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CONSIDERATIONS ON THE REINTRODUCTION AND RECOVERY OF THE ALAGOAS CURASSOW *MITU MITU* (LINNAEUS, 1766) FROM EXTINCTION USING A POTENTIALLY HYBRID CAPTIVE STOCK

Anita Wajntal, Sergio L. Pereira, & Erwin T. Grau

Department of Genetics and Evolutionary Biology, University of São Paulo, São Paulo, SP, Brazil. Rua do Matão 277, sala 216, São Paulo, Brazil, 05508-090. *E-mail*: sergiolp@gmail.com

Resumo. – Considerações sobre a reintrodução e recuperação do mutum do Alagoas *Mitu mitu* (Linnaeus, 1766) usando um estoque de cativeiro potencialmente híbrido. – O Mutum de Alagoas *Mitu mitu* é considerado extinto na natureza. Duas fêmeas e um macho coletados na natureza em 1979 reproduziram com sucesso em cativeiro, e em 1990, hibridações entre *M. mitu* e Mutum cavalo *M. tuberosum* foram efetuadas para tentar aumentar o sucesso reprodutivo do plantel. Em junho de 2008, 130 aves estavam presentes em dois aviários. Nós sequenciamos duas regiões do DNA mitocondrial dos dois estoques cativos do Mutum de Alagoas. Identificamos inequivocamente aves híbridas que possuem haplótipo de *M. tuberosum*. Contudo, a não ser que o registro original de acasalamentos seja recuperado, não há meio confiável de identificar os *M. mitu* "puros" para propósito de acasalamento em cativeiro e reintrodução. Baseado em um estudo independente que identificou características fenotípicas diagnóticas de *Mitu mitu*, nós sugerimos que as ações de conservação devem focar em espécimens com caracteres fenotípicos diagnósticos de *M. mitu* e que não tenham contribuição genética mitocondrial de *M. tuberosum.* Embora tenhamos detectado pouca variabilidade genética entre as aves cativas, o crescimento constante da população cativa sugere que depressão genética por endocruzamento e hibridações não afetaram o sucesso reprodutivo. Reintroduções destas aves potencialmente híbridas na área original de ocorrência do Mutum de Alagoas, onde não ocorre nenhuma outra espécie de mutum hoje em dia, pode ser a única esperança de preencher o nicho ecológico deixado vago pela espécie. Um programa educacional envolvendo comunidades locais para proteger as futuras reintroduções de mutuns e conservar seu habitat restaurado é considerado fundamental.

Abstract. – The Alagoas Curassow *Mitu mitu* is considered extinct in the wild. Since 1979, two females and a male caught in the wild have bred successfully in captivity, and, in 1990, hybridizations between *M. mitu* and Razor-billed Mitu *M. tuberosum* were performed. By June 2008, there were around 130 living birds in two different aviaries. We sequenced two regions of the mitochondrial DNA of both captive stocks of Alagoas Curassows. We unequivocally identified hybrids that have haplotype typical of *M. tuberosum*. However, unless the original studbook can be recovered there is no confident way to discriminate "pure" *M. mitu* birds for breeding and reintroduction purposes. Allied with morphological data gathered in an independent study, we suggest that conservation actions need to focus on specimens with diagnostic phenotypic characters of *M. mitu*, and avoid birds with mitochondrial genetic contribution of *M. tuberosum.* Although we have detected low levels of genetic variability among captive birds, the steady increase of the captive population suggests that inbreeding depression and hybridization are not a reproductive hindrance. Reintroduction of some of these potential hybrid birds in the original area of occurrence of the Alagoas Curassow may be the only hope to fill in the ecological niche left vacant. An educational program involving local communities to conserve future reintroduction of curassows and their restored habitat is highly recommended. *Accepted 12 November 2009.*

Key words: *Mitu mitu*, Cracidae, extinct, reintroduction, hybrids.

