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ESTRUTURA POPULACIONAL, DIVERSIDADE GENÉTICA, ÁREA DE DISTRIBUIÇÃO E CONSERVAÇÃO DO CARDEAL-AMARELO – *GUBERNATRIX CRISTATA* (VIEILLOT, 1817) (AVES, PASSERIFORMES, EMBERIZIDAE).

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Área de Concentração: Biologia Comparada

Orientador: Prof. Dr. Thales Renato Ochotorena de Freitas

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Tese intitulada “ESTRUTURA POPULACIONAL, DIVERSIDADE GENÉTICA, ÁREA DE DISTRIBUIÇÃO E CONSERVAÇÃO DO CARDEAL-AMARELO - *GUBERNATRIX CRISTATA* (VIEILLOT, 1817) (AVES, PASSERIFORMES, EMBERIZIDAE)” apresentada por **Claiton Martins Ferreira** como parte dos requisitos para obtenção do grau de Doutor em Biologia Animal, Área de Concentração Biologia Comparada, aprovada em 12/08/2010 pela Comissão Examinadora:

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DEDICATÓRIA

Para todas as aves que necessitam urgente de planos de conservação, especialmente o cardeal-amarelo.

Para a minha amada esposa, Regina. Verdadeira companheira e amiga, que me deu muito de seu apoio em todos os momentos dessa caminhada. Te amo.

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RESUMO

Estrutura populacional, diversidade genética, área de distribuição e conservação do cardeal-amarelo En – *Gubernatrix cristata* (Vieillot, 1817) (Aves, Passeriformes)(Emberizidae)

Gubernatrix cristata, o cardeal-amarelo, é uma espécie de ave rara do Pampa. Ele tem uma distribuição geográfica restrita ao sul da América do Sul (Uruguai, Argentina e sul do Brasil) e é exclusivo deste bioma. Quatorze viagens de campo foram feitas de Abril de 2006 a março de 2009, em uma tentativa de encontrar indivíduos e coletar amostras para análise genética. Desenvolvemos dez marcadores microssatélites isolados a partir desta espécie e caracterizamos a sua variabilidade alélica. O número de alelos observados em cada locus variou de 4 a 14, com uma média de 7,5 alelos por locus. Os microssatélites mostraram-se úteis em revelar os níveis de diversidade de *G. cristata* e, portanto, podem ser usados para explorar a estrutura genética das populações dispersas ao longo de sua distribuição geográfica atual. Um total de 72 amostras de cardeal-amarelo foi usado neste estudo, 59 foram de amostras contemporâneas e 13 amostras de espécimes de museu. Nós acessamos a diversidade genética da espécie através dos dez loci polimórficos de microssatélites nucleares do *G. cristata* desenvolvidos nesse estudo e através de um fragmento do gene ND2 do DNA mitocondrial. Encontramos apenas três haplótipos com diversidade nucleotídica e haplotípica respectivamente igual a π = 0,00277 e *Hd* = 0,6219. O F_{st} (0,00340) e *Nm* (73,18) mostraram uma fraca estruturação com pouca diferenciação genética. A estatística Fs de Fu foi significativamente diferente de zero (2,248) e D de Tajima foi positivo (2,17506). Ambos os testes mostraram um indicativo de evidência provável de uma diminuição no tamanho da população e/ou seleção equilibradora. As análises usando o "*no admixture model*" e um grande burnin (500000) não resultaram em clustering dos indivíduos. Devido a esse resultado nenhuma atribuição individual a uma área geográfica foi possível. O presente estudo auxilia na compreensão das necessidades de conservação do cardeal-amarelo, fornecendo informações sobre a diversidade genética e estruturação populacional ao longo de sua área de distribuição. Embora não tenhamos encontrado estruturação dentro da nossa área de estudo, é preciso mais estudos, analisando a diversidade genética e estruturação populacional em toda a área de distribuição da espécie, e adicionando mais amostras de animais silvestres da população de La Pampa e de Corrientes. O mapa da área de distribuição do cardeal-amarela parece estar ultrapassado devido à situação cada vez mais crítica que a espécie está enfrentando na natureza. Dada a recente falta de conhecimento sobre sua distribuição e seu estado de conservação em seu habitat natural, decidimos modelar a distribuição potencial da espécie. A principal idéia por trás dessa informação era propor estratégias para sua conservação. Para melhor alcançar esse objetivo,

focamos nossa pesquisa especificamente em a) localizar as populações silvestres desta espécie no Rio Grande do Sul, Uruguai e Argentina, e b) identificar as áreas de ocorrência do cardeal-amarelo que poderiam ser transformadas em áreas protegidas. Todos os registros georeferenciados de *G. cristata* foram extraídos de diferentes fontes: através de trabalho de campo, peles de museu, literatura e bases de dados da Internet. Para modelar a distribuição da espécie, foram selecionadas 20 variáveis ambientais e o aplicativo de modelagem Maxent v. 3.3.2. Mapas de distribuição histórica e atual de *G. cristata* foram criados usando DIVA GIS. O modelo que melhor prediz o índice relativo de adequação ambiental para a espécie é o modelo onde a AUC resultante sobre dados de testes foi 0,868. O resultado do teste de jackknife acerca da importância das variáveis mostrou que a variável ambiental com maior ganho quando usada isoladamente é bio1_sa_30s_cut (temperatura média anual) que, portanto, parece ter as informações mais úteis por si só. A variável ambiental que mais diminui o ganho quando é omitida é alt_sa_30s_cut (altitude) que, portanto, parece ter o máximo de informação que não está presente nas outras variáveis. Se o objetivo de pesquisas futuras do cardeal-amarelo em sua área de distribuição for detectar outras populações, então as áreas preditas em ter elevada adequabilidade relativa no modelo espacial são um bom ponto de partida para a pesquisa mais direcionada. A pressão da caça sobre a espécie é tão grande que o cardeal-amarelo hoje em dia parece ser encontrado apenas em áreas de difícil acesso. Muitas das áreas adequadas para a espécie mostrada pelo modelo podem ainda conter populações e também podem ser usadas como áreas-chave para futuras reintroduções dentro do programa de conservação do cardeal-amarelo.

ABSTRACT

Population Structure, genetic diversity, distribution range and conservation of yellow cardinal En – *Gubernatrix cristata* (Vieillot, 1817) (Aves, Passeriformes)(Emberizidae)

Gubernatrix cristata, the yellow cardinal, is a rare bird species from the Pampas grassland. It has a restricted geographical distribution in southern South America and is unique to this Biome (Uruguay, Argentina, and Southern Brazil). Fourteen field trips were made from April 2006 to March 2009 in an attempt to find individuals and collect samples for genetic analysis. We developed ten microsatellite markers isolated from this species and the characterization of their allele variability. The number of alleles observed for each locus ranged from 4 to 14, with an average of 7.5 alleles per locus. The microsatellites proved to be useful in revealing levels of diversity in *G. cristata,* and thus can be used to explore the genetic structure of scattered populations across its present geographic range. A total of 72 yellow cardinal samples was taken in this study, 59 were from contemporary specimens and 13 samples were from museum specimens. We accessed the species genetic diversity through ten polymorphic nuclear microsatellite loci of *G. cristata* and a ND2 mtDNA fragment. We found only three haplotypes with nucleotide and haplotype diversity equals to π = 0.00277 and *Hd* = 0.6219. The F_{st} (0,00340) and *Nm* (73,18) shown a weak structuring with little genetic differentiation. Fu's Fs statistic was significantly different from zero (2,248) and Tajima's D was positive (2,17506). Both tests showed likely evidence indicating a decrease in population size and/or balancing selection. Analyses using the “no admixture model” and a larger burn-in (500000) yielded no clustering of individuals. Due to this result no individual assignment to any geographical area was possible. The present study assists in understanding the conservation needs for yellow cardinal by providing information on the genetic diversity and population structuring along its distribution range. Although we found no structuring within our study area, further study is needed, examining the genetic diversity and population structuring throughout the species’ range, adding more samples from wild animals from La Pampa population and Corrientes. The yellow cardinal distribution range map seems to be outdated because of the increasingly critical situation the species is facing in nature. Given the recent lack of knowledge regarding its distribution and its state of preservation in its natural habitat we decided to model the species potential distribution. The main idea behind this information was to propose strategies for their conservation. To better achieve that target we specifically focused our research on a) locating wild populations of this species in Rio Grande do Sul, Uruguay and Argentina, and b) identifying areas of occurrence of yellow cardinal that could be turned into protected areas. We extracted all georeferenced *G.*

cristata records from different sources: field working, museum skins, literature, and internet datasets. To model the species distributions, 20 environmental predictors were selected. To model the species distributions we selected the modeling application Maxent v. 3.3.2. Historical and current distribution maps of *G. cristata* were created using DIVA GIS. The model that best predicts the relative index of environmental suitability for the species is the model where resulting AUC on test data was 0.868. The results of the jackknife test of variable importance showed that the environmental variable with highest gain when used in isolation is bio1_sa_30s_cut (Annual Mean Temperature), which therefore appears to have the most useful information by itself. The environmental variable that decreases the gain the most when it is omitted is alt_sa_30s_cut (Altitude), which therefore appears to have the most information that isn't present in the other variables. If the objective of future surveys of the yellow cardinal in its distribution range is to detect other populations, then areas predicted to have high relative suitability in the spatial model is a good starting point for further targeted survey. The hunting pressure on the species is so great that the yellow cardinal nowadays seems to be only found in areas of difficult access. Many of the areas suitable for the species shown by the model may still contain populations and can also be used as key areas for future reintroductions into the yellow cardinal conservation program.

CAPÍTULO UM

Introdução

1.1. Estruturação da Tese e Objetivos

A tese está estruturada na forma de artigos. Para facilitar a consulta, foi dividida em cinco capítulos.

Capítulo Um – Introdução: Esse capítulo fornece informações detalhadas sobre a espécie e área de estudo, bem como métodos gerais utilizados nesta pesquisa. A primeira parte do capítulo consiste em uma descrição detalhada do cardeal-amarelo (*Gubernatrix cristata*), fornecendo informações sobre aspectos da taxonomia, morfologia, distribuição geográfica, ecologia, comportamento e uma visão geral do estado de conservação da espécie e as ameaças que enfrenta em seu ambiente natural. A segunda parte fornece informações sobre as ameaças enfrentadas pelo cardeal-amarelo. A terceira parte apresenta os métodos gerais utilizados na pesquisa e a quarta parte apresenta uma síntese dos resultados encontrados.

Capítulo Dois – Primeiro artigo: Esse capítulo apresenta o artigo apresentado durante o exame de qualificação do doutorado e que foi publicado esse ano na revista *Molecular Ecology Resources*.

Capítulo Três – Segundo artigo: Esse capítulo apresenta o artigo que aborda a estrutura populacional e diversidade genética de *G. cristata*. Após a defesa o mesmo será submetido à revista *Animal Conservation*.

Capítulo Quatro – Terceiro artigo: Esse capítulo apresenta o artigo sobre a distribuição potencial modelada para a espécie. Após a defesa o mesmo será submetido à revista *Diversity and Distribution*.

Capítulo Cinco – Conclusão: Esse capítulo sintetiza as conclusões gerais resultantes da tese e recomenda ações para a conservação da espécie.

Referencias – As referências bibliográficas seguem o estilo da APA (*American Psychological Association*), como sugerido nas orientações ao autor de ambas as revistas - *Animal Conservation* e *Diversity and Distribution*.

Além da introdução e conclusão em Português foi também incluída uma versão em Inglês desses dois capítulos bem como dos agradecimentos. Esse cuidado foi tomado em virtude dos colaboradores canadenses dessa tese.

Nosso principal objetivo foi determinar o status das populações de *Gubernatrix cristata* dentro de sua área de distribuição, especialmente aquelas encontradas no Rio Grande do Sul. Nos focamos em aspectos da biologia reprodutiva, estrutura populacional e fluxo gênico. A principal idéia por trás dessa informação foi propor estratégias para sua conservação. Para melhor atingir

esse alvo, especificamente, buscamos centrar nossa pesquisa em a) localizar as populações selvagens desta espécie no Rio Grande do Sul, Uruguai e Argentina, b) comparar as diferentes populações em termos de variabilidade genética e para verificar o fluxo gênico entre elas, utilizando marcadores moleculares tanto nucleares (microssatélites) quanto mitocondriais (ND2), c) identificação das populações mais importantes em termos de variabilidade genética, onde os esforços de conservação deveriam ser concentrados, e d) identificação de áreas de ocorrência de cardeal-amarelo que poderiam ser transformadas em áreas protegidas.

1.2. O cardeal-amarelo

Gubernatrix cristata é classificado como uma ave da ordem Passeriformes Linnaeus, 1758, Subordem Passeri Linnaeus, 1758, Parvordem Passerida Linnaeus, 1758, Família Emberizidae Vigors, 1825, e a única espécie do gênero *Gubernatrix* (Bencke, 2001; CBRO, 2009). Na literatura (Sclater, 1888; Hudson, 1920; Wetmore, 1926) foram encontradas *Gubernatrix cristatella* e *Coccothraustes cristata* como sinonímias de *Gubernatrix cristata*.

O macho tem peito e costas amarelo brilhante e crista, linha dos olhos e garganta pretos (Fig. 1.1). Possui ainda a sobrancelha e faixa malar amarelas enquanto que na fêmea são brancas, com um peito cinzento (Belton, 1994; Sick, 1997). O macho mede entre 19,2-20,1 cm e pesa cerca de 46g (Belton, 1994; Sick, 1997).

Durante a estação reprodutiva o macho defende seu território atacando de forma agressiva outros machos. Usando um macho de cativeiro como chamariz, esse comportamento territorialista passou a ser usado pelos caçadores como uma forma de capturá-los (Fontana et al. 2003). O acasalamento ocorre na primavera austral, de novembro a fevereiro, com ninhos contendo 2 a 3 ovos azuis esverdeados e manchados de preto (Fig. 1.2) com um período de incubação de cerca de 13 dias (Belton, 1994). Em um trabalho de campo realizado em janeiro de 2007 em Lavalleja, Uruguai, encontramos um ninho (Fig. 1.3) em uma árvore de cerca de 4m de altura conhecida como Viraró, *Ruprechtia salicifolia* da família Polygonaceae.

A alimentação da espécie consiste principalmente de grãos, embora quando filhotes são alimentados basicamente de insetos (Sick, 1997).

O cardeal-amarelo é uma espécie de ave rara do Pampa. Ele tem uma distribuição geográfica restrita ao sul da América do Sul e é exclusivo desse bioma (Uruguai, Argentina e sul do Brasil) (Azpiroz, 2003). Esta espécie tem sofrido um dramático declínio populacional ao longo de sua área de distribuição e seu estado de conservação é definido como ameaçado de extinção (En)

(*Endangered* da sigla em inglês) (BirdLife International, 2000; Fontana et al., 2003).

Na Argentina é agora raro exceto muito localmente (Fraga, 1997). Pode ser encontrada em San Luis, Buenos Aires, La Pampa e Río Negro (especialmente entre General Conesa, San Antonio Oeste e Viedma). Há populações importantes em Corrientes (Pay Ubre e Estancia San Antonio), Entre Ríos (recentemente na área de Montiel, Ceibas e Estancia La Choza) e Córdoba [recentemente em Chancaní (Chebez et al., 1998)] (Di Giacomo, 2005). Ao mesmo tempo, Córdoba apresenta uma redução enorme da população de cardeal-amarelo, apesar de alguns novos registros (Kopta, 1999). Há registros anteriores a 1975 de Salta, Tucumán, Santiago del Estero, Santa Fe e San Juan, e foi registrado sem detalhes para Formosa, Chaco, La Rioja e (erroneamente) em Misiones (Chebez, 1996). Matteucci e colaboradores (2007) afirmaram que a zona dos *bajos submeridionales* é a área mais importante em Santa Fé uma que é a zona onde se concentram a maior densidade de cardeal-amarelo no Chaco argentino. Os autores, entretanto não dizem o quão grande é a população nem se está diminuindo.

No Uruguai, a espécie foi historicamente encontrada em 13 departamentos, mas recentemente apenas em Paysandú, Río Negro, Florida, Rocha, Lavalleja e Artigas (Azpiroz, 2003; BirdLife International, 2000; Lapitz, 2010; Martins-Ferreira, 2007). Em 1999, a população uruguaia de cardeal-amarelo foi estimada em menos de 300 indivíduos, concentrados principalmente na Bacia do Rio Uruguai (Azpiroz em litt. 1999).

Dois indivíduos coletados no Paraguai em 1905 eram provavelmente aves de gaiolas que escaparam do cativeiro (Di Giacomo, 2005).

No Sul do Brasil (Rio Grande do Sul) já era considerado raro na década de 1970 e 1980 (Belton, 1985). Desde então, ele sofreu uma drástica queda e agora se acredita que esteja limitado a locais restritos da Serra do Sudeste, próximo a fronteira com o Uruguai e na ponta Oeste do Rio Grande do Sul (Fontana et al., 2003). Há um registro de um macho de *G. cristata* em um parque urbano de Porto Alegre (Parque Mascarenhas de Moraes) em outubro/novembro de 2005 (Scherer *et al*., 2010). Este registro não representa qualquer extensão de sua distribuição real, uma vez que é muito provavelmente uma fuga do cativeiro como concluíram os próprios autores do trabalho.

