História evolutiva e conservação de duas espécies de sapinho-da-barriga-vermelha
Melanophryniscus dorsalis e *Melanophryniscus montevidensis*.

Jorge Sebastião Bernardo Silva

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Orientador: Prof. Dr. Thales Renato Ochotorena de Freitas.

Comissão Examinadora

Prof. Dr. Natan Medeiros Maciel (UFG)

Prof. Dra. Fabiana Gonçalves Barbosa (FURG)

Prof. Dr. Fernando Gertum Becker (UFRGS)

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"Estamos sozinhos no universo. A humanidade não espera auxílio algum do exterior, por isso nossa ajuda, tal como ela existe, deve vir de nossos próprios recursos. Como indivíduos, devemos aproveitar ao máximo o pequeno período de tempo que temos, pois é um privilégio estarmos aqui. Devemos aproveitar a oportunidade apresentada pela nossa boa sorte e suprir nossas breves memórias, antes de morrermos, com o conhecimento de por que, e onde, existimos."

(Richard Dawkins)

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RESUMO:

O processo de especiação é lento e gradual e a não completa compreensão deste processo ao longo da história do conhecimento nos remete a problemas clássicos sobre a classificação dos organismos. Neste trabalho, conduzimos um estudo sobre os padrões filogeográficos e evolutivos de duas espécies irmãs parapátricas de sapinhos de barriga-vermelha *Melanophryniscus dorsalis* e *M. montevidensis*, listadas como mundialmente ameaçadas segundo a IUCN , além de um estudo aplicado com o uso de modelos de nicho ecológico identificando áreas de interesse a conservação para ambas as espécies. Através dos modelos consensos baseados nos algoritmos BIOCLIM, GARP e Máxima entropia e da análise de "*Hotspot*" áreas de alta adequabilidade ambiental foram identificadas para ambas as espécies.

A validação em campo de modelos preliminares indicou a ausência de indivíduos em locais de alta adequabilidade. Expedições a campo também revelaram uma série de novos pontos de ocorrência, entre os limites da distribuição, e a confirmação de registros historicamente omitidos por listas regionais, nacional e mundial de espécies ameaçadas. Para este estudo propomos cinco "*hotspots*" de grande interesse na conservação e preservação de ambas as espécies.

Os dados moleculares, obtidos através do Cyt*b* e COI, indicam claramente um processo de especiação ocorrendo com *M. dorsalis* e *M. montevidensis* com um ancestral comum para ambas as espécies datando entre 3 e 3,5 milhões de anos. A rede da haplótipos, para ambas as espécies nos dois marcadores, é do tipo estrela com um haplótipo central e uma série de outros com uma ou poucas mutações partindo do haplótipo central, indicando expansão demográfica e também demonstrando alta variabilidade. A população mais ao norte de *M. dorsalis,* na localidade de Mar Grosso no município de Laguna (SC), merece uma atenção especial por se tratar de uma Unidade de Manejo (UM) por seus indivíduos apresentarem uma

frequência única de alelos. Segundo a definição de UM, a estratégia indicada para esta população é a delimitação de uma área específica e monitoramento da população.

Os resultados obtidos neste trabalho provêm um conjunto de informações importantes em nível específico para a implementação de um banco de dados nacional sobre espécies ameaçadas. Os dados gerados neste estudo também serão integrados ao Plano Nacional de Ação para a conservação e preservação dos anfíbios e répteis ameaçados de extinção da região sul do Brasil (PAN SUL) gerido pelo Instituto Chico Mendes de conservação da Biodiversidade.

Palavras-chave: delimitação de espécies, especiação, evolução, conservação, modelos consensos, "*hotspot*".