INTRODUCTION

The Alagoas Curassow *Mitu mitu* is the most endangered cracid species. It used to occur in a limited area of Atlantic Forest in northeastern Brazil that quickly became replaced by sugar cane plantations for alcohol and sugar production. In an effort to save the species from extinction, Pedro Nardelli captured the last known individuals living in the species' historical native area in 1979 and brought them to his private aviary, Zoobotânica Mário Nardelli (ZMN) (Nardelli 1993). He soon started a breeding program, separating the five surviving birds in a pair and a trio of two females and a male. The trio reproduced successfully in captivity, and by 1990 there were 19 *M. mitu* individuals in the aviary. Nardelli reports that cross-species hybridization occurred between *M. mitu* and the Razor-billed Curassow *M. tuberosum* in 1990, aiming to increase the breeding efficiency of the captive stock. Nardelli's aviary was closed in 1999 and the 44 curassows alive at that time in the facility were split in two lots. Twenty birds were sent to the Criadouro Científico e Cultural Poços de Caldas (CCCPC) owned by Moacyr de Carvalho Dias and 24 were sent to Fundação CRAX (FC) owned by Roberto Azeredo. Further details of the history on the captive breeding of *M. mitu* can be found elsewhere (Nardelli 1993, Grau *et al.* 2003, Silveira et al. 2004; Bianchi 2008). Unfortunately, the studybook was not made available at the time of the dismemberment of Zoobotânica Mário Nardelli and specific information on the crosses performed and their outcome are unknown. Hence, all birds born after 1990 can be considered as potentially hybrids between *M. mitu* x *M. tuberosum*.

We had the opportunity to visit CCCPC in 2000 and collected blood samples from 20 birds from Nardelli's original stock. Applying molecular tools among these 20 extant birds and a DNA sample extracted from a *M. mitu* museum skin dated from 1951, and considering the three birds born before 1990 as possibly pure *Mitu mitu* specimens descendants of the birds collected by Nardelli in the wild in 1979, we validated that the level of mtDNA divergence between *M. mitu* and *M. tuberosum* is consistent with species-level taxonomic status (Grau *et al.* 2003). Additionally, we discriminated between birds with greater genetic similarity to *M. mitu* or to *M. tuberosum,* and identified three hybrid birds by the presence of mitochondrial DNA (mtDNA) sequences (haplotype) typical of *M. tuberosum*, nine birds of mixed genetic contribution, and five birds with higher probability of being pure or having a higher genetic constitution of the Alagoas Curassow genome (Grau *et al.* 2003). As the three birds born before 1990 and held at CCCPC (= assumed to be possibly "pure" *M. mitu*) were already older adults, we recommended that these birds and those with high genetic similarity to these birds (Table 1) should be preferentially involved in the breeding program (Grau *et al.* 2003). This strategy aimed at rescuing most of the *Mitu mitu* genome present in the three birds captured from the wild that successfully bred in captivity in Nardelli's facilities.

Silveira *et al.* (2004) surveyed 15 localities in the area of the historical distribution of the Alagoas Curassow in northeastern Brazil. Although they did not find any evidence of the existence of wild population of this species, they identified suitable sites for future release of captive born birds back to the wild, and reported positive local landowner support for the planned reintroductions.

Here, our study is directed to 1) extend the genetic analysis to include the birds born at ZMN which were later transferred to FC, 2) compare the results with those birds trans-

TABLE 1. *Mitu mitu* individuals born at ZMN and later relocated to FC and CCCPC; a museum skin (MZUSP) is also included (for acronyms see text). Possibly "pure" *M. mitu* are marked with an *, and CCCPC birds with higher genetic similarity to *M. mitu* as previously detected (Grau *et al.* 2003) are marked with **. Hybrids are identified by the presence of "tuberosum" haplotype. YoB = year of birth; UAG ID = identity of the blood and DNA samples deposited at the LGEMA, IBUSP; CR = control region.