Gubernatrix cristata é encontrado em bosques (incluindo bosques de *Prosopis*), planícies, matagais e estepes arbustivas, em altitudes de cerca de 700 m acima do nível do mar (Di Giacomo, 2005). Há uma população reprodutiva no Rio Grande do Sul, Brasil, no Parque Estadual do Espinilho (Damiani et al., 2009). Este parque é uma extensão da província do Espinal, unidade fitogeográfica que descreve um grande arco irregular em território argentino, a partir do centro da cidade de Corrientes e Entre Ríos, na costa atlântica, sul da província de Buenos Aires e passando

pela região central de Santa Fé, Córdoba, San Luís e La Pampa (Marchiori, 2004). Membro do domínio Chaqueño o Espinal se separa da Província Chaqueña pela ausência de espécies do gênero *Schinopsis* (Anacardiaceae) (Marchiori 2004). Dos seus três distritos fitogeográficos, o do "Nandubay", situado mais ao norte (ou nordeste do arco), inclui o Parque do Espinilho e distingue-se pela associação de *Prosopis affinis* e *P. nigra*. Na Mesopotâmia argentina, o "Espinal" também pode incluir: o chañar (*Geoffroea decorticans*), sombra-de-touro (*Jodina rhombifllia*) e até mesmo duas palmas - a jataí (*Butia yatay*) e carandaí (*Trithrinax campestris*) - além das espécies anteriormente citadas (Cabrera & Willink, 1973).

1.3. As Ameaças

Devido aos raros avistamentos do cardeal-amarelo nos últimos anos e sua situação ruim em áreas adjacentes no Uruguai, a população da serra do sudeste parece estar isolada (Martins-Ferreira, 2007). A única população reprodutiva selvagem conhecida no Brasil de *G. cristata* parece estar limitada ao Parque Estadual do Espinilho e arredores (Damiani et al., 2009).

A exploração constante para o mercado de aves de gaiola continua a ser a ameaça mais significativa. A destruição e a fragmentação de seu habitat foram enumeradas como possíveis ameaças adicionais para a espécie (Chebez, 1994; BirdLife International 2000). A principal razão por trás do tráfico é que é um pássaro de bonito colorido e canto melodioso. O crescente registro na Argentina de híbridos entre diuca (*Diuca diuca*) e cardeal-amarelo parece ser uma resposta à escassez de indivíduos do sexo masculino na população da espécie (Bertonatti & Guerra, 1997, 2001). Até a publicação do Livro Vermelho da Fauna Ameaçada de Extinção do Rio Grande do Sul (Fontana et al., 2003), sabia-se que o cardeal-amarelo era capturado principalmente nos municípios de Piratini e Pinheiro Machado. Após as intensas buscas a campo realizadas durante a execução desse trabalho, pode-se acrescentar ainda a essa lista os munícipios de Herval, Pedras Altas e Lavras do Sul. No Rio Grande do Sul, boa parte da paisagem original do sul e oeste foi convertida em plantações de arroz e pastagens para o gado. Entretanto, não se até que ponto essas atividades afetam a espécie (Fontana et al., 2003). Presumivelmente, o seu habitat sofreu com a conversão para pastagem de gado, extração de madeira para lenha e mobiliário (Chebez, 1994) e, especialmente, pela arborização rápida com plantios de eucalipto.

Hudson (1920) já atestava que suas qualidades como belo pássaro com voz alta e musical fizeram dele um pássaro de gaiola preferido. Loydi (2008) encontrou oito indivíduos de cardeal-amarelo em pet shops da cidade de Bahía Blanca, Província de Buenos Aires, em 2004 e

mais 3 em 2007. A comercialização da espécie ainda continua. Pereira e Brito (2005) encontraram *G. cristata* sendo vendido na região metropolitana do Recife em feiras livres. Pessino e Titarelli (2006) disseram que é uma ave ainda muito valorizada como pássaro de estimação e sugeriram uma ação conjunta entre as três províncias do limite sul da sua distribuição (Buenos Aires, Río Negro e La Pampa) como forma de diminuir a pressão sofrida pelas populações da espécie. Dois indivíduos foram avistados em dezembro de 1996 em Río Negro, onde era uma espécie comum que foi diminuindo devido à caça (Seewald e Perez, 2009). Uma ameaça sofrida pelas aves em geral em comunidades locais pobres, incluindo espécies ameaçadas de extinção, é o uso delas como meio de subsistência através da venda em autoestradas (Pautasso, 2003).

1.4. Métodos gerais

1.4.1. Métodos em Genética da Conservação

Atualmente muitas espécies enfrentam os problemas da redução severa de suas áreas de distribuição causadas pela fragmentação e, em casos mais extremos, a perda total dos seus hábitats devido ao crescimento da atividade humana nas últimas décadas. Tanto a perda do hábitat quanto o isolamento dos fragmentos remanescentes, pode reduzir o tamanho populacional a níveis tão baixos que a extinção local pode ser atingida (Gaines *et al*., 1997; Lacy, 1997). Dois processos têm uma grande importância no aumento do risco de extinção em pequenas populações. Em primeiro lugar, processos não genéticos, tanto no nível demográfico quanto ambiental, podem afetar parâmetros tais como a reprodução, mortalidade, distribuição por classes de idade e proporção sexual seguidas de flutuações no tamanho populacional (Goodman, 1987; Shaffer, 1987). Segundo, quanto menor o tamanho populacional, mais suscetível ficam as populações a processos genéticos, tais como deriva genética e endocruzamento, seguidos de perda da variabilidade genética populacional ou aumento da homozigose (Shields, 1993; Hartl & Clark, 1997; Broders *et al.*, 1999) e do efeito de depressão por endocruzamento, ou seja, diminuição do *fitness* da descendência por efeito do cruzamento entre aparentados. Esses processos genéticos conhecidos como erosão genética, podem causar degradação de importantes características da espécie como a sobrevivência, crescimento e reprodução (Shields, 1993; Hedrick *et al.*, 1996).

Apesar de que a discussão a respeito do real impacto da depressão por endocruzamento em populações naturais ainda existe, e que os padrões observados em animais de cativeiro podem ser bem diferentes daqueles achados na natureza (Hoelzel *et al.*, 1993; Craig, 1994) a maioria dos planos de conservação atuais monitoram, tanto o tamanho, quanto a estrutura populacional e a variabilidade genética das espécies (Lande, 1995; Gaines *et al*. 1997).

A ferramenta molecular mais usada neste tipo de abordagem são as análises de DNA mitocondrial (mtDNA) que permitem o seguimento de rastros genealógicos além dos limites genéticos entre as populações, espécies e grupos taxonômicos mais elevados.

Outras vantagem no uso das informações obtidas a partir da análise da diversidade genética: redução do risco de extinção reduzindo o endocruzamento e perda da diversidade genética; identificação de populações importantes, do ponto de vista de variabilidade genética; resolução da estrutura populacional; definição de unidades de manejo dentro das espécies; detecção de hibridizações; permite amostragem não-intrusiva para análise genética; possibilita a definição de sítios para reintrodução e escolha da melhor população para reintrodução; permite uma melhor compreensão da biologia da espécie (Avise *et al*., 1987a; Gaines *et al*., 1997; Lacy, 1997; Warning, 1994, 2000; Frankham *et al*., 2002; Sutherland *et al*., 2004).

Isto levou a melhor compreensão de biogeografias regionais e áreas de endemismo, bem como à elaboração de propostas prioritárias para a conservação das biodiversidades taxonômicas e locais (Rojas, 1995; Moritz & Bermingham, 1998; da Silva & Patton, 1998; Firestone *et al*., 1999; Jerusalinsky, 2001).

A maioria das espécies distribuem-se em populações geograficamente estruturadas, muitas das quais podem experimentar pouco ou nenhum contato por longos períodos de tempo. Outras espécies, entretanto, podem estar caracterizadas por expansões recentes, o que determina estreitas conexões genealógicas entre as populações resultantes (Ibrahim *et al.*, 1996).

Características demográficas históricas e contemporâneas podem afetar a estrutura espacial de populações co-específicas e com ela influenciar nas genealogias matrilineares em vários sentidos. Um importante desafio dos estudos filogeográficos é decifrar os fatores demográficos passados e presentes que produzem um determinado padrão de relações genealógicas (Avise, 2000).

Duas metas fundamentais da genética da conservação são a prevenção de problemas associados ao endocruzamento e à deriva genética em pequenas populações e a descrição da estrutura populacional com a finalidade de identificar unidades de conservação. A genética molecular tem proporcionado numerosas técnicas de acesso à variabilidade genética dentro e

entre as populações, tanto através de polimorfismos de proteínas quanto de DNA (Avise, 1994). Por serem altamente polimórficos e possuírem altas taxas de mutação (dentre outras características), os marcadores moleculares mais utilizados em estudos populacionais e filogeográficos são o DNA mitocondrial e os *loci* de microssatélites.

O genoma mitocondrial de animais é haplóide e está formado por uma dupla fita circular entre 15000 e 17000 pares de bases de comprimento estando presente em centenas a milhares de cópias por célula (Meyer, 1993; Li & Graur, 2000).

Tipicamente cada genoma de DNA mitocondrial (mtDNA) consiste de 37 genes funcionais sem longos espaços intergênicos. Estes *loci* codificam para 22 RNAs de transferência, dois RNAs ribossômicos e 13 RNAs mensageiros para a síntese de subunidades de proteínas específicas envolvidas na fosforilação oxidativa e o transporte de elétrons (Avise, 2000).

O mtDNA é simples em estrutura e econômico em tamanho sendo que o único grande fragmento não codificante (de aproximadamente 1000 pares de bases) é a região controladora (CR - *Control Region*) da molécula que tem função regulatória na dinâmica da molécula e é predominantemente rica em bases AT (Brown, 1985). Nos vertebrados esta região é referida como *D-loop* (*Displacement-loop*) devido à formação de uma estrutura de fita tripla que gera deslocamentos dinâmicos no começo da replicação do mtDNA (Brown et al., 1986).

Devido à rápida evolução o mtDNA torna-se um bom marcador para o estudo de diferenciações genéticas recentes por acumular substituições de base, inserções e deleções com uma taxa média de 5 a 10 vezes mais rápida que o DNA nuclear cópia simples (Brown *et al*., 1979). Várias hipóteses têm sido levantadas na tentativa de explicar a rápida evolução do DNA mitocondrial: alta taxa de mutação devido à ineficiência dos mecanismos de reparo de DNA, alta exposição aos radicais livres (mutagênicos) produzidos no interior da mitocôndria ou ao rápido *turnover* dentro das linhagens celulares. Também o fato do DNA não estar complexado com proteínas histonas, que são evolutivamente conservadas e poderiam limitar a taxa evolutiva do DNA nuclear (Avise, 2000). Outra vantagem do mtDNA, como marcador para o estudo de diferenciações genéticas recentes, é a freqüência extremamente baixa de transposições, inversões, rearranjos e recombinação (Brown, 1985). Isto permite a caracterização de linhagens filogenéticas sem a ambigüidade causada pela recombinação meiótica que ocorre nos genes nucleares.

1.4.2. Esforço de Campo e de Laboratório

Foram feitas 14 viagens de campo de Abril de 2006 a março de 2009 ao Rio Grande do Sul e Uruguai, em uma tentativa de encontrar indivíduos e coletar amostras para análise genética. Cada viagem durou em média 10 dias o que resultou num total de cerca de 1680 horas de campo, incluindo observações, transectos e capturas em redes de neblina.

Todos os exemplares foram anilhados com anilhas fornecidas pelo CEMAVE/ICMBio (Centro Nacional de Pesquisa para a Conservação de Aves Silvestres). Na Argentina, uma visita foi feita ao Museo de Ciencias Naturales Bernardino Rivadavia onde, a partir de oito peles escolhidas foram coletadas amostras de tecido, uma de cada província onde a espécie ocorria, e uma do Uruguai. Um total de 97 amostras de cardeal-amarelo foram usadas neste estudo (Apêndice A.1) sendo que apenas 72 funcionaram adequadamente.

Uma biblioteca de DNA genômico enriquecido em microssatélites de *Gubernatrix cristata* foi construída e dez loci de microssatélites polimórficos nucleares (com repetições de 2 bp) foram selecionados com base nos níveis de polimorfismo e tamanho dos produtos de amplificação. Os Primers desenhados e as condições de PCR estão descritos no capítulo 2.

Foi extraído DNA de amostras de pena utilizando o protocolo Alkali para extração de DNA e foram sequenciados vários loci de DNA mitocondrial (mtDNA) utilizando primers de aves degenerados de Sorenson (2003) para determinar se variabilidade genética suficiente estava presente para análise. A região controle, ATPase8 e ND2 foram escolhidas, uma vez que estas regiões têm se mostrado úteis em estudos de genética de populações de aves (Burg & Croxall, 2001; Oyler-McCance et al., 2005; Sorenson, 2003). Porções do gene ND2 foram amplificadas com sucesso, mas não foi possível amplificar a região controle e ATPase8. Devido à baixa qualidade do DNA extraído de várias amostras, o gene foi amplificado em dois pedaços e correu quatro reações de seqüenciamento (ambas as fitas de cada produto de PCR). Os primers usados e as condições de PCR estão descritos no Capítulo 3.

1.4.3. Métodos para Modelagem de Distribuição

Todos os registros georeferenciados de *Gubernatrix cristata* foram extraídos de diferentes fontes: registros em GPS durante trabalhos de campo, peles de museu, literatura e bases de dados da Internet. No total este conjunto foi composto por 172 registros

georreferenciados pertencentes a indivíduos, casais ou um pequeno número de aves vistas na zona onde os registros foram tomados.

Para modelar a distribuição da espécie, foram selecionados 20 indicadores ambientais. Foi feito o download de 19 variáveis bioclimáticas (1950 - 2000) e altitude (em m) do conjunto de dados do WORLDCLIM (http://www.worldclim.org) para a América do Sul na resolução de 30 arco-segundo (~ 1km) (Hijmans et al ., 2005a).

BIOCLIM (usando a plataforma DIVA-GIS) e MaxEnt foram escolhidos para nossos dois modelos preditivos biogeoclimáticos separados. DIVA-GIS é um plataforma baseada em SIG do modelo BIOCLIM (Hijmans et al., 2005b) que se encaixa num envelope de habitat mínimo da espécie em um espaço climático multidimensional. MaxEnt é um programa desenvolvido para a modelagem de entropia máxima de distribuição geográfica das espécies (Phillips et al., 2006) que expressa a adequação de cada célula da grade em função das variáveis ambientais nessa quadrícula. Alto valor de funções indica condições adequadas preditas para a espécie. Estes modelos foram selecionados por acomodarem os dados de presença apenas (Elith et al., 2006).

1.5. Resultados gerais

Em populações sofrendo um estrangulamento genético, a diversidade genética observada excede a diversidade genética esperada em equilíbrio sob a suposição de equilíbrio mutação-deriva. A hipótese nula para o excesso de heterozigosidade foi testada usando o teste Wilcoxon de Rank de Sinal Fornecido. As probabilidades no âmbito do Modelo de Alelos Infinitos (IAM), Mutação de Duas Fases (TPM) e Mutação Step-wise (SMM), foram respectivamente: (P <0. 05273), (P> 0,09668) e (P <0,42285). A hipótese nula é aceita no modelo SMM apenas, o que implica que *Gubernatrix cristata* não sofreu qualquer estrangulamento genético recente. Os valores estimados do excesso de heterozigosidade e suas probabilidades de teste do sinal foram 5,89 (P <0,15033) para o IAM, 5,90 (P <0,15148) para o TPM e 5,91 (P <0,38936) para o SMM. A hipótese de equilíbrio mutação-deriva também não foi aceita com base no teste de sinal sob os modelos de mutação.

Somente 67 amostras foram seqüenciadas com sucesso para o fragmento ND2 alvo (seqüências dos haplótipos foram depositadas no GenBank sob números de acesso HQ15712 a HQ15714). Um total de 349 pb foram alinhados em amostras de *G. cristata* contendo sítios informativos parcimoniosos e variáveis e um total de três haplótipos (H1 - H3). Entre os haplótipos H1 e H2 75 passos mutacionais foram encontrados, ao passo que somente 34 entre H2 e H3.

Medidas de variabilidade genética tais como diversidade haplotípica (*Hd*), variação nucleotídica estimada com base no número de sítios segregantes (π) e o número médio de diferenças em pares de populações amostradas (k) são mostrados no Capítulo 3. Estimativas de diversidade nucleotídica e haplotípica na amostra foram relativamente elevadas, com π = 0,00277 e *Hd* = 0,6219. A análise de network não revelou padrão de estrutura algum. O F_{st} (0,00340) e *Nm* (73,18) mostraram uma estruturação fraca com pouca diferenciação genética. Falta de apoio para a hipótese de expansão demográfica foi o resultado de diferentes testes realizados para testar a teoria neutra da evolução molecular: teste estatístico D* de Fu e Li (0,71916) não apresentaram significância estatística (P> 0,10), bem como não foi significativo o teste estatístico F* de Fu e Li (1,34814) (P> 0,10). Estatística Fs de Fu foi 2,248 o qual foi significativamente diferente de zero e é uma evidência provável indicando uma diminuição no tamanho da população e/ou seleção equilibradora. D de Tajima foi positivo (2,17506), o que significa baixos níveis de polimorfismos de freqüência alta e baixa, indicando uma diminuição no tamanho da população e/ou seleção equilibradora.

