCMAACCGT CTCATYGAG-3′) (Muñoz-Fuentes et al. 2005). PCR reactions were performed in final volumes of 10 ul, containing approximately 25 ng of genomic DNA, 2.5 µM $MgCl_2$; 2.5 µM dNTPs; 5 M of each primer; 4 mg/ml BSA; 1 × buffer and 0.5 unit of Taq DNA polymerase tube. The reactions


**Fig. 1** Map of Brazil with localities of DNA sample sources for Brazilian Merganser (*Mergus octosetaceus)*, representing all known remnant populations. (Map done by Gisele Dantas, and Brazilian Merganser drawn by Marcelo Ianhez)

were conducted in an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, hybridization of primers at 55 °C for 30 s, and chain extension at 72 °C for 45 s, and one final extension step at 72 °C for 7 min. Cytochrome b (Cytb) was amplified by primers L1481 (F: 5′-CCATCCAACATCTCAGCATGATGAAA-3′) and H 160,565 (5′-GTCTTCAGTTTTTGGTTTACAAGAC-3′). (Kocher et al. 1989; Lougheed et al. 2000). PCR reactions were performed in final volumes of 20 µl, containing approximately 25 ng of genomic DNA, 1.5 µM $MgCl_2$; 2.5 µM dNTPs; 5 M of each primer; 4 mg/ml BSA; 1 × buffer, and 0.5 unit of Taq DNA polymerase tube. The reactions were conducted with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, hybridization of primers at 55 °C for 30 s, and chain extension at 72 °C for 45 s, and one final extension step at 72 °C for 7 min.

Nuclear intron from skeletal Muscle Gene Receptor tyrosine kinase (MUSK) was amplified by primers MUSK13F (5′-CTTCCATGCACTACAATGGGAAA-3′) and MUSK13R (5′-CTCTGAACATTGTGGATCCTCAA-3′) (Clark and Witt 2006). PCR reactions were performed in final volume of 21 µL, and 50 ng of genomic DNA, 2.5 µM $MgCl_2$; 2.5 uM dNTPs; 0.5 pmol of each primer; 1 × buffer and 0.5 unit of Taq DNA polymerase tube. The thermal program used has an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, hybridization of primers at 55 °C for 30 s, and chain extension at 72 °C for 45 s and over a final extension step at 72 °C for 7 min. PCR products were cleaned by precipitation using 20% polyethyleneglicol with 2.5 M NaCl (Paithankar and Prasad 1991). The sequencing reactions were performed using standard protocol by BIGDYE v.3.1, performed in automatic sequencer ABI 3130.

Five microsatellite (MOCC3, MOCD4, MOCH3, MOCH5, APH08) were amplified following conditions described for Maia et al. (2017). For genotyping, 1 µl of each amplified product was added to a solution composed 0.25 µl molecular weight marker GeneScan ROX-500 and 7.75 Hi-Di Formamide, performed in an automatic sequencer ABI 3130.

The sequence alignments in SeqScape v5 and haplotype diversity (*h*) and nucleotide diversity (π) were estimated in DNAsp v.5.10.01 (Librado and Rozas 2009). Microsatellites were checked in GeneMapper software, null allele, and dropout was verified by MICROCHECKER 2.2.3 (Van-Oosterhout et al. 2004). The program version GenAlEx 6501 (Peakall and Smouse, 2006) was used to calculate the allele frequency, number of alleles (Na), effective number of alleles (Ne), expected heterozygosity (He), observed heterozygosity (Ho), the alleles fixation index (F), and the FST pairwise population. In addition to variation in the composition of microsatellites, individuals were evaluated by PCoA (Principal Coordinates Analysis). In addition, microsatellites data were used in the software STRUCTURE program. 2.3.4 (Pritchard et al. 2000) and Structure Harvester (Earl and Vonholdt 2012) to estimate distinct groups inside the populations (delta K) considering ten independent runs were performed with K assumption ranging between 1 and 10, parameters 100,000 interactions of MCMC in a period of burn-in of 10,000 interactions. BOTTLENECK V.1.2 (Cornuet and Luikart 1996) was used to check if there are bottleneck effects of influence on the pattern of genetic variability of populations, under the hypothesis of mutation-drift equilibrium. The assumed mutation models were the model of alleles Infinite (IAM) and Mutation by Step Model (SMM). The distribution of allele frequency (-Shift Mode) was generated to verify the signs of such a bottleneck. The statistical test used to assess the significance of differences was the Wilcoxon sign rank test, to have greater statistical power when used for less than 20 loci, and small number of samples.

## Results

Control region DNA fragments were obtained from 58 individuals (579 bp), Cytochrome b from 32 individuals (846 bp), and MUSK (560pb) fragments from 56 samples covering all three regions. MUSK, Cytb, and Control regions showed only one haplotype for all samples that included the three remaining populations of Brazilian Merganser, thereby revealing no genetic variations between them.