Breeder	Band	YoB	UAG ID	cyt *b* haplotype	CR haplotype	Sex	Reference
MZUSP	37188	-	C242	mitu	mitu1	-	Grau *et al.* (2003)
FC	02ZMN83*	1983	C274	mitu	mitu1	M	this study
FC	01ZMN85*	1985	C277	mitu	mitu1	F	this study
FC	02ZMN85*	1985	C283	mitu	mitu1	M	this study
FC	01ZMN85a*	1985	C281	mitu	mitu2	M	this study
FC	01ZMN85b*	1985	C290	mitu	mitu2	M	this study
FC	03ZMN89*	1989	C271	mitu	mitu1	F	this study
FC	01ZMN89	1989	C286	tuberosum	tuberosum	F	this study
FC	03ZMN90	1990	C273	mitu	mitu1	F	this study
FC	07ZMN92	1992	C278	mitu	mitu1	F	this study
FC	01ZMN92	1992	C279	mitu	mitu1	M	this study
FC	08ZMN92	1992	C288	mitu	mitu1	F	this study
FC	07ZMN93	1993	C276	mitu	mitu1	F	this study
FC	04ZMN94	1994	C272	mitu	mitu1	M	this study
FC	02ZMN94	1994	C285	tuberosum	tuberosum	M	this study
FC	08ZMN95	1995	C284	mitu	mitu1	F	this study
FC	06ZMN95	1995	C289	tuberosum	tuberosum	F	this study
FC	03ZMN96	1996	C291	tuberosum	tuberosum	M	this study
FC	03ZMN97	1997	C280	mitu	mitu1	F	this study
FC	02ZMN97	1997	C275	tuberosum	tuberosum	F	this study
FC	03ZMN98	1998	C282	mitu	mitu1	F	this study
FC	05ZMN98	1998	C287	mitu	mitu1	M	this study
CCCPC	03ZMN84*	1984	C211	mitu	mitu2	M	Grau *et al.* (2003)
CCCPC	02ZMN87*	1987	C202	mitu	mitu1	F	Grau *et al.* (2003)
CCCPC	02ZMN89*	1989	C201	mitu	mitu1	M	Grau *et al.* (2003)
CCCPC	09ZMN91**	1991	C199	mitu	mitu1	M	Grau *et al.* (2003)
CCCPC	07ZMN91	1991	C203	mitu	mitu1	M	Grau *et al.* (2003)
CCCPC	08ZMN91	1991	C218	mitu	mitu1	F	Grau *et al.* (2003)
CCCPC	03ZMN92**	1992	C200	mitu	mitu1	F	Grau *et al.* (2003)
CCCPC	06ZMN92**	1992	C208	mitu	mitu1	F	Grau *et al.* (2003)
CCCPC	04ZMN92	1992	C212	tuberosum	tuberosum	F	Grau *et al.* (2003)
CCCPC	02ZMN92	1992	C215	tuberosum	tuberosum	M	Grau *et al.* (2003)
CCCPC	01ZMN93	1993	C207	mitu	mitu1	M	Grau *et al.* (2003)
CCCPC	04ZMN93	1993	C217	mitu	mitu1	M	Grau *et al.* (2003)
CCCPC	03ZMN94**	1994	C205	mitu	mitu1	M	Grau *et al.* (2003)
CCCPC	07ZMN95	1995	C216	mitu	mitu1	F	Grau *et al.* (2003)
CCCPC	01ZMN95	1995	C209	tuberosum	tuberosum	M	Grau *et al.* (2003)
CCCPC	02ZMN96	1996	C204	mitu	mitu1	F	Grau *et al.* (2003)
CCCPC	04ZMN96	1996	C214	mitu	mitu1	F	Grau *et al.* (2003)
CCCPC	01ZMN97	1997	C210	mitu	mitu1	F	Grau *et al.* (2003)
CCCPC	04ZMN98**	1998	C206	mit	mitu1	F	Grau *et al.* (2003)
CCCPC	01ZMN98	1998	C213	mitu	mitu1	M	Grau *et al.* (2003)

ferred to CCCPC (Grau *et al.* 2003), and 3) seek to identify those birds that are most genetically similar to the original trio of *Mitu mitu* that formed the founder population for the captive-born individuals in Nardelli's aviary. These birds should be used in a captive breeding program designed to maximize the recovery of the genetic constitution present in the original native stock. As the ultimate aim is reintroduction, and the species appears to be extinct in the wild, we discuss the effects of reintroducing potentially hybrid as well as highly inbred Alagoas Curassows within their historical area of distribution.

MATERIAL AND METHODS

We visited FC in June of 2003 and collected blood samples from the brachial vein of each of the surviving 21 birds that were originally transferred from Nardelli's collection. Each sample was transferred into two 1.5 ml flasks containing absolute ethanol. We visited the CCCPC collection in August 2003 and drew blood from the 17 surviving birds. During these visits, phenotypic features were carefully examined and registered for each bird in both aviaries by L. F. Silveira. Birds born locally at CCCPC and FC that were not part of Nardelli's original lot were not examined.

Mitochondrial sequences have been profusely used in population genetics due to its higher rate of evolution as compared to sequences from the nuclear genome, and hence, have been used largely to discriminate between species. We chose to amplify fragments of 390 bp of cyt b and 370 bp of the hypervariable control region by PCR and sequenced them as described in Grau *et al.* (2003).

DNA fingerprinting is a useful technique to provide a glimpse of the overall nuclear genetic variation based on neutral minisatellite markers, and for unambiguous individual identification at the molecular level. For technical reasons DNA samples of all the birds were sent to Dr. Sergio Danilo Pena (GENE), Belo Horizonte, Brazil, who kindly performed the DNA fingerprinting experiment as previously described (Pena *et al.* 1990) and sent us the resulting images for analysis.