Uma vez que a origem geográfica exata de muitos dos indivíduos amostrados (45%) de cardeal-amarelo é desconhecida ou incerta (muitos dos indivíduos que foram amostrados de zoológicos ou de particulares, ou que vieram de apreensões policiais não foram associados a informações confiáveis sobre a sua localização exata de captura), a análise do Structure (Falush *et al*., 2003) seria particularmente relevante porque isso permitiria o agrupamento de indivíduos, sem a necessidade de uma informação geográfica *a priori*. Cinco corridas independentes para 1 a 6 populações, utilizando o modelo admixture e freqüências alélicas afins entre as populações (Falush et al. 2003), foram executadas no programa Structure. Os resultados de todas as corridas (mostrado no capítulo 3) foram praticamente os mesmos sugerindo que não houve aglomeração de indivíduos. Devido a esse resultado nenhuma atribuição individual a qualquer área geográfica foi possível.

A distribuição das diferenças da variação haplotípica observada foi unimodal revelando uma partida da variação esperada em uma população constante (k = 0,965, variação de k = 0,7205, r = 0,1319 estatística de irregularidade).

Os mapas com uma discussão detalhada dos resultados sobre os modelos de distribuição da espécie são encontrados no Capítulo 4.

A partir da execução desse projeto avançamos o conhecimento sobre *Gubernatrix cristata*, principalmente no que se refere ao seu status populacional dentro de sua área de distribuição. Conseguimos determinar claramente onde a espécie ainda sobrevive no Brasil e quais

são, de fato, os melhores locais para se investir na sua conservação. A partir desse projeto, definiram-se ações para o manejo da espécie em cativeiro. Foram estabelecidas parcerias e novos contatos que possibilitaram a centralização de todos os exemplares oriundos de apreensão no RS em um local: o zôo de Gramado. Esse foi o primeiro passo para a criação de um programa de reprodução em cativeiro da espécie, bem como um protocolo sanitário nacional e o engajamento de instituições públicas, privadas e ONGs para a conservação de *G. cristata*. Um dos impactos mais significativos do projeto foi o estímulo à criação do Plano de Ação do Cardeal-Amarelo, uma vez que enquanto não há um projeto efetivo de conservação para uma espécie ameaçada não há como definir ações por parte do ICMBio. Algumas dessas ações, embora ainda não tenha sido publicado o plano, já foram executadas e outras estão sendo realizadas. O Projeto Cardeal-Amarelo iniciou em 2005 como um projeto-piloto na região de Pinheiro Machado e Pedras Altas. Ele tomou forma em 2006 e 2007 com o apoio da Fundação O Boticário de Proteção à Natureza. E seguirá ainda por muitos anos à frente, pois muitas questões ainda precisam ser respondidas.

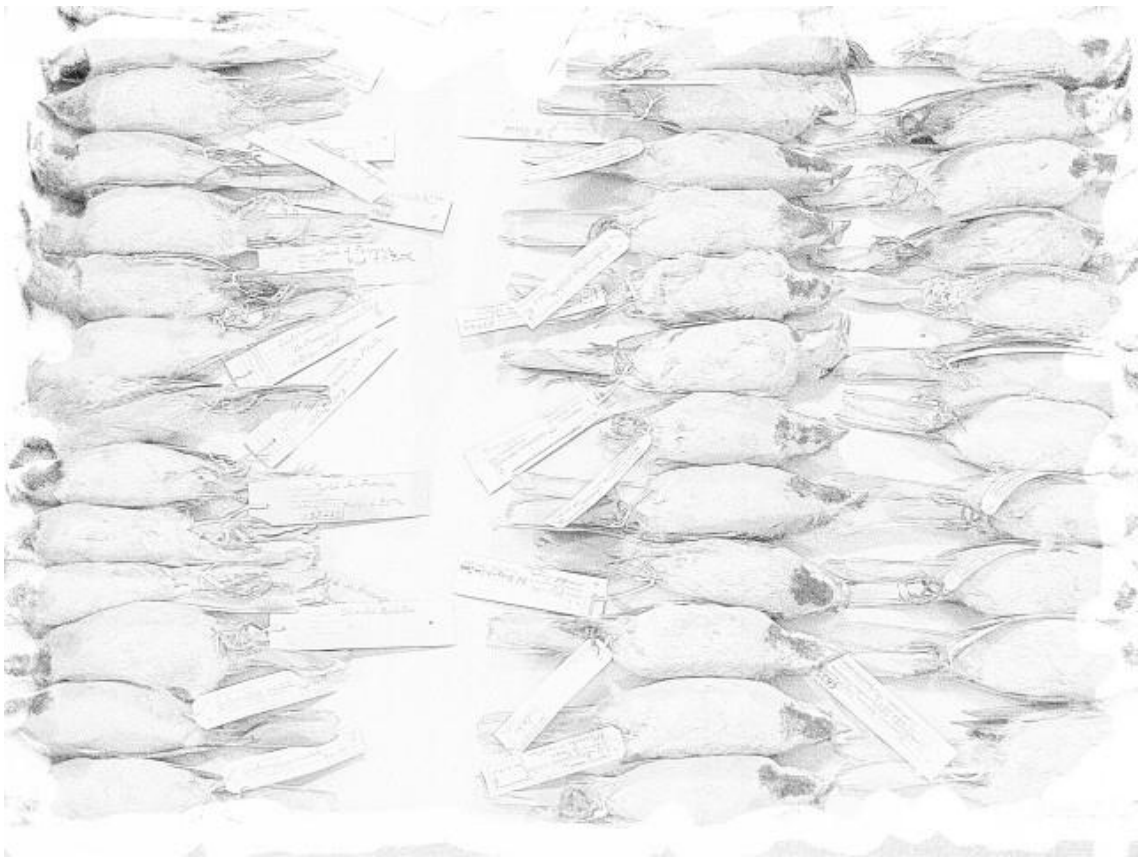
Figura 1.1. Macho de *Gubernatrix cristata*. Departamento Lavalleja, Uruguay em 19.03.2009. Foto de Claiton Martins-Ferreira

Figura 1.2. Ovos de *Gubernatrix cristata* encontrados em um ninho ativon. Departamento Lavalleja, Uruguay. Foto de Alvaro Riccetto.

Figura 1.3. Ninho de *Gubernatrix cristata* encontrado abandonado e coletado. Departamento Lavalleja, Uruguay em 22.01.2007. Foto de Claiton Martins-Ferreira

CAPÍTULO DOIS

Isolation and characterization of 10 microsatellite loci in the Yellow Cardinal *Gubernatrix cristata* En



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Isolation and characterization of 10 microsatellite loci in the Yellow Cardinal *Gubernatrix cristata* En

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Abstract *Gubernatrix cristata* is a rare and endangered bird species from the South American Pampas grassland with a restricted and patchy distribution. Here we report the development of ten microsatellite markers isolated from this species and the characterization of their allele variability. The number of alleles observed for each locus ranged from 4 to 14, with an average of 7.5 alleles per locus. The microsatellites proved to be useful in revealing levels of diversity in *G. cristata,* and thus can be used to explore the genetic structure of scattered populations across its present geographic range.

Keywords *Gubernatrix cristata* · Genetic diversity · Microsatellites · Endangered species · Pampas grassland · Mithochondrial DNA ·

Introduction

Gubernatrix cristata, the Yellow Cardinal, is a rare bird species from the Pampas grassland. It has a restricted geographical distribution in southern South America and is unique to this Biome (Uruguay, Argentina, and Southern Brazil). This species has undergone a dramatic population decline across its range and its conservation status is defined as endangered (Fontana *et al*. 2003). The remaining populations are small and isolated (BIRDLIFE INTERNATIONAL, 2000, 2009). The destruction and fragmentation of its habitat have been enumerated as possible additional threats for the species, as well as the extreme pressure of capture to supply the traffic in wild animals (Chebez 1994; BIRDLIFE INTERNATIONAL, 2000, 2009). The aim of this study was to develop a set of polymorphic microsatellite markers (*Simple Sequence Repeat* – SSR) for *G. cristata* to describe the genetic structure of these endangered populations. These are the first microsatellites isolated for the Yellow Cardinal. Thirteen field trips were made from April 2006 to May 2008 in an attempt to find individuals and collect samples for genetic analysis. In Rio Grande do Sul (the southernmost Brazilian state), just three Yellow Cardinals were found in a single locality (Espinilho State Park). In Uruguay, three separate field trips to different localities located only five individuals at a farm in Minas, Lavalleja Department. In Argentina, a visit was made to the *Museo de Ciencias Naturales Bernardino Rivadavia* from which tissue samples of eight skins were collected, one from each province where

the species has occurred, and one from Uruguay. The other samples came from specimens seized by the Environmental Police and are of unknown origin.

Material and Methods

Total DNA was extracted from 42 fresh samples of feathers or blood stored on FTA Cards (Whatman) of *G. cristata*, (using methods in Medrano *et al.* 1990; Eguchi and Eguchi, Y. 2000; Bello, N. *et al.* 2001), and from 13 museum skin samples (using methods in Mundy N. I. *et al.* 1997). Microsatellites were identified from genomic libraries partially enriched for the nucleotide repeats (CT)n and (GT)n using biotinylated oligonucleotide sequences linked to magnetic particles and recovered by streptavidin (KIJAS et al., 1994; BILLOTE et al., 1999). DNA fragments enriched with microsatellites were ligated in pGEM-T Easy vector (Promega), as described by the manufacturer, and used to transform cells of E. coli XL1Blue competent (Stratagene). A total number of 96 colonies were obtained and sequenced using the BigDye v3.1 Terminator kit and an ABI 3100 Genetic analyzer (Applied Biosystems). From these sequences, 40 clones were found to contain SSR motifs. Following some simple rules to design efficient PCR primers (WEISING, K et al. 2005) not all positive clones were useful for primer design. Forward and reverse sequences were aligned in SeqMan (DNASTAR packet), and primers were designed for 23 loci using the software PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For each SSR locus, the forward primer was synthesized with a 5' 19 bp long M13 tail (5'-CACGACGTTGTAA AACGAC-3') which functioned as an adaptor for a fluorescently labeled primer. Following the method of Schuelke (2000), three primer amplifications were performed using a locus-specific forward M13 tailed primer, a locus specific reverse primer, and a universal fluorescent-labelled M13 primer, labeled with either 6-FAM or VIC (Applied Biosystems). All the PCR amplifications were made under the following conditions: 94ºC for 4 min, followed by 36 cycles of 94ºC for 30 s, 54ºC for 25 s, and 72ºC for 30 s, with a final extension of 7 min to 72ºC. The amplification volume was 12.5 µl and consisted of: 3 µl of DNA, 6.67 µl H2O, 1.25 µl buffer (10X:100 mM Tris-HCl, pH 8.3, 500 mM KCl, 25 mM $MgCl_2$, 0.1% gelatin), 0.28 µl dNTPs (10 mM), 5 pmol reverse primer, 2.5 pmol forward primer, 5 pmol universal M13 primer, and 0.25 U Taq polymerase (Amersham Pharmacia Biotech). The amplified products were run on an ABI 3100 Genetic Analyzer along with the LIZ 500 size standard (Applied Biosystems). The STRand v3.7 software was used for peak scoring. ARLEQUIN 3.11 (EXCOFFIER et al. 2005) was used to calculate the observed (HO) and the expected (HE) heterozygosities, to test if there was deviation from Hardy-Weinberg equilibrium (HWE), and to test if there was linkage disequilibrium among all pairs of loci. MICRO-CHECKER (Van Oosterhout et al. 2004) was used to quantify the possible genotyping errors and null alleles. Ten SSRs were found to be polymorphic, although four of them presented null alleles according to

MICRO-CHECKER (GcrisC02, GcrisE02, GcrisF12, and GcrisH09) and ARLEQUIN (Table 1). In the remaining six loci, linkage disequilibrium among any pairs of loci, genotyping mistakes because of the presence of null alleles, allele dominance, or stuttering were not detected. The number of alleles per locus varied from 4 to 14 with an average of 7.5 alleles per locus. The observed heterozygosity (HO) varied from 0.126 to 0.893 and the expected (HE) heterozygosity from 0.130 to 0.930, with means and standard errors of 0.6824 $\pm$ 0.1144 and 0.7132 $\pm$ 0.0272 respectively (Table 1) (GenBank accession numbers: GU237061 to GU237070). The deviation from Hardy-Weinberg equilibrium presented by 4 loci may be the result of a sampling bias. Since the species is rare and most of the material used was from seizures made by the Environmental Police, we cannot determine if all of the samples represent the same population. Samples could represent several differentiated populations combined that may be the cause of departures from HWE. For the four loci that had null alleles and departed from HWE due to heterozygote deficiencies, we estimated null allele rates (Table 2). The high level of heterozygosity observed indicates that the species has not suffered any severe bottleneck, even though this conclusion should be regarded with caution because of the low sample size due to the rarity of the species in the wild, and also because of the low DNA quality in many of these samples due to the material collected. In terms of *ex situ* conservation, these data allow us to conclude that creating a *G. cristata* captive breeding program would have a high probability of success since the species is not suffering from low levels of genetic diversity as shown by the observed heterozygosity. The primers proved to be useful in revealing levels of diversity in *G. cristata*, and can be used to explore the genetic structure of scattered populations across its present geographic range.

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Table 1. *Gubernatrix cristata* microsatellite loci characteristics

Locus	Primer Sequences[a]	Repeat	Alleles	Size (bp)	H_O	H_E	PIC values[b]
GcrisC02	F: TCACCCCTTTGGTTGCTTAC	(CA)7	4	247	0,267	0,438*	0,373
	R: TCAGGGGTGTGTCTCTCCTT						
GcrisC08	F: AGCCACTCATCCGATTTGTT	(AC)8	6	174	0,911	0,791	0,748
	R: AGTCTTGGCTGCCTGTCATT						
GcrisE02	F: TTGGAGACCTTGTTGGTGTG	(CA)8	4	227	0,229	0,710*	0,648
	R: TCACACTGGGCACATGAGAT						
GcrisF02	F: TTCACTGGGTTCACAGAAAGG	(CA)12	14	190	0,833	0,893	0,873
	R: GCTGTCGTCATTGTTCAGGA						
GcrisF12	F: AAGATGTGCCTTTGGTCTGG	(AC)9 (CA)7	6	230	0,600	0,784*	0,734
	R: CCATTGCAGAAATGTCCTGA						
GcrisG10	F: CAGGATCCTCTGCCATGTCT	(CA)7	4	236	0,130	0,126	0,122
	R: TTTTCCCTTTTAACGCCAAG						
GcrisH06	F: GAGAGAAACCAGGTGCTTCG	(CA)9	10	230	0,735	0,830	0,799
	R: GTTTTAAGGCTGGGGACACA						
GcrisH07	F: GTGTGACTTGTTCCCCTTCC	(CA)7 (AC)5	6	213	0,739	0,755	0,709
	R: ACAGGAGCAGCCAGTTGAAT						
GcrisH09	F: CAAGGTGTTGTGGAGCCTTT	(CA)7	10	236	0,702	0,834*	0,802
	R: GCAACCAGCACATGAAATTG						
GcrisH12	F: AAGCGTGACCATGAAAAATGT	(CA)7	5	249	0,232	0,700	0,637
	R: TTCATCTGCCCTCCTTGTTC						

[a] All forward primers were M13-tailed at the 5' end. Significant departures from HWE: * P < 0.001

[b] Polimorphism Information Content

Table 2. Estimates of null allele rates of four loci that departed from HWE using three methods of null allele estimation.

Locus	Null Present	Oosterhout	Chakraborty	Brookfield 1
C2	yes	0,2512	0,4278	0,1662
E2	yes	0,3045	0,4823	0,2682
F12	yes	0,1072	0,1248	0,0966
H9	yes	0,0694	0,0751	0,0631

CAPÍTULO TRÊS

Lack of genetic population structure and evidence for population shrinkage in the endangered yellow cardinal, Gubernatrix cristata

Article to be submitted to Animal Conservation Journal.

Lack of genetic population structure and evidence for population shrinkage in the endangered yellow cardinal, Gubernatrix cristata

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Abstract

Gubernatrix cristata, the Yellow Cardinal, is a rare bird species from the Pampas grassland. It has a restricted geographical distribution in southern South America and is unique to this Biome (Uruguay, Argentina, and Southern Brazil). Fourteen field trips were made from April 2006 to March 2009 in an attempt to find individuals and collect samples for genetic analysis. A total of 72 yellow cardinal samples was taken in this study, 59 were from contemporary specimens and 13 samples were from museum specimens. We accessed the species genetic diversity through ten polymorphic nuclear microsatellite loci of *Gubernatrix cristata* and a ND2 mtDNA fragment. We found only three haplotypes with nucleotide and haplotype diversity equals to $\pi = 0.00277$ and *Hd* = 0.6219. The F_{st} (0,00340) and *Nm* (73,18) shown a weak structuring with little genetic differentiation. Fu's Fs statistic was significantly different from zero (2,248) and Tajima's D was positive (2,17506). Both tests showed likely evidence indicating a decrease in population size and/or balancing selection. Analyses using the "no admixture model" and a larger burn-in (500000) yielded no clustering of individuals. Due to this result no individual assignment to any geographical area was possible. The present study assists in understanding the conservation needs for Yellow Cardinal by providing information on the genetic diversity and population structuring along its distribution range. Although we found no structuring within our study area, further study is needed, examining the genetic diversity and population structuring throughout the species' range, adding more samples from wild animals from La Pampa population and Corrientes.

Keywords: Endangered species, Genetic diversity, *Gubernatrix cristata*, Mithochondrial DNA, Microsatellites, ND2 gene, Pampas grassland.