ABSTRACT

The speciation process is commonly slow and gradual and the incompletely understand of this process throughout the history of knowledge leads us to the classic problems of the organism classification. In this work, we conducted a study on the phylogeographic and evolutionary patterns of two parapatric sister species of red-bellied-toads *Melanophryniscus dorsalis* and *M. montevidensis*, listed as globally threatened according to the IUCN, as well as an applied study using ecological niche models to identify areas for conservation interest for both species. Through consensus models based on algorithms BIOCLIM, GARP and Maximum Entropy and the hotspot analysis, areas of high environmental suitability were identified for both species.

Field validation of preliminary models also indicated the absence of individuals in areas of high suitability. Field expeditions also revealed new occurrence points within the boundaries of the distribution, and confirmation records historically omitted by regional, national and global lists of threatened species. For this study we propose five hotspots of great interest in the conservation and preservation of both species.

Molecular data obtained through Cyt*b* and COI, clearly indicate the speciation process occurring with *M. dorsalis* and *M. montevidensis* with a common ancestor to both species dated between 3 and 3.5 million years. The haplotype network for both markers in both species are star-like which consist of a center haplotype with a series of haplotyps with one or few mutations starting from the central haplotype indicates demographic expansion and also shows high variability. The north population found in *M. dorsalis*, at the locality of Mar Grosso in the city of Laguna, needs special attention because it is a Management Unit (MU) by his individuals present a single frequency alleles. According to the definition of MU, the strategy indicated for this population is the delimitation of a specific area and population monitoring.

The results obtained in this study provide important information set on specific level for the implementation of a national database on endangered species. The generated data in this study will also be integrated into the National Plan of Action for the conservation and preservation of reptiles and amphibians threatened by extinction in southern Brazil (PAN SUL) managed by the Chico Mendes Institute for Biodiversity Conservation.

Keywords: Species-splitting, speciation, evolution, conservation, consensus models, hotspots*.*

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1. Introdução geral

1.1. Especiação

A definição de especiação mais adequada é aquela que descreve como o processo evolutivo pelo qual uma espécie é formada. Este processo pode ser uma transformação gradual de uma espécie em outra, anagênese, ou pela divisão de uma espécie em duas, cladogênese. Há quatro formas principais de especiação: alopátrica, simpátrica, parapátrica e peripátrica (Ridley, M. 2006). A especiação pode ser tão sutil quanto à descrita por Uy e colaboradores (2009) destacando que a mudança na cor da plumagem, usada para reconhecimento entre subespécies de aves papa-moscas-monarca *Monarcha castaneiventris*, está associada a uma única substituição de aminoácido no receptor de Melanocortina 1 que os torna melânicos. Esse exemplo claramente aponta para uma especiação vinculada ao reconhecimento sexual de determinada espécie, entretanto muitas espécies apresentam variação na sua forma, tamanho e cor, portanto simplesmente apresentar variação não caracteriza indivíduos com determinada característica, como espécies distintas.

Apesar de estudos como o de Uy e colaboradores (2009) o processo de especiação é lento e gradual, ao menos aos nossos olhos, e tal situação levou muito tempo a ser percebida como real a nossa compreensão, uma vez que muitos paradigmas sociais foram um entrave para o desenvolvimento do tema durante séculos (Dawkins, 2006). Um dos primeiros esboços de uma árvore evolutiva, demonstrando relações filogenéticas, foi apresentado por Charles D. Darwin no seu livro *First Notebook on Transmutation of Species* de 1837 (Figura 1). Darwin, que era teólogo, tinha consciência das consequências históricas sobre ideias como aquelas apresentadas por ele, porém sabendo que Alfred R. Wallace estava desenvolvendo a descrição de uma teoria similar foi forçado a publicar o conjunto de suas teorias em 1859 no famoso

25   livro *On the Origin of Species by Means of Natural Selection, or The Preservation of Favoured*

26   *Races in the Struggle for Life*. Interessante é que Darwin, apesar do título de sua obra, não

27   descreve em nenhum momento no seu livro o nascimento de uma espécie, entretanto a obra é