RESULTS

The analysis of mtDNA sequences (Table 1) revealed that among the 21 birds kept at FC five had typical sequences (haplotypes) of *M. tuberosum*, 14 had the most common haplotype of typical *M. mitu* found among the birds of CCCPC, and two had a second haplotype found in one bird at CCCPC (Grau *et al.* 2003). The two *M. mitu* haplotypes differ by one substitution in the control region and by 20 substitutions compared to the *M. tuberosum* haplotype. The less frequent *M. mitu* haplotype was present in males only. Unexpectedly, one bird supposedly born in 1989, prior to the reported starting date of the hybridization between *M. mitu* and *M. tuberosum*, had the mitochondrial haplotype of *M. tuberosum*. Thus, considering both aviaries, eight birds were identified unequivocally as being the result of past hybridization as they received the typical maternal mtDNA of *M. tuberosum*.

For the DNA fingerprinting analysis of the FC birds, seven birds with birth date before 1990 registered on their bands were initially considered as pure *M. mitu*. Comparison between band sharing coefficients of the five hybrid birds from FC (as detected through mtDNA typical of *M. tuberosum*) and "pure" *Mitu mitu* birds (born before 1990) did not show any significant differences. These results contrast with the significant results we detected previously in the CCCPC aviary when similar comparisons between birds born before 1990 versus those born afterwards were performed with a different DNA fingerprinting mini-satellite probe (Grau *et al.* 2003). Our estimates (Band Sharing Coefficient = 0.64 ± 0.07; Heterozygosity = 0.23; Sundt *et al.* 1994) show that the FC captive stock is highly inbred.

DISCUSSION

Genetic characterization of captive Alagoas Curassows. We characterized the genetic profile of 21 Alagoas Curassows kept at FC using sequencing of mtDNA and DNA fingerprinting in a similar way we analyzed the stock held at CCCPC (Grau *et al.* 2003). The analysis of mtDNA sequences indicated that eight birds held at both FC and CCCPC could be identified unequivocally as being the result of hybridization in the past for having mtDNA typical of *M. tuberosum*. Moreover, the hybridization between *M. mitu* and *M. tuberosum* may have started prior to 1990, as one hybrid bird has a band with a birth date of 1989. However, we cannot discard the possibility that bands have been mistakenly switched between birds in the past. The presence of two *M. mitu* mtDNA haplotypes among birds born in captivity until 1985 suggests that both original wild-caught females had different haplotypes and both bred successfully in captivity. On the other hand, DNA fingerprint profiles revealed no significant differences between hybrids and presumably "pure" (= born before 1990) birds among the birds kept at FC, in contrast to a similar analysis performed previously for CCCPC (Grau *et al.* 2003). The analysis of genetic variability among a sample of birds from FC revealed a population with very low genetic variability. This finding is not surprising, as most birds held in captivity today are the descendants of three birds caught in the wild.

There is no reason to assume that the mtDNA has any influence on phenotypic features, which are usually of autosomal inheritance. Unfortunately, several nuclear markers (RAG-1, RAG-2, c-mos, and intron 7 of beta-fibrinogen gene) tested by one of us (SLP) in curassows do not have enough genetic variation to discriminate between species of *Mitu*, thus potential paternal contribution of *M. tuberosum* cannot be ruled out. These nuclear loci are likely not associated with morphological differentiation between species. Other molecular markers, such as microsatellites, could be potentially used to detect hybrids, but would hardly identify pure *M. mitu* birds with a reasonable degree of confidence.

Silveira *et al.* (2004) analyzed and recorded morphological characters of 38 birds from both facilities. They have recognized the presence of four typical *M. mitu* features: presence of a bare auricular patch, a bicolored bill, central rectrices black or nearly black , and lateral rectrices with tawny tips. Among these 38 birds, 27 had the complete *M. mitu* phenotype, nine birds had some of these features absent and were considered as hybrids, and the records for two birds were ambiguous and should be re-checked. Explicit recommendations on which birds should have priority in the breeding program rest on the research team that evaluates morphological traits. However, until suitable diagnostic nuclear markers are developed to complement the results of morphological analysis we conclude that in the current captive lot of *M. mitu* the phenotype of a given bird that does not harbor mtDNA typical of *M. tuberosum* is the most appropriate criterion of selection since it reflects a higher contribution of the *M. mitu* genome. The captive breeding program should concentrate on increasing the genetic contribution of these individuals, especially on the offspring of the males with the less frequent mtDNA haplotype, aiming the preservation of the autosomal contribution of the second original female taken from the wild. Contrary to the suggestion in Silveira *et al.* (2004) that some birds are indeed pure *M. mitu*, we believe that even those birds with a typical *M. mitu* mtDNA genotype and all phenotypic features of *M. mitu* should be considered as potentially hybrids, as long as there is no information on their ancestral history.