Introduction

Gubernatrix cristata, the Yellow Cardinal, is a rare bird species from the Pampas grassland. It has a restricted geographical distribution in southern South America and is unique to this Biome (Uruguay, Argentina, and Southern Brazil) (Azpiroz, 2003). This species has undergone a dramatic population decline across its range and its conservation status is defined as endangered (BirdLife International, 2000; Fontana *et al*. 2003). In Argentina, it is now rare except very locally (Fraga, 1997). In Uruguay, it was historically known from 13 departments, but recently from only Paysandú, Río Negro, Florida and Rocha (BirdLife International, 2000; Azpiroz, 2003). In Southern Brazil (Rio Grande do Sul State) it was already considered rare in the 1970s and 1980s (Belton, 1985). Since then it suffered a dramatically decline and now is believed it is confined to restricted spots in the SE hills, near Uruguayan border and at W tip of Rio Grande do Sul (Fontana *et al*. 2003). Once the lack of yellow cardinal sightings in the last few years and its bad situation in the adjacents areas in Uruguay, the SE hills population seems to be isolated or even extinct. (Martins-Ferreira, 2007). The remaining populations are small and isolated (BirdLife International, 2000). The only breeding wild population known in Brazil of *G. cristata* seems to be limited to Espinilho State Park (*Parque Estadual do Espinilho*) and surroundings (Damiani *et al*., 2009). The destruction and fragmentation of its habitat have been enumerated as possible additional threats for the species, as well as the extreme pressure of capture to supply the traffic of wild animals (Chebez 1994; BirdLife International, 2000). The main reason behind the traffick is that bird is a colorful and singing passerine. The male has a bright yellow chest and back with black crest, eye line and throat. It has the full eyebrow and malar stripe yellow while the female are white with a gray breast (Belton, 1994; Sick, 1997). During the breeding time the male defends its territory aggressively atacking other males. That behavior turns to be a way how poachers capture them using a captive male as decoy (Fontana *et al*. 2003). Hudson (1920) already atested that its qualities as beautiful bird with loud and musical voice made it a favourite cage bird. The growing record of hybrids among yellow cardinal and common diuca finch (*Diuca diuca*) in Argentina seems to be a response to the shortage of male individuals in the

population of the species (Bertonatti & Guerra, 1997, 2001). With the null hypothesis that Yellow Cardinal has not a population structure throughout its distribution range, we decide to investigate that. Our aim was to determine the population status of *Gubernatrix cristata* across its distribution range, taking in consideration the population structure, genetic diversity and gene flow. Only with such information in hand we would be able to propose a specific conservation strategy to the species.

Methods

Sampling

Fourteen field trips were made from April 2006 to March 2009 in an attempt to find individuals and collect samples for genetic analysis. In Rio Grande do Sul (the southernmost Brazilian state), just four Yellow Cardinals were found in a single locality (Espinilho State Park). In Uruguay, four separate field trips to different localities found only six individuals in Minas, Lavalleja Department, five in one farm and one in another land. All of them were ring banded with rings provided by CEMAVE/ICMBio (National Research Center for the Conservation of Wild Birds). In Argentina, a visit was made to the Museo de Ciencias Naturales Bernardino Rivadavia from which tissue samples of eight skins were collected, one from each province where the species has ocurred, and one from Uruguay. Five more samples came from museum specimens: one from *Museu de Zoologia da Universidade de São Paulo* (MZUSP, São Paulo, Brazil), two from *Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul* (MCN/FZBRS, Porto Alegre, Brazil) and two from Royal Ontario Museum (ROM, Toronto, Canada) (Fig. 1). The rest of the samples came from specimens seized by the Environmental Police and are of unknown origin. A total of 97 yellow cardinal samples was taken in this study (Appendix A.1). Some of which having been maintained (but not bred) in captivity by zoos, conservation institutions, or private individuals. Of these, 25 presented a poor DNA quality that wasn't possible to proceed with any analysis. From 72 samples that worked properly 59 were from contemporary specimens. The other 13 samples were from museum specimens (years 1905–1975) selected from across the range, but with a special emphasis on the population in Argentina since many samples with unknown

geographical data were supposed to come. Blood samples were stored in FTA Cards (Whatman) and feathers were placed in 95% ethanol solution. Museum tissues samples had their DNA extracted using the DNeasy Tissue Kit (QIAGEN) with the appropriate protocol according to the manufacturer for each type of tissue (Mundy, N. I. *et al.*, 1997). Contamination with modern DNA or PCR products was monitored by including two extraction blanks in every extraction round and prevented by performing all museum samples extractions in a dedicated 'clean' laboratory, kept free of good quality DNA and PCR products.

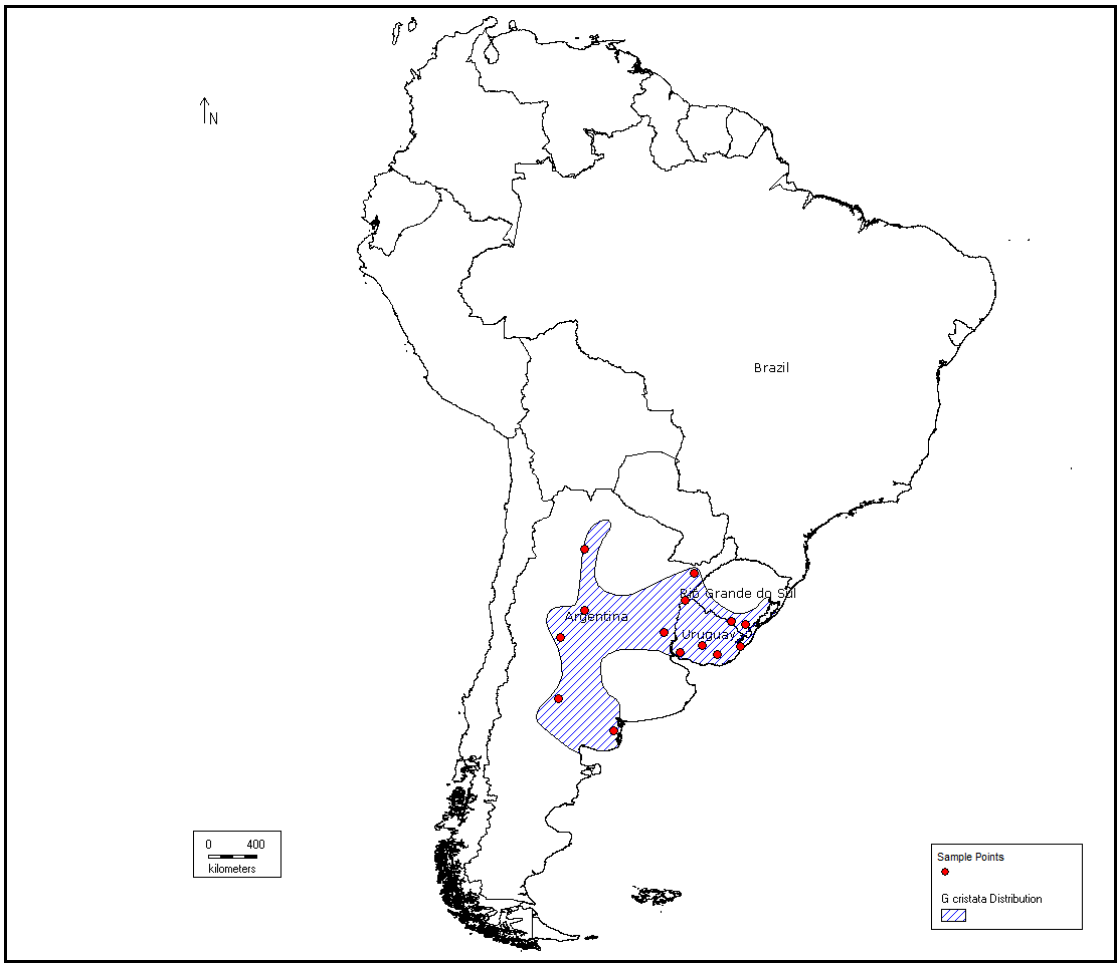

Figure 1. Map showing the points of collected samples from wild individuals or from museum skins across *G. cristata* distribution range. Polygon used with permission from Birdlife.

Laboratory Analyses

A microsatellite-enriched genomic DNA library of *Gubernatrix cristata* was constructed and ten polymorphic nuclear microsatellite loci (with 2-bp repeats) were selected on the basis of polymorphism levels and size of amplification products. All samples were genotyped using a polymerase chain reaction targeting different loci (Table 1). Primers and PCR conditions are given in Molecular Ecology Resources Primer Development Consortium (2010). PCR products were purified, cycle sequenced on both strands, and run on an ABI 3730 sequencer.

We extracted DNA from feather samples using the Alkali protocol for DNA extraction and attempted to sequence several mitochondrial DNA (mtDNA) loci using degenerate avian primers from Sorenson (2003) to determine if sufficient genetic variability was present for analysis. The control region, ATPase8, and ND2 were chosen, as these regions have proven useful in population genetic studies of birds (Burg and Croxall, 2001; Sorenson, 2003; Oyler-McCance *et al.,* 2005). We successfully amplified portions of the ND2, but were unable to amplify control region and ATPase8. Given the lower quality of DNA extracts from many samples we decided to amplify the gene in two pieces and run four sequencing reactions (both strands of each PCR product). The primers used were *ND2H* 5' CCT TGA AGC ACT TCT GGG AAT CAG A 3' (Tavares *et al*., 2006) - ND2F_Passerine 5' CCA YCC ACG AGC YAT TGA AGC 3' (new primer designed), and MetLTF 5' AAG CTA TCG GGC CCA TAC CCG 3' (Tavares *et al.*, 2006) - ND2R_Passerine 5' GCC ATG CRT TGG TYA TGC TNG AG 3' (new primer designed). Those primers were used to amplify a 349 base-pair region that contained the variable sites in ND2. We amplified DNA via the polymerase chain reaction (PCR) in 12,5 µl reaction volumes containing 2.0 µl DNA, 7.92 µl water, 0.5 µl (10 pmol) of each primer, and 1.58 µl PCR master mix, consisting of a 1.25 µl buffer (10X:100 mM Tris-HCl, pH 8.3, 500 mM KCl, 25 mM $MgCl_2$, 0.1% gelatin), 0.28 µl dNTPs (10 mM), and 0.05 U Taq polymerase (Amersham Pharmacia Biotech). Reactions were performed under the following conditions: initial denaturation at 94°C for three minutes, followed by 36 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 1 minute and 30 sec, and a final extension of 72°C for 7 minutes. The PCR products were then electrophoresed on a 1%

agarose gel containing ethidium bromide and visualized under UV light. Amplified segments were purified by excising bands from agarose gels and centrifuging each through a filter tip. The sequencing reactions were then electrophoresed on an ABI Prism 3100 automated sequencer (Applied Biosystems). We sequenced in both directions and sequences were aligned and assembled using ChromasPro V. 1.49 (Technelysium Pty Ltd) and aligned using the CLUSTAL W algorithm with default options, implemented in MEGA 4.0.2 (Tamura *et al*., 2007). Alignments were checked and edited by hand when necessary. The haplotype sequences were deposited in GenBank under accession numbers HQ15712 to HQ15714.

Table 1. *Gubernatrix cristata* microsatellite loci used in this study.

Locus	Repeat	Alleles	Size (bp)	H_O	H_E	GenBank no.
GcrisC02	(CA)7	4	247	0,267	0,438*	GU237061
GcrisC08	(AC)8	6	174	0,911	0,791	GU237062
GcrisE02	(CA)8	4	227	0,229	0,710*	GU237063
GcrisF02	(CA)12	14	190	0,833	0,893	GU237064
GcrisF12	(AC)9 (CA)7	6	230	0,600	0,784*	GU237065
GcrisG10	(CA)7	4	236	0,130	0,126	GU237066
GcrisH06	(CA)9	10	230	0,735	0,830	GU237067
GcrisH07	(CA)7 (AC)5	6	213	0,739	0,755	GU237068
GcrisH09	(CA)7	10	236	0,702	0,834*	GU237069
GcrisH12	(CA)7	5	249	0,232	0,700	GU237070

Significant departures from HWE: * P < 0.001

Statistical Analyses

We assessed the bottleneck history of these populations using Bottleneck v.1.2.02 (Cornuet & Luikart, 1996). This program was used to detect a heterozygote excess for individual populations, considering the two-phased model (TPM) of microsatellite mutation, a 70% stepwise-mutation model (SMM) and 30% infinite alleles model (IAM), and 1000 replications. Several other combinations of the

SMM:IAM ratio were tested to establish the sensitivity of these data to the mutational mechanism. The Wilcoxon signed-rank test was used to determine if the allele frequency distribution for a population exhibited significant heterozygote excess relative to model expectations. Bayesian clustering with software Structure v.2.3.2 (Pritchard *et al.*, 2000) was used to assign individuals to populations (K) based on posterior probabilities where K is unknown. The number of groups was set to 1–7 with 3 runs per K. Posterior probabilities were calculated for all K hypothetical populations. All analyses were based on 1,000,000 Markov Chain Monte Carlo iterations following a burn in of 50,000 iterations.

To measure mtDNA diversity, both haplotype diversity, *Hd*, and nucleotide diversity, π, and their standard deviations were estimated using DnaSP v5.10.00 (Librado & Rozas, 2009). To test for evidence of recent population expansion we calculated Fu's Fs (Fu, 1997) and Fu and Li's (1993) D* and F* statistics to compare with Fu's Fs. Thus, if Fs is significant and F* and D* are not, it is an indication of population expansion, while the opposite indicates selection (Fu, 1997). We also calculate Tajima's D (Tajima, 1989). We used ARLEQUIN 3.11 (Schneider et al., 2000) and DnaSP v5.10.00 (Librado & Rozas, 2009) to perform these calculations. We created a mismatch distribution of pairwise differences using DnaSP v5.10.00 (Librado & Rozas, 2009) to compare the expected distribution for a population. The distribution tends to be multimodal when populations are at equilibrium and unimodal in cases of recent demographic expansion or reduction (Rogers and Harpending, 1992). To graphically display the observed mismatch distribution compared to the expected distributions for populations in equilibrium and expansion, we used Roger's method of moments (Rogers, 1995) as calculated in DNASP. The topological relationship between the haplotypes was estimated using the program NETWORK 4.5.1.0 (http://www.fluxus-engineering.com) with median joining approach, for the three data sets (Bandelt *et al.*, 1999).

Results

Genetic Variation

Allelic variation for the ten nuclear microsatellite loci ranged between 4 and 14 alleles with an average of 7.5 alleles per locus. The observed heterozygosity (H_O) varied from 0.126 to 0.893 and the expected (H_E) heterozygosity from 0.130 to 0.930, with means and standard errors of 0.6824 $\pm$ 0.1144 and 0.7132 $\pm$ 0.0272 respectively (Table 1). Four loci exhibited a departure from Hardy-Weinberg expectation that may be the result of a sampling bias. Since the species is rare and most of the material used was from seizures made by the Environmental Police (Table 2), we cannot determine if all of the samples represent the same population.

Table 2. Geographical origin and source of the samples processed in our study.

Country	Samples	Source	Samples
Argentina	8	Captive Bred	27
Brazil	16	Police Seizure	27
Uruguay	16	Wild	5
Unknown	32	Museum Skin	13
Total	72	Total	72

Results of bottlenecks detection using Wilcoxon signed-rank and sign tests under Infinite Allele Model (IAM), Two-Phase mutation (TPM) and Step-wise mutation (SMM) are presented in Table 3. In bottlenecked populations, the observed gene diversity exceeds the expected equilibrium gene diversity under the assumption of mutation-drift equilibrium. The null hypothesis tested for heterozygosity excess using Wilcoxon sign-rank test provided (P < 0. 05273), (P > 0.09668) and (P < 0.42285) probabilities under the IAM, TPM, and SMM respectively. The null hypothesis is accepted under the SMM model only, implying that the *Gubernatrix cristata* has not experienced any recent genetic bottlenecks. The estimated values of the heterozygosity excess and their probabilities in sign test were 5.89 (P < 0.15033) for the IAM, 5.90 (P < 0.15148) for the TPM and 5.91 (P < 0.38936) for SMM. The null hypothesis of mutation-

drift equilibrium was not also accepted based on the sign test under mutation models.

Not all of the 97 sampled individuals were successfully sequenced for the target ATPase 8 or Control Region fragment. Only 67 were succesfully sequenced for the target ND2 fragment (haplotype sequences were deposited at GenBank under accession HQ15712 to HQ15714). A total of 349 bp were aligned in *G. cristata* samples contained two variable and parsimony informative sites and a total of three haplotypes (H1 – H3). Among H2 and H1 haplotypes 75 mutational steps were found whilst 34 among H2 and H3 (Fig. 2). Estimates of nucleotide and haplotype diversity in the sample were relatively high, with π = 0.00277 and *Hd* = 0.6219. The genealogical relationships between haplotypes were estimated in the median-joining networks illustrated in Figure 2. The network revealed no structure pattern at all. Haplotype 2 had the highest probability of represent ancient forms because of its central position. The F_{st} (0,00340) and *Nm* (73,18) shown a weak structuring with little genetic differentiation. The F_{st} analysis was done following Nei's (1978) classification where a value is considered low when $F_{st} < 0,05$; medium when $0,05 < F_{st} < 0,15$, and high when $F_{st} > 0,15$. Lack of support to a demographic expansion hypothesis was either obtained by different tests made to test the neutral theory of molecular evolution: Fu and Li's D* test statistic (0,71916) showed no statistical significance ($P > 0.10$) as well not significant was Fu and Li's F* test statistic (1,34814) ($P > 0.10$). Fu's Fs statistic was 2,248 which was significantly different from zero and is a likely evidence indicating a decrease in population size and/or balancing selection. Tajima's D was positive (2,17506) what signifies low levels of both low and high frequency polymorphisms, indicating a decrease in population size and/or balancing selection.