The microsatellite loci did not show evidence of null alleles or of drop out to MOCC3, MOCD4, MOCH5, Aph08, MOCH3 to PNCV and Serra do Salitre. However, PNSC samples yielded results which detected an excess of homozygosity for MOCD4, MOCH3 and MOCH5, and Aph08 that may represent the presence of null alleles. Low genetic variability also was observed in microsatellite loci at all populations (Table 1), with low allelic richness (1–4 alleles) and heterozygosity (0.00–0.32). Despite the low genetic diversity observed, it was possible to estimate population genetic differentiation and identify PNCV samples as the most differentiated population (Table 2). However, the observed genetic differentiation among populations in Brazilian Mergansers probably is due to their isolation by distance (Mantel test $r = 0.99$, $p < 0.001$). However, STRUCTURE analysis found three distinct clusters (groups), but these clusters were not associated with geographic localization of the samples. Thus, these analyses corroborate the low genetic structure to recovery by F-statistic (Fig. 2). In addition, BOTTLENECK indicated significant evidence of a possible bottleneck for the Serra da Canastra population for MOCD4, MOCH3, and APH08 under SMM.

**Table 1** Summary statistics from five microsatellites loci used for Brazilian Merganser from National Park Chapada dos Veadeiros (PNCV); Parque Nacional Serra da Canastra (PNSC), and Alto Paranaíba region (APR): sample size (N), number of alleles (Na), Shannon diversity (I), observed heterozygosity (Ho), expected heterozygosity (He), and significance of $\chi^2$ test. F = Fixation Index = (He—Ho) / He = 1—(Ho / He)

| Pop | Locus | N | Na | I | Ho | He | *p* value $\chi^2$ | *F* |
|---|---|---|---|---|---|---|---|---|
| PNCV | MOCC3 | 8 | 2 | 0.234 | 0.125 | 0.117 | 0.85 | − 0.06 |
| | MOCD4 | 6 | 2 | 0.451 | 0.333 | 0.278 | 0.62 | − 0.2 |
| | MOCH3 | 7 | 1 | 0.000 | 0.000 | 0.000 | Monomorphic | – |
| | MOCH5 | 8 | 2 | 0.483 | 0.125 | 0.305 | 0.09 | 0.59 |
| | Aph08 | 7 | 1 | 0.000 | 0.000 | 0.000 | Monomorphic | – |
| PNSC | MOCC3 | 50 | 2 | 0.611 | 0.320 | 0.420 | 0.09 | 0.23 |
| | MOCD4 | 43 | 4 | 0.630 | 0.047 | 0.344 | < 0.01 | 0.86 |
| | MOCH3 | 48 | 3 | 0.262 | 0.042 | 0.118 | < 0.01 | 0.64 |
| | MOCH5 | 51 | 2 | 0.262 | 0.042 | 0.118 | < 0.01 | 0.87 |
| | Aph08 | 44 | 4 | 0.559 | 0.114 | 0.279 | < 0.01 | 0.59 |
| SS | MOCC3 | 8 | 2 | 0.685 | 0.875 | 0.492 | 0.02 | − 0.77 |
| | MOCD4 | 8 | 1 | 0.000 | 0.000 | 0.000 | Monomorphic | – |
| | MOCH3 | 4 | 1 | 0.000 | 0.000 | 0.000 | Monomorphic | – |
| | MOCH5 | 5 | 1 | 0.000 | 0.000 | 0.000 | Monomorphic | – |
| | Aph08 | 7 | 2 | 0.257 | 0.143 | 0.133 | 0.83 | − 0.07 |

**Table 2** *Fst* (below hemimatrix) and geographic distance (above) among Brazilian Merganser population

| | PNCV | PNSC | APR |
|---|---|---|---|
| PNCV | | 550 | 665 |
| PNSC | 0.114 | | 80 |
| APR | 0.131 | 0.056 | |

## Discussion

Control region of mitochondrial DNA is considered to be the regions with the highest mutational rate, thus it was expected to find higher genetic diversity there than in nuclear intron (MUSK), but both markers showed only one haplotype. Low genetic diversity such as that observed in Brazilian Mergansers is expected to be found when a population has passed through an intense bottleneck or due to selective sweep on the specific loci (Frankham et al. 2002, Dantas et al. 2012). In addition, microsatellites loci also indicated low genetic diversity, with several loci monomorphic, corroborating the scenario of intense bottleneck on Brazilian Merganser. Our results do not corroborate the prior Brazilian Merganser study, which had reported finding two distinct mitochondrial lineages (cytochrome b and Dloop region) with high genetic differentiation (cytb-20 polymorphic sites and Dloop-24 polymorphic sites) (Villaça et al. 2012). All markers used in deriving our results (Dloop, Cytb and MUSK) revealed only one haplotype and seem to be more realistic for one species with restricted distribution and extreme habitat loss, whose world population is estimated at only 250 individuals (Birdlife 2016). One study on Common Mergansers (*Mergus merganser)* a species that is widely distributed across the northern hemisphere in both Europe and North America, showed high genetic variability in control region, showed 25 mutational points between haplo-groups (Canadian and Swiss) (Hefti-Gautschi 2009). On the other hand, a study of the Scaly-sided Merganser, *M. squamatus*, one of the most endangered of known Palearctic species of ducks revealed low nucleotide diversity and haplotype between individuals sampled (Solovyeva et al. 2011). We believe that genetic diversity observed by Villaça et al. (2012) could be the result of nuclear copying or heteroplasmy, that has been reported in other bird species (Gandolfi et al. 2017; Kvist et al 2003; Zhang and Hewitt 1996), since we used the same locations and even re-sequenced some Villaça et al. (2012) samples.