Considerations for reintroduction of the Alagoas Curassow. The historical distribution range of *M. mitu* in the Atlantic Forest in the state of Alagoas, in northeastern Brazil, has been heavily destroyed and replaced by sugar cane agriculture. Reasonable efforts are being invested by regional owners of the sugar cane plantations, NGOs, and governmental organizations to recover and protect the remnant patches of Atlantic Forest in this region. Local inhabitants still recall the presence of *M. mitu* in the forest around 30 years ago. Consequently the species has been used as a symbol to implement appropriate educational programs involving the local population in the protection of restored areas, as hunting and logging are still a threat to the regional biota and their associated fauna.

The introduction of potentially hybrid birds does not represent any additional menace to the biome as the species is extinct from nature and no other species of curassows have occurred in historical times in or around the area that is being recovered. The release of hybrid specimens has been advocated in the past when the endangered species may not have chances of survival on its own, when hybrid individuals cannot be distinguished from non-hybrids, or when intentional hybridization is the only chance of recovery (Allendorf *et al.* 2001). Thus, the hazards of genetic introgression of a hybrid genome to local populations of curassows are non-existent. On the contrary, the reintroduction of those birds may help to protect and restore the local biome and give the opportunity for a species with many characteristics of *M. mitu* to occupy its previous niche in the Atlantic Forest.

Based on a dataset of birds bred in captivity and mtDNA analyses, Price & Bovier (2002) report that more than half of crosses between species of the same genus produce fertile hybrids. They also estimated that loss of F1 fertility corresponds to lineages that diverged from 1 to 7 million years ago. These estimates were based on the standard cyt *b* clock (2% sequence divergence per million years) that result in younger estimates than those obtained by Bayesian algorithms. Based on 6488 bp of mtDNA, Pereira & Baker (2004) obtained 4% sequence divergence between *M. mitu* and *M. tuberosum* that correspond to a Bayesian divergence time estimate of 4.1 to 5.5 Mya. Despite the difference in the methodology used to estimate divergence times, this range falls within the acceptable diversification time for maintaining the fertility of hybrid birds (Price & Bovier 2002).

Haldane's rule states that "when in the F_1 offspring of two different animal races one sex is absent, rare or sterile, that sex is the heterozygous [heterogametic] sex" (Haldane 1922). Further data have shown that the hybrid sterility and inviability appears first in the heterogametic sex and in latter generations extends to the homogametic sex (Dobzhanki 1936, Muller 1942). In the studied lot of Alagoas Curassows no significant deviation of the sex ratio was present and, contrary to Haldane's rule, there was a slight excess of females (R. Azeredo, pers. com.).

Large out-crossing populations accumulate recessive deleterious genes that may result in reduced fecundity and viability, or inbreeding depression, when heterozygosity declines. However, in populations that are highly inbred for several generations, many of the deleterious alleles may have already been eliminated by natural selection (Templeton & Read 1984), and the negative effects of inbreeding depression may have been overcome. This may be the case for *M. mitu*. Its original wild population was probably small and highly inbred. The captive stock that originated from the breeding of one male with two females represents a highly inbred population (Grau *et al.* 2003, this study) and has since been increasing in size, with more than half of the 130 birds in captivity showing typical features of *M. mitu*.

In general, a population with a low genetic variability has a lower capacity of adapting to changing ecological and climatic conditions, lower resistance to disease, and is more susceptible to extinction. However, there are examples of successful reintroduction of a reduced number of birds from captive breeding (*Branta canadensis* in Scandinavia; Tegelström & Sjöberg 1995), as well as of natural populations with reduced variability as in many seabirds (Baumgarten *et al.* 2001, Lawrence *et al.* 2008). Cracids generally respond well to conditions of captivity and release (Azeredo 1996, Pereira & Wajntal 1999, Angulo 2008, Fournier & Janik 2008). Considering that much effort is being expended on reforestation and captive breeding, it is our opinion that it is worthwhile to proceed with the reintroduction of the available *M. mitu* descendents with *M. mitu* phenotypes. We also advocate that local communities need to be involved in this conservation program to increase the chances of a successful reintroduction. Likewise it is very important to keep a detailed record of crosses performed and their outcome (e.g., infertile and unhatched eggs, rate of chick survival, sex ratio, etc.) in both aviaries that harbor birds with the genomes of the Alagoas curassow.

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