Table 3. Genetic bottleneck detection using Wilcoxon signed-rank sign tests under Infinite Allele (IAM) , Two-Phase (TPM), and Step-wise (SMM) mutations models of microsatellite evolution

Test	Model		
	IAM	TPM	SMM
Wilcoxon signed-rank test:			
Probability of Heterozygosity excess	$P < 0.05273$	$P > 0.09668$	$P < 0.42285$
Sign test:			
Number of loci with heterozygosity excess			
Observed	8	8	5
Expected (probability)	5.89	5.90	5.91
Probability	$P < 0.15033$	$P < 0.15148$	$P < 0.38936$

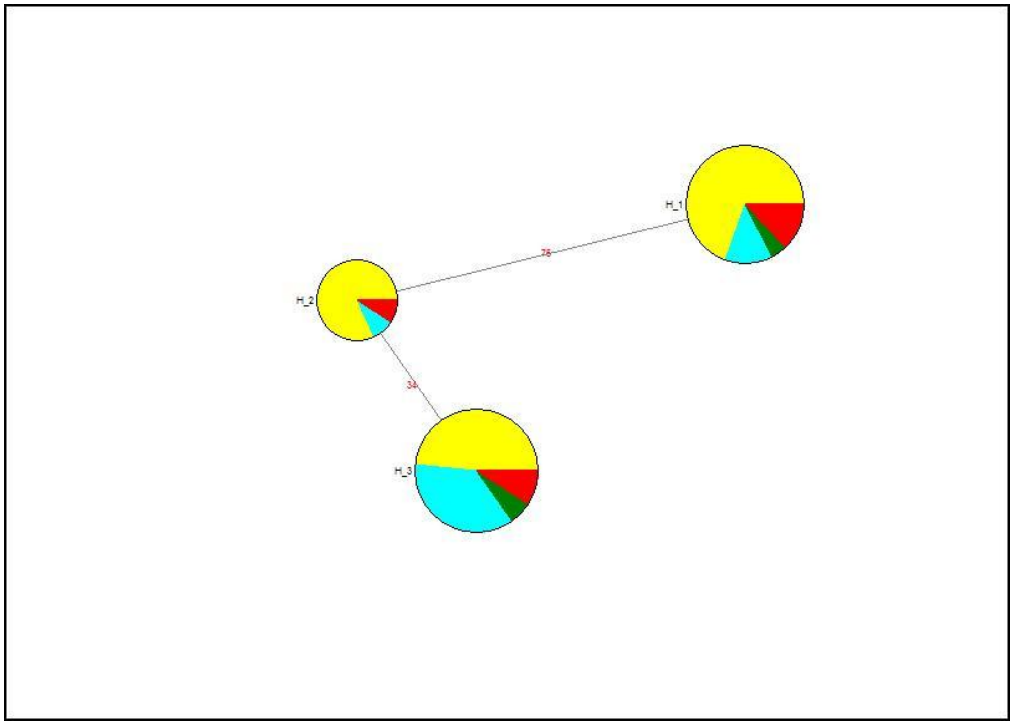

Figure 2. Genealogical relationships between haplotypes estimated in the median-joining networks. H_1, H_2 and H_3 – Haplotyes; 75 – mutational steps between H2 and H1; 34 – mutational steps between H2 and H3. Yellow – unknown geographical origin; Red – individuals from Argentina; Green – individuals from Brazil; Blue – individuals from Uruguay

Population Structure

As the exact geographical origin of many (45%) of the sampled Yellow cardinal individuals is unknown or uncertain (many of the individuals sampled in zoos or at privates, or got from police seizures were not associated to reliable information on their exact location of capture), the structure analysis would be particularly relevant because it would allows clustering of the individuals without the need for a priori geographical information. Three independent runs for one to seven populations using an admixture model and correlated allele frequencies among populations (Falush *et al.*, 2003), were run. Figure 3 shows the results for k equals to 3 populations as bar plot. Analyses using the "no admixture model" and a larger burn-in (500000) yielded very similar results (data not shown). The results for all runs were practically the same suggesting no clustering of individuals (Fig. 3). Due to this result no individual assignment to any geographical area was possible.

The mismatch distribution of observed haplotype variation was unimodal revealing a departure from expected variation under a Constant population size (Fig. 4) (k = 0,965, variance of k = 0,7205, Raggedness statistic r = 0,1319).

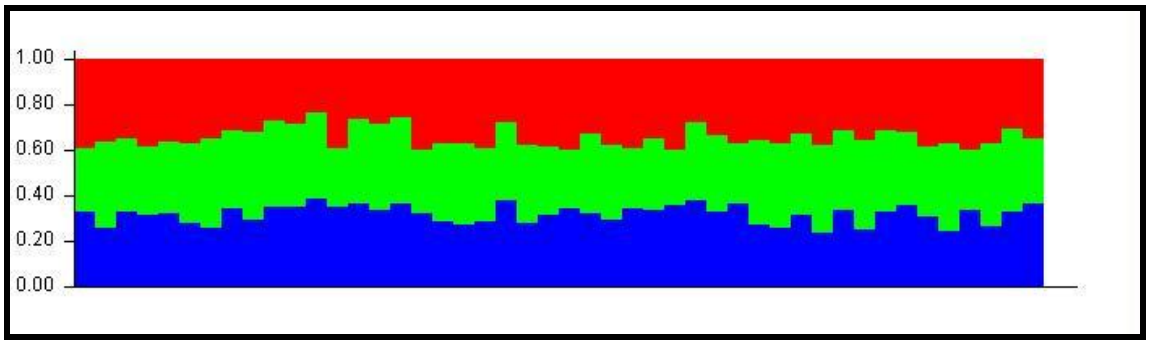

Figure 3. Results of Structure run. Here is depicted when k = 3 populations in bar plots.

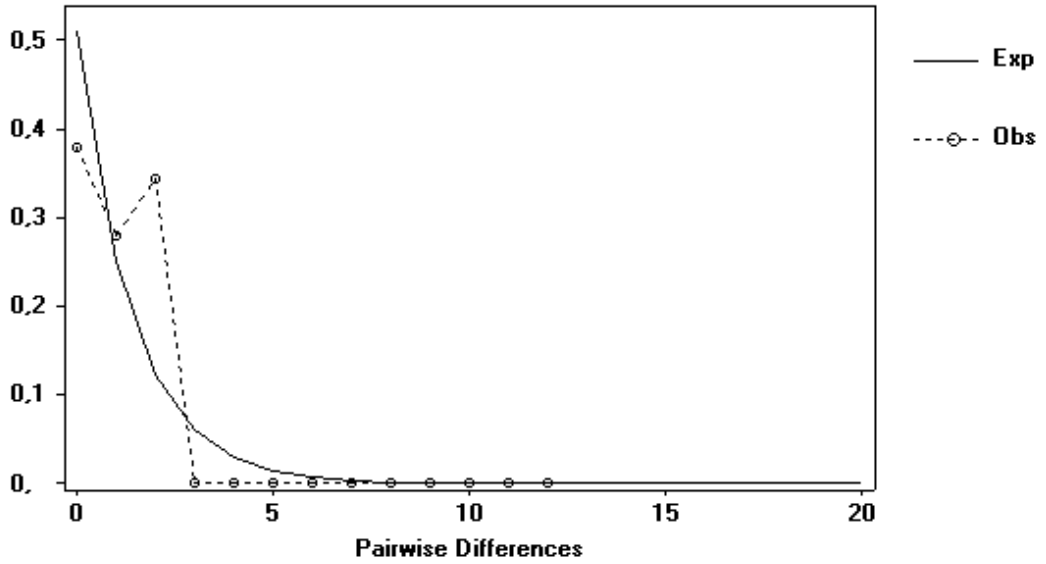

Figure 4. Mismatch distribution of the observed haplotype variation from 67 Yellow Cardinal mtDNA samples compared to the expected under a population expansion.

Discussion

Endangered birds usually have small and fragmented populations. A commonly concern about population studies is a proper sample size to rightly infer genetic diversity measures. Despite the relatively small sample size of this study it has been recommended that at least 20 to 30 individuals be sampled in microsatellite studies (Pruett and Winker, 2008). Our 72 sample size is more than enough to run studies about genetic diversity and population structure estimations. The findings about no structuring appear to be related to other issues.

The lack of population structure showed by microsatellites with low F_{st} and large *Nm* apparently tell us the history about only one huge population across Yellow cardinal range with an intense gene flow. The same is truth for ND2 mitochondrial DNA gene.

The fact that both Fu's Fs and Tajima's D indicates a decrease in population size and/or balancing selection shows us a possible explanation of what is happening.

The yellow cardinal has suffered an intense hunting pressure by almost one hundred years now (Hudson, 1920). This time period is not sufficient to be detected at the molecular level, however is enough to drastically reduce the species population size

or even driven to extinct some subpopulations (Martins-Ferreira, 2007).

The species had a wide distribution area in the past that has been reduced over time, both due to changes in habitat, as the hunting pressure. Today it seems to be limited to a narrow continuous irregular arc-shaped band in Argentina, from San Luís, passing through La Pampa, Córdoba, Santa Fé, Entre Ríos and Corrientes (Marchiori, 2004; Martins-Ferreira, 2007). Following the center south of Uruguay to the west end of Rio Grande do Sul, Brazil. That reduction in its distribution range to only the optimal environment for the species, also ended up reducing their genetic variability and, possibly, the haplotype diversity. The Whooping Crane (*Grus americana*) experienced a severe decline in their population size in early 1900. Reducing its population to 14 individuals in 1938, resulted in the loss of two thirds of its haplotype diversity. Only three haplotypes were identified in the current population (Glenn *et al*., 1999).

As shown in Whooping Cranes, a severe reduction in population size can cause a genetic bottleneck, resulting in loss of genetic diversity (Nei *et al.*, 1975). However, the impact of a bottleneck on population genetic diversity depends on how rapidly the population declines, the size of the bottleneck population and the duration of the bottleneck. When a population suffers a genetic bottleneck, rare alleles initially are lost, but the population can maintain diversity if the population recovery is rapid (Allendorf , 1986). If population abundance remains low, diversity can continue to be lost due to genetic drift (Allendorf ,1986). There are several possible explanations for the relatively high genetic diversity observed in Yellow Cardinals along Southern South America. First, the Yellow Cardinal decline may have been less severe than that of the Whooping Crane. The population status in the early 1900s is unknown, but never drop low before than today. Secondly, population recovery may have been sufficiently rapid so as not to have lost diversity due to genetic drift. Although Yellow Cardinal in Uruguay and Brazil has recently experienced several episodes of population decline, each time population recovery appears to have been rapid. Finally, recolonization by individuals that emigrated from areas where genetic diversity remained high, such as Argentina, could have contributed to the high genetic diversity. The observed genetic similarity within the region is consistent with an open population, as gene flow is expected to homogenize populations (Charlesworth, 2003). We detected no pattern in the distribution of haplotypes to suggest that genetic structuring exists in Yellow Cardinals

along its range.

Thus, our results support managing Yellow Cardinals along its distribution range as a single breeding population. It is possible that the Yellow Cardinal may form one large panmictic population. The existence of one large population could explain the high level of genetic diversity found in the population by allowing the effective population size to remain high during periods of population fluctuation and serve as a source of immigrants. Although the Yellow Cardinal's demographic history does not show drastic reductions in genetic diversity, the population size fluctuations appear to have had no detectable effects on the differentiation and frequency of haplotypes as evidenced by the mismatch distribution and Fu's Fs. The minimum spanning network (Fig. 2) shows that the 3 haplotypes are not closely related (within 75 and 34 mutational steps). A triangle-shaped phylogeny, as shown in Fig. 2, tell us only that a population has experienced a severe decline on its genetic diversity. The Yellow Cardinal population still appears smaller than historical accounts suggest. Historical accounts described Yellow Cardinal as common in Argentina in the 1900s (Hudson, 1920; Wetmore, 1926). Thus, it seems that the population may have being suffering a decline in its numbers from the last century until now.

Conclusions

The present study assists in understanding the conservation needs for Yellow Cardinal by providing information on the genetic diversity and population structuring along its distribution range.

Although we found no structuring within our study area, further study is needed, examining the genetic diversity and population structuring throughout the species' range, adding more samples from wild animals from La Pampa population and Corrientes.

The main implication for Yellow Cardinal conservation with these results is that a captive breeding program could be established on an international level, without much concern about preserving specific haplotypes, since all populations share the same ones.

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CAPÍTULO QUATRO

Potential Distribution Model to Yellow Cardinal *Gubernatrix cristata*: how much effort we should put to preserve this endangered bird?

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Potential Distribution Model to Yellow Cardinal *Gubernatrix cristata*: how much effort we should put to preserve this endangered bird?

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ABSTRACT

Aim The yellow cardinal distribution range map seems to be outdated because of the increasingly critical situation the species is facing in nature. Given the recent lack of knowledge regarding its distribution and its state of preservation in its natural habitat we decided to model the species potential distribution. The main idea behind this information was to propose strategies for their conservation. To better achieve that target we specifically focused our research on a) locating wild populations of this species in Rio Grande do Sul, Uruguay and Argentina, and b) identifying areas of occurrence of yellow cardinal that could be turned into protected areas.

Location Southern South America (Argentina, Uruguay and Southern Brazil)

Methods We extracted all georeferenced *Gubernatrix cristata* records from different sources: field working, museum skins, literature, and internet datasets. To model the species distributions, 20 environmental predictors were selected. To model the species distributions we selected the modeling application MaXent v. 3.3.2. Historical and current distribution maps of *G. cristata* were created using DIVA GIS.

Results The model that best predicts the relative index of environmental suitability for the species is the model where resulting AUC on test data was 0.868. The results of the jackknife test of variable importance showed that the environmental variable with highest gain when used in isolation is bio1_sa_30s_cut (Annual Mean Temperature), which therefore appears to have the most useful information by itself. The environmental variable that decreases the gain the most when it is omitted is alt_sa_30s_cut (Altitude), which therefore appears to have the most information that isn't present in the other variables.

Main conclusions If the objective of future surveys of the yellow cardinal in its distribution range is to detect other populations, then areas predicted to have high relative suitability in the spatial model is a good starting point for further targeted survey. The hunting pressure on the species is so great that the yellow cardinal nowadays seems to be only found in areas of difficult access. Many of the areas suitable for the species shown by the model may still contain populations and can also be used as key areas for future reintroductions into the yellow cardinal conservation program.

Keywords Gubernatrix cristata, Endangered species, Espinal, Pampa, Species Distribution Model.

INTRODUCTION

The Yellow Cardinal, is a rare bird species from the Pampas grassland. It has a restricted geographical distribution in Southern South America and is unique to this Biome (Uruguay, Argentina, and Southern Brazil) (Azpiroz, 2003). This species has undergone a dramatic population decline across its range and its conservation status is defined as endangered (BirdLife International, 2000; Fontana *et al*., 2003).

In Argentina, it is now rare except very locally (Fraga, 1997). It is found in San Luis, Buenos Aires, La Pampa and Río Negro (especially among General Conesa, San Antonio Oeste and Viedma). There are important populations in Corrientes (Pay Urbe and Estancia San Antonio), Entre Ríos (recently in the area of Montiel, Ceibas and Estancia La Choza) and Cordoba [recently in Chancaní (Chebez *et al.*, 1998)] (Di Giacomo, 2005). At the same time Cordoba present a huge lowering of Yellow Cardinal population, despite some new records (Kopta, 1999). There are records prior to 1975 from Salta, Tucumán, Santiago del Estero, Santa Fe and San Juan, and it was recorded with no details to Formosa, Chaco, La Rioja and (wrongly) in Misiones (Chebez, 1996).

In Uruguay, it was historically known from 13 departments, but recently from only Paysandú, Río Negro, Florida, Rocha, Lavalleja and Artigas (BirdLife International, 2000; Azpiroz, 2003; Lapitz, 2010; Martins-Ferreira, 2007). In 1999 the Uruguayan population of yellow cardinal was estimated less than 300 individuals, mostly concentrated in the Uruguay River Basin (Azpiroz *in litt.* 1999).

Two individuals collected in Paraguay in 1905 were probably caged birds escaped from captivity (Di Giacomo, 2005).

In Southern Brazil (Rio Grande do Sul State) it was already considered rare in the 1970s and 1980s (Belton, 1985). Since then it suffered a dramatically decline and now is believed it is confined to restricted spots in the SE hills, near Uruguayan border and at W tip of Rio Grande do Sul (Fontana *et al*., 2003).