Low genetic diversity has been reported in other threatened species that have gone through population bottlenecks, as in the case of *Leontopithecus rosalia*, the golden lion

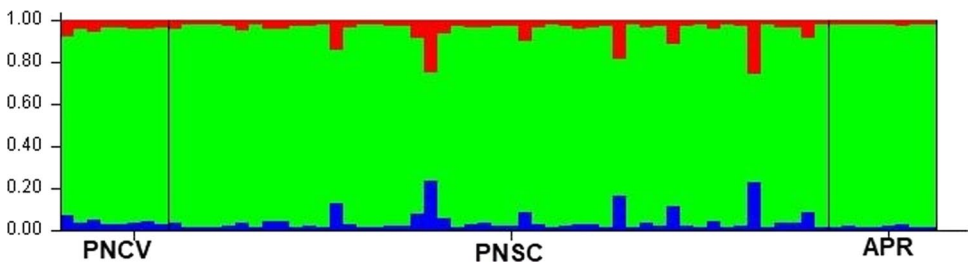


**Fig. 2** Bayesian STRUCTURE based on five microsatellites of *Mergus octocetaseus* population at Brazil, PNCV (National Park Chapada dos Veadeiros), PNSC (National Park Serra da Canastra), and APR (Alto do Paranaíba Region)

tamarin (Grativol et al 2001), *Terpsiphone corvina,* the Seychelles Paradise Flycatcher (Bristol et al 2013), and *Petroica traversi*, the black robin (Arden & Lambert 1997). Despite of the weak signal, we recovered on Bottleneck tests for Brazilian Merganser, we suggest stronger historical bottleneck probably occurred in this species since observed loss of genetic diversity in loci from both mitochondrial and nuclear, probably resulted from a demographic event. In addition, the bottleneck test is known to have limitations in detecting slower rates of population declines and work most efficiently to detect catastrophic population crashes (Cornuet and Luikart 1996; Goossens et al. 2006). The effects of population bottlenecks in natural populations are directly related to the intensity (proportion individuals of the population survival) and the event duration time (Kuo and Janzen 2004; Frankham et al. 2008). However, this critical factor can be minimized by increasing the number of markers sampled, it being suggested by Hogan et al. (2013) that using higher than 50 markers, for this genomic approach is fundamental to elucidate evolutionary history of endangered species. One other great problem of loss of genetic diversity is continuing decline of populations, despite protection of its remaining population areas, as observed in the North American spotted owl, *Strix occidentalis caurina* (Funk et al. 2010). In future, monitoring both population numbers and of genetic diversity of population stocks will be fundamental to conservation efforts of these endangered species. However, it is possible that the northern Brazilian Merganser species is already caught in an extinction vortex.

Despite the low genetic diversity, we observed it was possible to infer differentiation among remnant populations through microsatellites loci. Our outcome indicated that the Alto do Paranaíba region and the Serra da Canastra National Park, distant by about 80 km, showed low genetic differentiation. National Park of Chapada of Veadeiros distant 665 km of PNCV and 550 km to APR evidenced the greatest differentiation. Thus, we recommended that each population receive intense conservation attention and that this population as a whole be managed together as one unit. In addition, it is important to note that one of the remaining populations was not sampled in this study. Thus, the sampling effort in the Tocantins region is extremely important for the consolidation of observed patterns.

We concluded from our study and this genetic analysis context, that the Brazilian Merganser should be considered extremely endangered due to habitat degradation, low population size, and low genetic diversity. Thus, it is extremely important to conserve all remnant populations and execute an integrated management plan, using genetic tools for design of management strategies for breeding and reintroductions into the wild. However, it is essential to continue trying to develop new genetic markers to access evolutionary and demographic history to help retain or increase genetic diversity to help manage and avoid extinction of this species.

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**Author contributions** TAM, GPM analyzed data and designed the manuscripts. TAM, DPC, LRS developed laboratory procedures, LV, FR, FS given samples to study. All authors contributed to manuscript text.

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