The constant and chronic exploitation as songbird for the cage bird market remains the most significant threat. The destruction and fragmentation of its habitat have been enumerated as possible additional threats for the species, as well as the

extreme pressure of capture to supply the traffic of wild animals (Chebez, 1994; BirdLife International 2000). The main reason behind the traffick is that bird is a colorful and singing passerine. In Rio Grande do Sul, Yellow Cardinal is caught mainly in the municipalities of Piratini and Pinheiro Machado. The mischaracterization and destruction of habitat have been listed as potential additional threats to the species (Chebez, 1994; BirdLife International, 2000). In Rio Grande do Sul, most of the original landscape of the south and west has been converted into rice fields and pastures for cattle. However, it is unknown to what degree these activities affected the species. Presumably its habitat suffered with conversion for livestock grazing, timber extraction for firewood and furniture (Chebez, 1994) and especially by the rapid forestation with *Eucalyptus* plantations.

The species distribution range map better known and used can be accessed on the website of Birdlife (2010). This map, however, seems to be outdated because of the increasingly critical situation the species is facing in nature. Given the recent lack of knowledge regarding its distribution and its state of preservation in its natural habitat we decided to model the species potential distribution.

The main idea behind this information was to propose strategies for their conservation. To better achieve that target we specifically focused our research on a) locating wild populations of this species in Rio Grande do Sul, Uruguay and Argentina, and b) identifying areas of occurrence of yellow cardinal that could be turned into protected areas.

METHODS

Species Data

We extracted all georeferenced *Gubernatrix cristata* records from different sources: field working, museum skins, literature, and internet datasets. Fourteen field trips were made from April 2006 to March 2009 in an attempt to find individuals. In Rio Grande do Sul (the Southernmost Brazilian State), just four yellow cardinals were found in a single locality (Espinilho State Park). In Uruguay, four separate field trips to different localities found only six individuals in Minas, Lavalleja Department, five in one farm and one in another land. All of them were ring banded with rings provided by

CEMAVE/ICMBio (National Research Center for the Conservation of Wild Birds) and have samples collected for genetic analysis. In Argentina, a visit was made to the *Museo de Ciencias Naturales Bernardino Rivadavia* to better know which localities yellow cardinals were found, the distribution range, and past records. Nine records came from Belton's field trips (Belton, 1985) to Southern Brazil. One more record came from *Museu de Zoologia da Universidade de São Paulo* (MZUSP, São Paulo, Brazil), and one from Royal Ontario Museum (ROM, Toronto, Canada). Some other records were extracted from Red Data Book (Collar *et al*., 1992) and IBAS from Argentina (Di Giacomo, 2005). The remainder records were accessed through ORNIS data portal. In total this dataset comprised 172 georeferenced records belonging to individuals, couples or a small number of birds seen on the area where the records were taken. To avoid duplicate location names and confusion about co-ordinate system, a data cleaning was made (Hijmans *et al*., 1999). After that we got 150 georeferenced records used for training gain.

Environmental Predictors

To model the species distributions, 20 environmental predictors were selected. We downloaded the altitude (in m) and the 19 bioclimatic predictors (1950 - 2000) of the WORLDCLIM dataset (http://www.worldclim.org) for South America at 30 arc-second resolution (~ 1km) (Hijmans et al., 2005a). Data-layer manipulations were performed with DIVA GIS.

Environmental layers used: alt_sa_30s_cut (Altitude), bio1_sa_30s_cut (Annual Mean Temperature), bio2_sa_30s_cut (Mean Diurnal Range [Mean (period max-min)]), bio3_sa_30s_cut (Isothermality [P2/P7]), bio4_sa_30s_cut (Temperature Seasonality [Coefficient of Variation]), bio5_sa_30s_cut (Max Temperature of Warmest Period), bio6_sa_30s_cut (Min Temperature of Coldest Period), bio7_sa_30s_cut (Temperature Annual Range [P5-P6]), bio8_sa_30s_cut (Mean Temperature of Wettest Quarter), bio9_sa_30s_cut (Mean Temperature of Driest Quarter), bio10_sa_30s_cut (Mean Temperature of Warmest Quarter), bio11_sa_30s_cut (Mean Temperature of Coldest Quarter), bio12_sa_30s_cut (Annual Precipitation), bio13_sa_30s_cut (Precipitation of Wettest Period), bio14_sa_30s_cut

(Precipitation of Driest Period), bio15_sa_30s_cut (Precipitation Seasonality [Coefficient of Variation]), bio16_sa_30s_cut (Precipitation of Wettest Quarter), bio17_sa_30s_cut (Precipitation of Driest Quarter), bio18_sa_30s_cut (Precipitation of Warmest Quarter), bio19_sa_30s_cut (Precipitation of Coldest Quarter).

Species Distribution Model (SDM) building

To model the species distributions we selected the modeling application MaXent v. 3.3.2 (Phillips *et al*., 2006). MaxEnt was specifically developed to model species distributions with presence-only data, has shown to outperform most other modelling applications (Elith *et al*., 2006; Pearson *et al*., 2007), is least affected by location errors in occurrences (Graham *et al*., 2008), and best performs when few presence records are available (Wisz *et al*., 2008). MaxEnt was set to use all species presence records for model building (explained below), by setting the ''random test percentage'' to zero. The modelling rules were set to use auto features. As measure of SDM accuracy we used the threshold independent and prevalence insensitive area under the curve (AUC) of the receiver operating characteristic (ROC) plot (Fielding & Bell, 1997; McPherson *et al*., 2004; Raes & ter Steege, 2007), produced by MaxEnt. All measures of SDM accuracy require absences (Fielding & Bell, 1997). When these are lacking, as is the case here, they are replaced by pseudo-absences or sites randomly selected at localities where no species presence was recorded (Ferrier *et al*., 2002, Phillips *et al*., 2006). The random points were drawn from cells where in the past collections were made, and hence were corrected for any geographical sampling bias.

MaxEnt was implemented using version 3.3.2 of the software made freely available for download (http://www.cs.princeton.edu/~schapire/maxent). Recommended default values for convergence threshold (10^{-5}), maximum iterations (500), and 10,000 background points were accepted. The regularization value that is appropriate for the types of environmental variables used and the number of presence points was automatically selected by the program (used to reduce overfitting), as was the selection of environmental variables that is also dependent on the number of presences. Linear and quadratic features were considered. The output of MaxEnt is a

continuous variable ranging from 0 to 1, where high values indicate higher relative suitability.

These models were selected to accommodate the presence-only data and span a range of modeling platforms (Elith *et al*., 2006).

Using different datasets and scenarios allows us capacity to bracket predicted futures based on an ensemble of models that capture the extremes.

To select the highest performing yet most accurate predictive model, the species was tested in different ways.

We initially used all default features of the program. We also build models using bootstrap replicated type run with 20 replicas. The bootstrapping method was used to obtain an estimate of the AUC for the model. This procedure is recommended when an independent data set is lacking, and provides the least biased estimate of predictive performance of any of the model evaluation methods that are based on resampling (Wintle *et al*., 2005b).

To all models we selected random seed otherwise the generated models would be the same.

The default method of determining AUC for the MaxEnt model was also applied, after partitioning the data by randomly assigning 30% of occurrences to an evaluation (test) data set, and the remaining 70% to a training data set. Additionally, 10,000 pixels were drawn randomly from the study area as background samples.

Predicted habitat, as well as mean AUC scores, improved with using fewer, better-performing parameters.

Parameters were evaluated in a jackknife test in the MaxEnt program to assess individual parameter contribution, which had to be positive to include the parameter. Final models were run with all the data.

The final selection criteria for MaxEnt were (a) lack of significant strong relationships, (b) AUC value of >0.8, and (c) positive contribution to the model outcome in an internal jackknife test procedure.

Model evaluation

The predictive performance of the model was evaluated using the area under the receiver operating characteristic curve (ROC). A ROC curve is a plot of true-positive cases (or sensitivity) against corresponding false-positive cases (or 1 – specificity) across a range of threshold values (Fielding & Bell, 1997). The area under the curve (AUC) provides a measure of discrimination ability, and varies from 0.5 for a model with discrimination ability no better than random, to 1.0 for a model with perfect discriminatory ability (Pearce & Ferrier, 2000). The specificity is defined using predicted area, rather than true commission (Phillips *et al*., 2006). This implies that the maximum achievable AUC is less than 1. If test data is drawn from the Maxent distribution itself, then the maximum possible test AUC would be 0.903 rather than 1.

Although this evaluation technique typically requires presence and absence data, it has also been applied to presence-only data by substituting the absences with randomly selected pseudo-absences (Graham & Hijmans, 2006; Hernandez *et al*., 2006; Lütolf *et al*., 2006; Phillips *et al*., 2006). We similarly use it to evaluate and compare the resulting MaxEnt models.

Distribution Maps

Historical and current distribution maps of *G. cristata* were created using DIVA GIS (Figure 1). IBAs (Important Bird Areas) and conservation units points (Parks, Private Reserves, etc.) were used to compare the species distribution and protected areas across its range. Each area received a different color according to its protection degree: red for no protection, yellow for partial protection and green for total protection.

Our dataset was divided into current and historical records, according to the date on which they were collected. Historical records were considered from the first record of a collected yellow cardinal (1802) until 1989. The recent records were considered from 1990 to the present. This distinction was made for two reasons: 1) to have a clear idea of how diminished the area of occurrence and in which ways and 2) because of intense hunting pressure experienced by species, define the last 20 years as a current distribution, in an environment where a population could be extinct in that period, it is quite reasonable.

Thus we created maps showing the yellow cardinal historical distribution and the recent one. On top of these data we also used data from the Birdlife IBAs (Important Bird Areas) that was proposed to the region, and the existing map for the species distribution (Birdlife, 2010). The birdlife data was partially used, only for comparison purposes, in full agreement with birdlife guidelines and disclaimers.

We build a map based on these data records to the proposed update of the map currently in use. From the model generated by MaxEnt we built a map that would show environments probably best suited to meet the species needs or to use as future areas for reintroduction programs.

RESULTS

Maxent

From the generated models, those which had the best response in both the AUC as in the test gain were generated with the following settings used during the run:
103 presence records used for training, 43 for testing.
10103 points used to determine the Maxent distribution (background points and presence points).
Environmental layers used: all of them.
Regularization values: linear/quadratic/product: 0.050, categorical: 0.250, threshold: 1.000, hinge: 0.500
Feature types used: product linear quadratic hinge threshold
Random seed: true
Random test points: 30
Auto feature: false

A representation of the resultant best Maxent model is shown in Fig. 1. This output represents logistic values where the value of a given grid cell (or pixel) is the sum of that cell and all other cells with equal or lower probability, multiplied by 100 to give a percentage (Phillips *et al*., 2006). The image uses colors to indicate suitable conditions of the predicted probability. Red indicates high probability of suitable

conditioning for the species; green indicates typical conditions of those where the species is found and lighter shades of green indicating low predicted probability of suitable conditions (Philips *et al*., 2008).

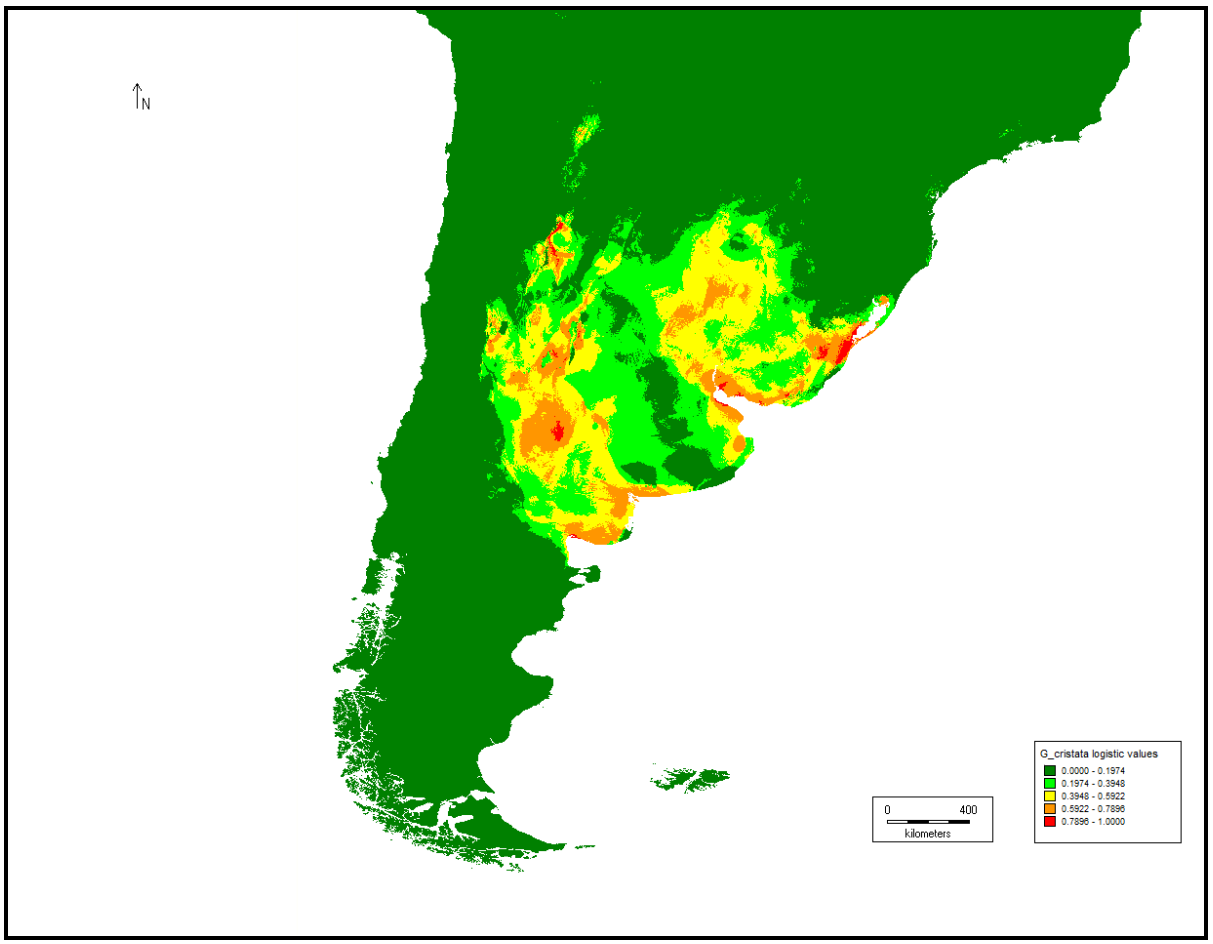

Figure 1. Model output.

Figure 2 shows the results of the jackknife test of variable importance. The environmental variable with highest gain when used in isolation is bio1_sa_30s_cut (Annual Mean Temperature), which therefore appears to have the most useful information by itself. The environmental variable that decreases the gain the most when it is omitted is alt_sa_30s_cut (Altitude), which therefore appears to have the most information that isn't present in the other variables.

All the other models made with the complete set of variables had an AUC scoring greater than 0.8, means that no noticeable different was find taking any of the parameters off the model.

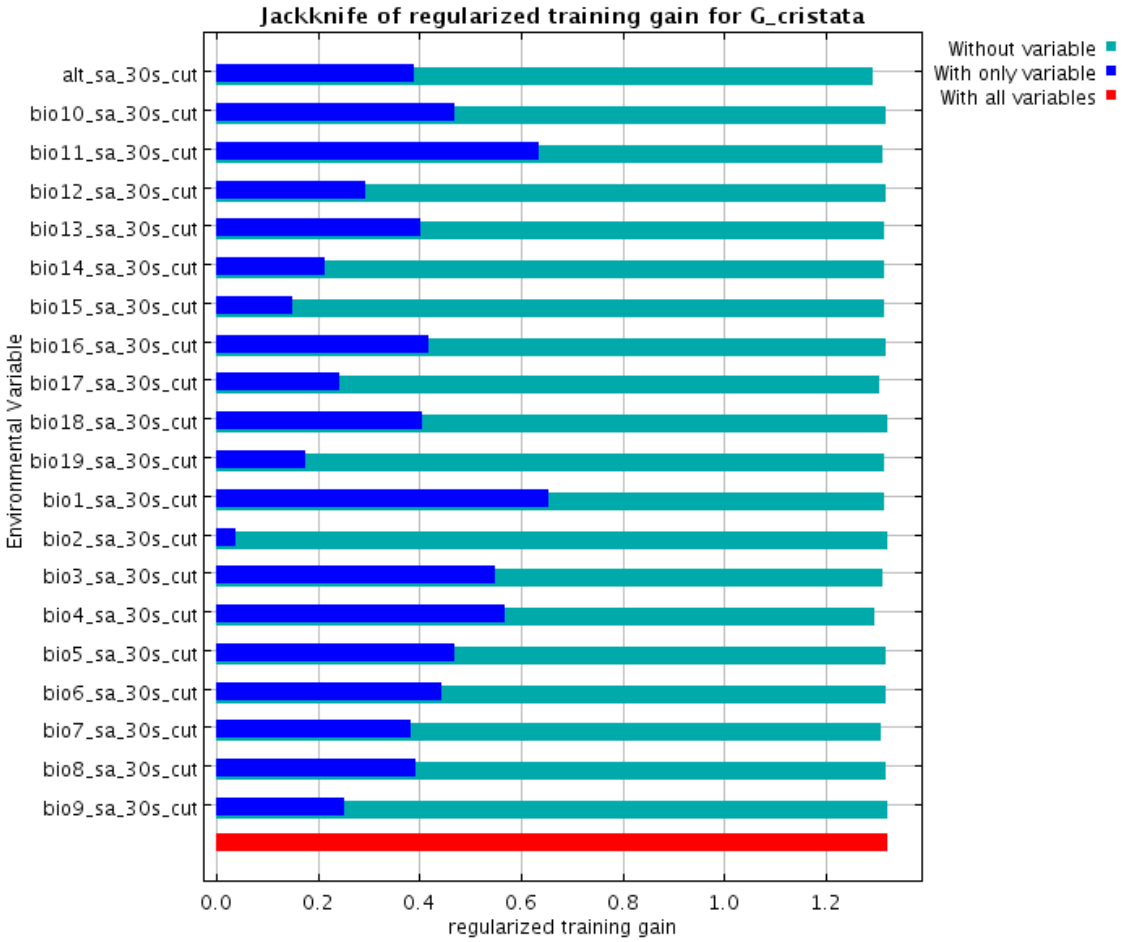

Figure 2. Jackknife test of variable importance of regularized training gain.

The Maxent model with independent data test yielded an AUC of 0.889 for the test data.

The models generated with the independent test file were very similar to models generated with the file in which 30% were separated for testing, demonstrating that both the number of points were enough to generate good models as the points themselves have no bias associated to them.

The bootstrapped estimates of the ROC curve for the model resulted in an averaged training AUC for the 20 replicate runs of 0.961, with standard deviation of 0,004 (Fig. 3).

The model that best predicts the relative index of environmental suitability for the species is the model shown in Figure 1. Specificity is defined using the area instead of the commission predicted true. This implies that the maximum AUC achieved is less

than 1. In other words, as the test data was obtained from the Maxent distribution itself, and then the maximum possible test AUC was 0.903 instead of 1. So, the resulting AUC of this model on test data was 0.868 (Fig. 4).

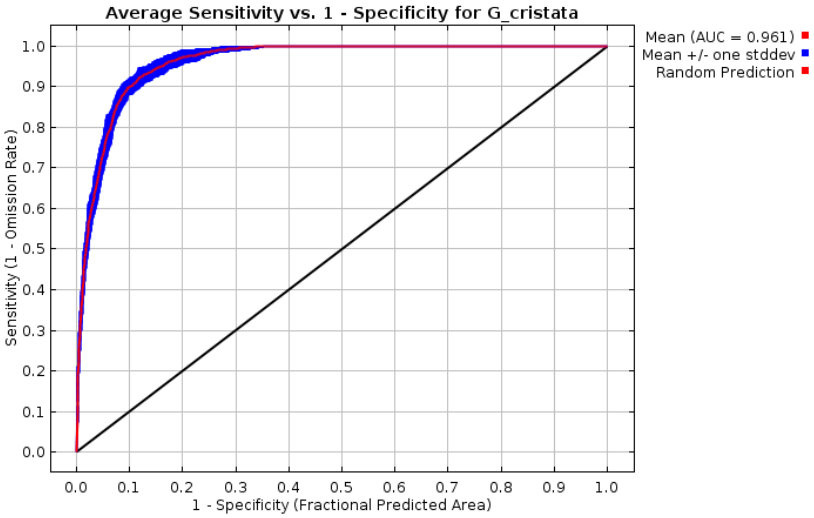

Figure 3. Receiver operating characteristic (ROC) curve for the data. The average training AUC for the replicate runs is 0.961.

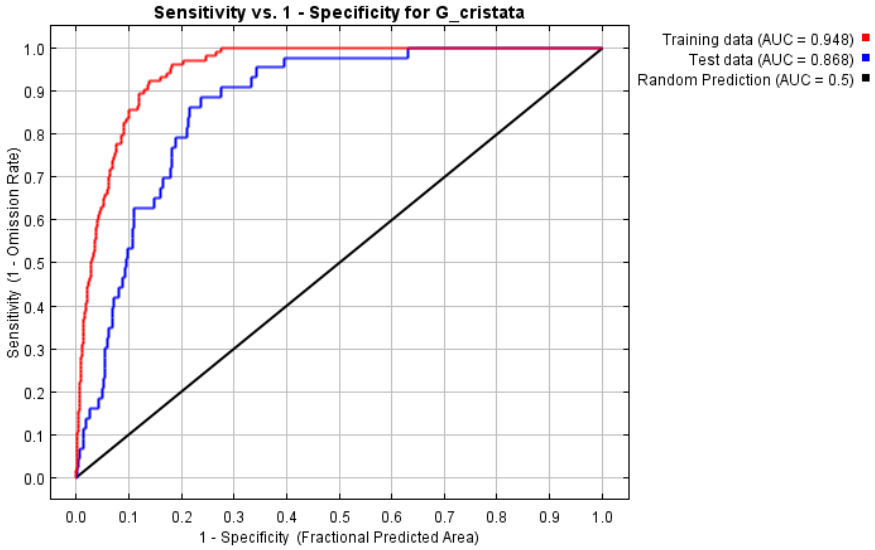

Figure 4. Receiver operating characteristic (ROC) curve for the data. The test AUC is 0.868.

Distribution Maps

A map of the historical records of *G. cristata* is shown in fig. 5 and another one with recent records is shown in fig. 6. Both maps were designed plotting together with suggested areas as IBAs by BirdLife and already existing conservation units. A distinction was made between categories of protection for each unit by assigning a different color: red for areas without any protection, yellow for areas with partial protection and green for fully protected areas (most times within state or federal parks).

A polygon based on the current species records was created and plotted on the map with the model generated by Maxent (Fig. 7).

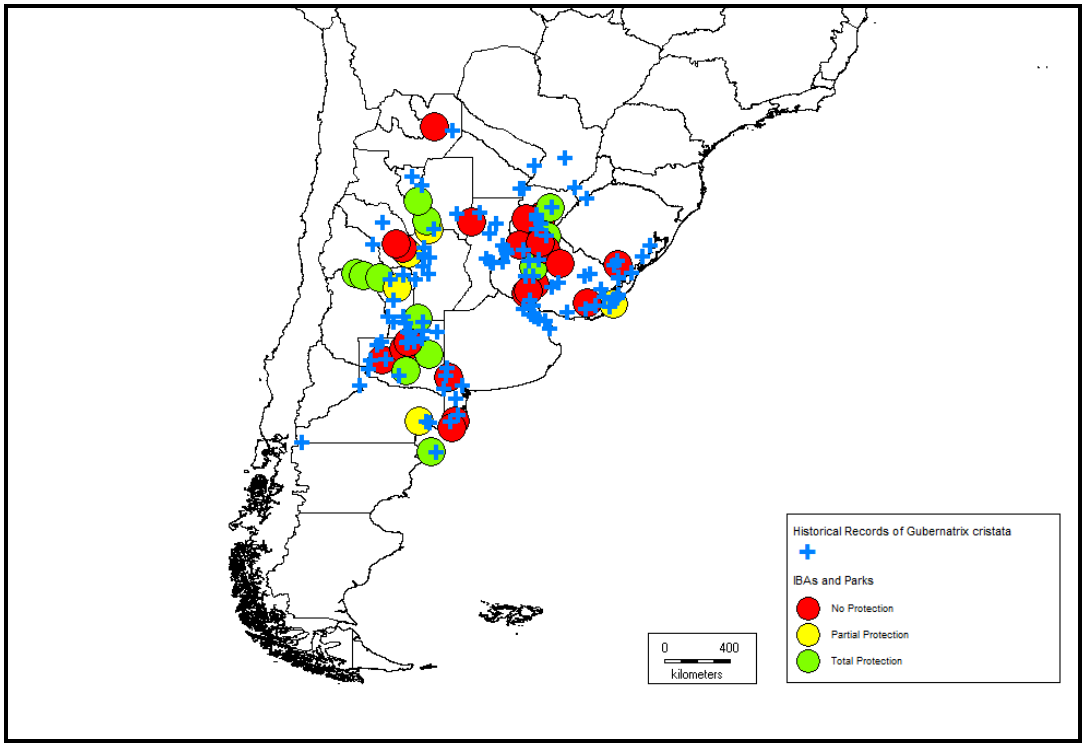

Figure 5. Map showing the historical records of *Gubernatrix cristata* with plotted IBAs and Parks across its range.

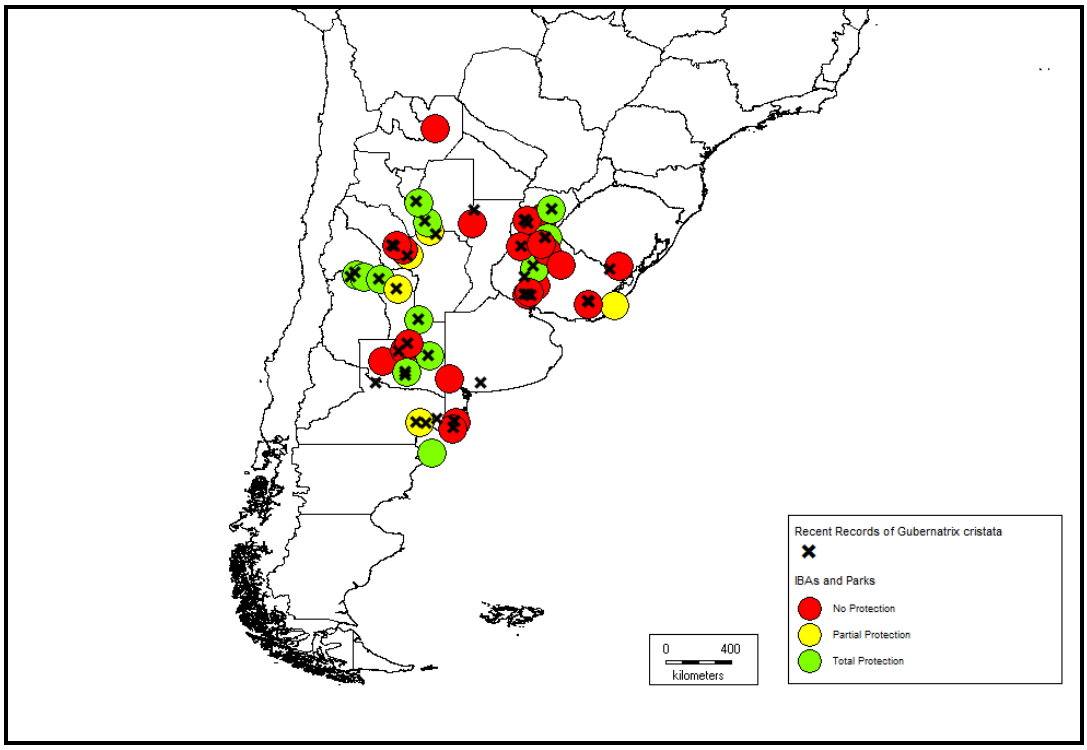

Figure 6. Map showing the current records of *Gubernatrix cristata* with plotted IBAs and Parks across its range.

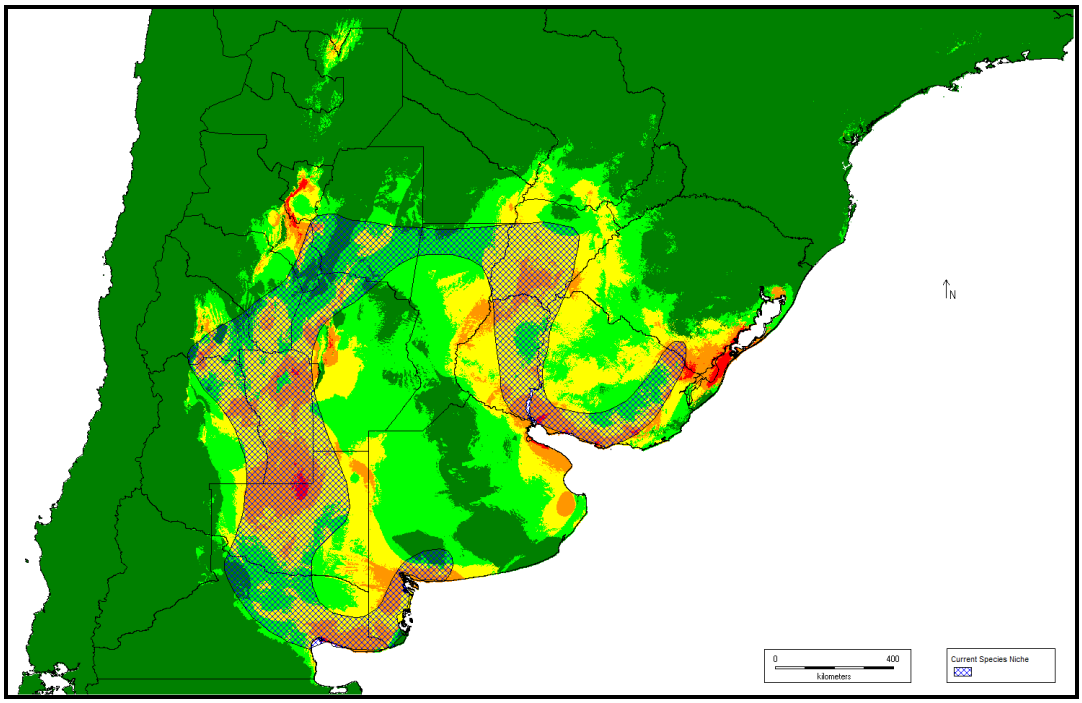

Figure 7. Map showing the species distribution model with a plotted polygon of current records.

DISCUSSION

Habitat suitability

This study sought to explore environmental attributes that may define habitat suitability of the yellow cardinal at a broad spatial scale.

The model agrees on the relationship between yellow cardinal occupancy and each environmental variable. Even though Mean Diurnal Range (bio2_sa_30s_cut) was included in the MAXENT model, Fig. 2 shows that this variable contributed almost no information to the model. The positive associations with elevation, temperature seasonality, and precipitation of driest quarter, suggest that the yellow cardinal prefers areas not high in altitude (below 1000m), with a temperature seasonality between 4ºC and 30 ºC and precipitation below 300 mm a quarter. Those conditions are according to what is found in the ecoregion of Espinal (Marchiori, 2004; Parodi, 2002).

A very low association with increasing temperature diurnal range also suggests that this species prefers habitats with low daily temperature variations. This apparent selection for low variations in temperature is again likely to be related to the vegetation type associated with this ecoregion.

Modelling approach

One problem often encountered when dealing with a rare species, is that the number of presence sites are likely to be relatively small and this then limits the number of candidate variables that should be used in model development. A high ratio of candidate models to the number of species observations can lead to overfitting of the model, i.e. inclusion of spurious variables (Burnham & Anderson, 2002; Harrell, 2001), thereby emphasizing the importance of using expert scientific knowledge in the a priori selection of variables. A plausible variable set would be in the bounds of the rule of thumb of Harrell (2001) where no more than n/10 variables should be included in the final model, where n is the total sample size or, in this case, this number would be 15. As none of the variables decrease strongly the fit of the model (fig. 2) nor any

publications says which ones are the best, we decided to model with the complete set of variables (20).

While MAXENT uses background information, pixels without species records are not interpreted as absences (Phillips et al., 2006), and therefore this method may not suffer from biases resulting from the inclusion of false absences in the model. A study by Elith et al. (2006) comparing various methods for modelling presence-only data showed that MAXENT outperformed most other methods when evaluated using independent presence and absence data. MAXENT has also been shown to be superior to other approaches that use presence data only (i.e. not requiring pseudo-absences) such as Domain, GARP, and BIOCLIM (Elith et al., 2006; Hernandez et al., 2006; Pearson et al., 2007; Phillips et al., 2006). Even though post hoc evaluation of predictive performance should involve a comparison with independent data (Fielding & Bell, 1997; Manel et al., 1999; Pearce & Ferrier, 2000; Scott et al., 2002), with studies involving rare species, such data are difficult and costly to collect. Resampling techniques, such as bootstrap used in the current study, are popular methods for dealing with the lack of an independent data set, as is the MAXENT approach of partitioning the data set into training and test data (Guisan & Zimmermann, 2000). A further complication when evaluating model performance with presence-only data is the difficulty of evaluating false-positive prediction errors (Hernandez et al., 2006). The approach used in this study where ROC plots were generated using presence and randomly selected pseudo-absence data (or background samples) is one solution to this problem. But here the AUC is interpreted as a measure of the ability of the model to discriminate between suitable habitat and a random background sample, rather than suitable and unsuitable habitat (as would be the case with real absence data) (Hernandez et al., 2006; Phillips et al., 2006). The similarity of the AUC derived from the ROC plots for MAXENT model suggests that it performed well and values greater than 0.8 also indicates that its ability to discriminate suitable habitat from random was high. The good performance of this presence-only method suggests that the environmental variables used here describe the habitat where the species occurs within its range quite well. However, assuming the species is in disequilibrium, there are clearly additional factors not included in the variable set that account for the inability of the bird to occupy all suitable habitats. This highlights one of the problems

of distributional models, as most rely on environmental attributes, and rarely are factors such as dispersal limitations or interspecific interactions included in such models.

Distribution Maps

When analyzing the species historical records map, we noticed that yellow cardinal is distributed over a wide area, covering different phytogeographical zones (Di Giacomo, 2005). Many of these records were taken in areas without any protection (Fig. 5). When this map is compared with that of recent records is clear the shrinkage that populations have suffered in recent years. This shrinkage makes an irregular arc shape along the species distribution, resembling much to the arc-shape made by the ecoregion Espinal (Marchiori, 2004; Parodi, 2002). When the polygon drawn from the recent record is plotted on the map generated from the model of potential distribution (Fig. 7) this arc is also formed. What can be analyzed from this is an apparent association of the yellow cardinal to a particular vegetation type. Throughout its geographic range and especially in the area where the arc forms, the characteristic vegetation type is savanna steppe, with abundant plants of the genus *Prosopis* (Marchiori, 2004; Di Giacomo, 2005). It is not surprising that this type of environment is the last refuge of the yellow cardinal, since it is a type of vegetation that also occurs in areas of difficult access.

CONCLUSION

While models derived from occurrence information have been shown to overestimate the extent of suitable habitat compared to models based on presence and absence data (Zaniewski et al., 2002; Brotons et al., 2004; Engler et al., 2004; Pearce & Boyce, 2006), predictive modelling is usually an iterative process, and this model can be used as a starting point to approximate habitat suitability, and hence guide future surveys for further data collection and model refinement. If the objective of future surveys of the yellow cardinal in its distribution range is to detect other

populations, then areas predicted to have high relative suitability in the spatial model (Fig. 1) is a good starting point for further targeted survey.

The hunting pressure on the species is so great that the yellow cardinal nowadays seems to be only found in areas of difficult access. Many of the areas suitable for the species shown by the model may still contain populations and can also be used as key areas for future reintroductions into the yellow cardinal conservation program.

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BIOSKETCH

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Author contributions: This study was conceived by C.M.F., who programmed and implemented the modeling experiments, analyzed the results and prepared the manuscript. The study was supervised by T.R.O.F. All authors discussed the results and commented on the manuscript.

CAPÍTULO CINCO

CONCLUSÕES E RECOMENDAÇÕES PARA A CONSERVAÇÃO DA ESPÉCIE

5.1. Viabilidade do cardeal-amarelo no RS, Uruguai e Argentina

A partir das expedições feitas durante a execução do projeto, pudemos constatar que as melhores áreas para serem transformadas em UCs, no RS, se localizam na serra do sudeste, mais especificamente no entorno da cidade de Herval, na região da várzea do rio Jaguarão, localidade de Passo São Diogo. Naquela área encontramos uma fitofisionomia muito semelhante àquela encontrada em áreas que ainda possuem populações de cardeal-amarelo, tanto na Argentina quanto no RS (Parque Estadual do Espinilho). A região de Herval já foi incluída dentro da IBA RS08 – Região de Pinheiro Machado e, de acordo com nossos dados, essa região possuiria ainda hábitats adequados para espécie. Embora não tenhamos avistado o cardeal-amarelo nas cidades vizinhas de Pedras Altas e Pinheiro Machado, é importante estimular a criação de uma rede de RPPNs naquela região. Tanto para a proteção de alguns exemplares errantes ou para um programa de reintrodução, conforme mostra o modelo de distribuição potencial para a espécie. Como a região sofreu muito, e ainda sofre, com o impacto da coleta/caça da avifauna, há que se estabelecer um programa de educação ambiental junto à comunidade, paralelo à criação de UCs e de programa de reintrodução/translocação da espécie.

Outra área que cabe destacar é o entorno do Parque Estadual do Espinilho. Embora o parque já seja uma Unidade de Conservação, carece de melhor fiscalização. Os relatos de avistamentos anteriores ao nosso reportam a observação de *G. cristata* também em áreas fora do parque. Há vários hábitats interessantes para a espécie tanto dentro quanto fora do parque, o que torna aquela região como um todo extremamente importante para a sobrevivência da espécie.

Encontramos também áreas adequadas para a proteção integral da espécie no Uruguai, no interior do município de Minas, Departamento de Lavalleja, onde as áreas de proteção seriam dentro de propriedades particulares, estimulando a fiscalização por meio dos próprios fazendeiros, que se mostraram dispostos a colaborar com a conservação da espécie.

As áreas mais adequadas para se encontrar cardeal-amarelo na Argentina seriam em Corrientes, Entre Ríos, sul da província de Buenos Aires, Río Negro, La Pampa, San Luís, Córdoba e Tucumán. Muitas dessas áreas tem registros apenas

históricos de cardeal-amarelo, como é o caso de Tucumán, embora aparentemente ainda tenham áreas adequadas para a espécie. Áreas adequadas mas que não possuam mais populações da espécie seriam áreas potenciais para integrarem um futuro programa de reintrodução da espécie.

O status de conservação de *Gubernatrix cristata* é avaliado mundialmente pela IUCN como Em Perigo; no Brasil é considerado Criticamente em Perigo e no RS, Em Perigo. Os resultados obtidos no projeto nos levam a concluir que deveria ser feita uma reavaliação desse status, tanto para o RS, passando a Criticamente em Perigo, quanto em nível mundial, passando também para a categoria de Criticamente em Perigo.

A caça intensa continua sendo a principal pressão contra as populações na natureza e foi o principal fator na provável extinção da espécie na região da serra do sudeste. Eliminando essa pressão e estimulando a criação de UCs é possível que *G. cristata* volte a recolonizar a região.

A principal implicação para a conservação da espécie a partir da falta de estruturação populacional é que um programa conservacionista de reprodução em cativeiro tem grandes chances de obter sucesso sem a preocupação extra de manter uma diversidade haplotípica associada a áreas geográficas.

Os ambientes encontrados no RS (Herval e Pedras Altas) apresentam espécies do gênero *Scutia* sp. e *Prosopis* sp.. No Parque Estadual do Espinilho (PEE) predominam *Prosopis* sp,, *Celtis sp., Acacia* sp. e *Aspidosperma quebracho-blanco.* No PEE a formação tipo parque domina a paisagem, enquanto que em Herval e Pedras Altas o mesmo não ocorre. Isso se deve, provavelmente, ao pastejo de gado intenso nas áreas de fazendas e não protegidas, encontradas no sul do RS.

Em Corrientes, Argentina, a fitofisionomia é bastante semelhante aquela encontrada no PEE, com espécies similares. No Departamento de Lavalleja, Uruguai, onde foram capturados exemplares de cardeal-amarelo, a fitofisionomia encontrada é bastante similar à do sul do RS. Não parece haver uma correlação direta entre a presença/ausência de *Gubernatrix cristata* e a presença/ausência de gado. Essa correlação está mais relacionada, possivelmente, ao uso do hábitat pela ave, tanto para construção de ninhos quanto para alimentação. Ou seja, onde há determinadas espécies botânicas é possível encontrar cardeal-amarelo.

O que se observou em campo, além do descrito acima, é que a pressão de caça

causou a rarefação da espécie, mesmo nos hábitats considerados ideais.

Os resultados do trabalho também nos levam a concluir que a espécie está fortemente associada ao ambiente conhecido como savana tipo parque. O mapa construído a partir dos registros históricos da espécie e do modelo de distribuição potencial mostra claramente essa associação quando avaliamos os registros mais recentes da espécie. Ou seja, o cardeal-amarelo ainda sobrevive nas áreas mais remotas e com vegetação tipo espinilho. Ao compararmos os mapas da distribuição histórica com o mapa da distribuição recente de *G. cristata* pode-se concluir claramente que houve uma redução significativa da área de distribuição da espécie.

5.2. Recomendações para a conservação: um primeiro passo para a construção de um plano internacional para a conservação do cardeal-amarelo no Sul da América do Sul.

A partir dos resultados encontrados durante a execução do projeto, recomendamos as seguintes ações para a conservação da espécie:

1) Parceria multi-institucional (órgãos governamentais, universidades, ONGs) para o estabelecimento de um protocolo sanitário de manejo da população em cativeiro da espécie em nível nacional;
2) Parceria multi-institucional para o estabelecimento oficial de um programa de reprodução em cativeiro com a criação e manutenção de um *studbook* da espécie;
3) Acompanhamento e monitoramento dos indivíduos encontrados em vida livre com o intuito de conhecer melhor a respeito da biologia reprodutiva, uso do hábitat e comportamento da espécie a fim de dar bases para a elaboração de uma análise de viabilidade populacional;
4) Prosseguimento com as análises genéticas incluindo amostras das populações de La Pampa e de outras províncias argentinas, possivelmente através de um programa de parceria internacional, que possibilitarão o conhecimento mais aprofundado a respeito das populações em vida livre ainda existentes;
5) Designar unidades de conservação de proteção integral ao longo da distribuição da espécie dentro da Argentina, Uruguai e RS, naqueles locais que se mostraram mais adequados para a sobrevivência de cardeal-amarelo e que ainda não possuem unidade

de conservação alguma ou se possuem, não é de proteção integral;

6) Proteger bosques de *Prosopis* sp. no sul e oeste do RS, oeste e norte do Uruguai e centro e sul da Argentina;

7) Coibir o tráfico de animais silvestres, que é efetivamente a maior ameaça para a sobrevivência da espécie na natureza, utilizando tanto medidas legais quanto socioeducativas através de campanhas de educação ambiental das populações do entorno de áreas onde ainda existem cardeais-amarelos na natureza, especialmente os municípios de Barra do Quaraí, no RS e Bella Unión, no Uruguai;

8) Envolver os criadores particulares de cardeal-amarelo no programa de conservação da espécie. Muitas dessas pessoas têm boas intenções. São apenas mal orientadas. O contingente de criadores de cardeal-amarelo dentro do SISPASS/IBAMA não é algo a se descartar.

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7. ANEXOS

7.1. Appendix A.1. *Gubernatrix cristata* samples used in this study.

Sample #	Sample Code	Sex	Country	Locality	Date	Source	Tissue type	mtDNA Samples	SSRs Samples	Haplotypes
1	PCA 002	F	ND	Canoas Zoo	12. mai. 2006	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_1
2	PCA 003	M	ND	Canoas Zoo	12. mai. 2006	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
3	PCA 004	M	ND	Canoas Zoo	23. set. 2006	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
4	PCA 005	M	ND	Canoas Zoo	23. set. 2006	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_1
5	PCA 006	M	ND	Canoas Zoo	23. set. 2006	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
6	PCA 007	M	ND	Canoas Zoo	23. set. 2006	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
7	PCA 008	F	ND	Canoas Zoo	23. set. 2006	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
8	PCA 009	F	ND	Canoas Zoo	23. set. 2006	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
9	PCA 010	M	ND	Canoas Zoo	5. out. 2006	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_1
10	PCA 011	F	ND	Canoas Zoo	7. abr. 2007	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
11	PCA 012	?	ND	Canoas Zoo	7. abr. 2007	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
12	PCA 051	F	ND	Canoas Zoo	7. abr. 2007	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
13	PCA 052	M	ND	Canoas Zoo	22. jun. 2007	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_1
14	PCA 053	F	ND	Canoas Zoo	22. jun. 2007	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
15	PCA 054	M	ND	Canoas Zoo	22. jun. 2007	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_3
16	PCA 055	?	ND	Canoas Zoo	22. jun. 2007	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_1
17	PCA 056	?	ND	Canoas Zoo	22. jun. 2007	Police Seizure	Feathers/Blood	mtDNA	SSR	Hap_1
18	001/02	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3
19	002/02	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_1
20	003/02	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3
21	004/02	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_1
22	005/02	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3
23	006/02	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3
24	007/02	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3
25	0800/04	?	Uruguay	Colonia	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3
26	932/06	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3
27	453/07	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3
28	458/07	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3
29	459/07	?	Uruguay	Durazno	8. mai. 2008	Captive Bred	Blood	mtDNA	SSR	Hap_3

7.1. Appendix A.1. *Gubernatrix cristata* samples used in this study.

Sample #	Sample Code	Sex	Country	Locality	Date	Source	Tissue type	mtDNA Samples	SSRs Samples	Haplotypes
30	G 63205	M	Uruguay	Lavalleja	22. jan. 2007	Wild	Feathers/Blood	mtDNA	SSR	Hap_1
31	G 63206	?	Uruguay	Lavalleja	22. jan. 2007	Wild	Feathers/Blood	mtDNA	SSR	Hap_3
32	G 63207	?	Uruguay	Lavalleja	22. jan. 2007	Wild	Feathers/Blood	mtDNA	SSR	Hap_3
33	G 63209	F	Brasil	PEE/RS	24. mai. 2008	Wild	Blood	mtDNA	SSR	Hap_3
34	G 63210	F	Brasil	PEE/RS	24. mai. 2008	Wild	Blood	mtDNA	SSR	Hap_3
35	BRIAO 001	M	Brasil	Pedras Altas/RS	19. abr. 2006	Captive Bred	Feathers	mtDNA	SSR	Hap_1
36	BRIAO 002	M	Brasil	Bagé/RS	19. abr. 2006	Captive Bred	Feathers	No	SSR	-
37	PCA 001	M	ND	Canoas Zoo	12. mai. 2006	Police Seizure	Feathers	mtDNA	SSR	Hap_1
38	190907	?	ND	Canoas Zoo	19. set. 2007	Police Seizure	Feathers	mtDNA	SSR	Hap_1
39	URU 001	?	ND	Private Owner	30. jun. 1905	Captive Bred	Feathers	mtDNA	SSR	Hap_1
40	URU 002	?	ND	Private Owner	30. jun. 1905	Captive Bred	Feathers	mtDNA	SSR	Hap_1
41	URU 003	?	ND	Private Owner	30. jun. 1905	Captive Bred	Feathers	mtDNA	SSR	Hap_1
42	URU 004	?	ND	Private Owner	30. jun. 1905	Captive Bred	Feathers	mtDNA	SSR	Hap_1
43	MACN 384a	M	Argentina	Tucumán	7. abr. 1905	Museum Skin	Footpads	mtDNA	SSR	Hap_3
44	MACN 826a	M	Argentina	San Luis	15. dez. 1925	Museum Skin	Footpads	No	SSR	-
45	MACN 1462a	M	Uruguay	Colonia	15. abr. 1927	Museum Skin	Footpads	mtDNA	SSR	Hap_2
46	MACN 3388a	M	Argentina	Cordoba	13. mai. 1933	Museum Skin	Footpads	mtDNA	No	Hap_2
47	MACN 43559	M	Argentina	Entre Ríos	21. abr. 1961	Museum Skin	Footpads	mtDNA	SSR	Hap_3
48	MACN 48179	M	Argentina	Corrientes	26. dez. 1962	Museum Skin	Footpads	mtDNA	SSR	Hap_3
49	MACN 49697	M	Argentina	La Pampa	6. jun. 1963	Museum Skin	Footpads	mtDNA	SSR	Hap_1
50	MACN 51721	M	Argentina	Buenos Aires	18. abr. 1967	Museum Skin	Footpads	mtDNA	SSR	Hap_1
51	MZUSP 70522	M	Brasil	Arroio Xuí/RS	20. jul. 1966	Museum Skin	Footpads	No	SSR	-
52	MCN 01682	M	Brasil	Arroio Quaraí-chico/RS	12. jul. 1973	Museum Skin	Footpads	No	SSR	-
53	MCN 01683	M	Brasil	Pedras Altas/RS	25. mar. 1975	Museum Skin	Footpads	No	SSR	-
54	ROM 59090	M	Argentina	Cordoba	2. jun. 1913	Museum Skin	Skin	mtDNA	SSR	Hap_1
55	ROM 22.1.2.4	M	ND	Metro Toronto Zoo	?	Museum Skin	Skin	mtDNA	SSR	Hap_3
56	P 2151	?	ND	Private Owner	.2006	Police Seizure	Muscle	mtDNA	SSR	Hap_3
58	PCA014	M	ND	Gramado Zoo	24. abr. 2009	Police Seizure	Feathers/Blood	mtDNA	No	Hap_2
59	PCA015	M	ND	Gramado Zoo	24. abr. 2009	Police Seizure	Feathers/Blood	mtDNA	No	Hap_2
60	PCA016	M	ND	Gramado Zoo	24. abr. 2009	Police Seizure	Feathers/Blood	mtDNA	No	Hap_1
63	PCA057	F	ND	Gramado Zoo	24. abr. 2009	Police Seizure	Feathers/Blood	mtDNA	No	Hap_2
64	PCA058	F	ND	Gramado Zoo	24. abr. 2009	Police Seizure	Feathers/Blood	mtDNA	No	Hap_2

7.1. Appendix A.1. *Gubernatrix cristata* samples used in this study.

Sample #	Sample Code	Sex	Country	Locality	Date	Source	Tissue type	mtDNA Samples	SSRs Samples	Haplotypes
65	PCA059	F	ND	Gramado Zoo	24. abr. 2009	Police Seizure	Feathers/Blood	mtDNA	No	Hap_2
67	PCA061	M	ND	Gramado Zoo	24. abr. 2009	Police Seizure	Feathers/Blood	mtDNA	No	Hap_2
75	DA32105	F	Brasil	CITOCEL	3. abr. 2008	Captive Bred	DNA	mtDNA	No	Hap_3
77	DA32107	M	Brasil	CITOCEL	3. abr. 2008	Captive Bred	DNA	mtDNA	No	Hap_1
78	DA32108	M	Brasil	CITOCEL	3. abr. 2008	Captive Bred	DNA	mtDNA	No	Hap_1
79	DA32109	F	Brasil	CITOCEL	3. abr. 2008	Captive Bred	DNA	mtDNA	No	Hap_1
84	DA32387	F	Brasil	CITOCEL	20. mai. 2008	Captive Bred	DNA	mtDNA	No	Hap_3
87	DA33531	M	Brasil	CITOCEL	18. dez. 2008	Captive Bred	DNA	mtDNA	No	Hap_3
88	DA33532	M	Brasil	CITOCEL	18. dez. 2008	Captive Bred	DNA	mtDNA	No	Hap_2
89	DA33793	F	Brasil	CITOCEL	20. jan. 2009	Captive Bred	DNA	mtDNA	No	Hap_2
90	DA33794	F	Brasil	CITOCEL	20. jan. 2009	Captive Bred	DNA	mtDNA	No	Hap_2

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