28   singular e o marco inicial sobre o tema.
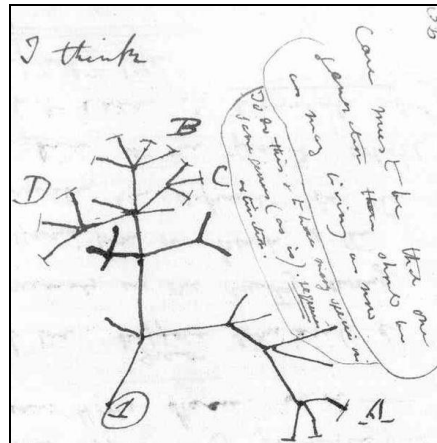
29

30   **Figura 1**: Primeiro esboço de uma árvore evolutiva feito por Darwin. Fonte: *First Notebook on*

31   *Transmutation of Species* (1837)

32

33   Os processos de especiação são descritos em diversos livros texto e em uma série de artigos e

34   não é nosso foco discutirmos sobre esse tema, entretanto, situações observadas sobre a

35   classificação dos organismos e a forma como isso ocorre nos remete a problemas clássicos na

36   compreensão sobre especiação e classificação. Tradicionalmente sistematas utilizam critérios

37   específicos para a classificação dos organismos através de observações morfológicas. Com o

38   advento de métodos moleculares as classificações ficaram cada vez mais acuradas, sanando

39   um problema recorrente na classificação clássica que é a distinção entre **sinal filogenético** e

40   **convergência adaptativa**.

41   Indiferente ao método de classificação adotado, o táxon espécie é tratado como uma variável

42   **categórica** por sistematas, quando na verdade espécie é uma variável **continua** (Butlin et al

9

43 2008) e a utilização do critério de categorizar espécie como variável discreta é facilmente

44 explicado, pois o objetivo é compreender as relações filogenéticas entre um conjunto de

45 espécies e para isso existe a necessidade de que o táxon espécie seja analisado como variável

46 categórica. O critério de tratar espécie como uma variável categórica não tira o mérito do

47 trabalho de sistematas, pelo contrário, pouco poderia ser feito sem a informação basal sobre a

48 posição de uma espécie ou de um conjunto de espécies em relação aos demais organismos.

49 Diversos exemplos nos ajudam a entender de forma clara os problemas associados com

50 classificação morfológica. Pode-se citar o caso da espécie de serpente *Tantilla equatoriana*

51 (Wilson e Mena, 1980) que foi descrita com base em poucos exemplares e que após serem

52 comparadas com os exemplares tipos de outra espécie *Tantilla melanocephala* (Linnaeus,

53 1758) foram colocadas em sinonímia, pois se tratavam da mesma espécie (Greenbaum et al.,

54 2004). Outro exemplo interessante, ocorrente no estado do Rio Grande do Sul, é o das

55 espécies de anuros *Leptodactylus gracilis* (Duméril e Bibron, 1841) e *Leptodactylus plamani*

56 Ahl, 1936. Estas espécies não são distinguíveis morfologicamente (Kwet et al., 2010),

57 entretanto são claramente considerados táxons distintos tendo como base, principalmente, os

58 cantos de anúncio de cada espécie (Kwet et al, 2010). Situações como essas, em um primeiro

59 momento consideradas problemáticas para sistematas, ao longo do desenvolvimento do

60 conhecimento são melhores compreendidas por esses profissionais e de grande interesse em

61 estudos sobre especiação, sendo um dos focos de interesse da presente tese.

62

63 **1.2. Espécies alvo, ameaças e problemática**

64 Os organismos alvo deste trabalho são os sapinhos-de-barriga vermelha das espécies

65 *Melanophryniscus dorsalis* (Mertens 1933) e *M. montevidensis* (Phillipi 1902), anfíbios de

66 pequeno porte (entre 20 e 27mm) (Kwet et al. 2005) que ocorrem no Sudeste da América do

67   Sul (Uruguai e Sul do Brasil). *Melanophryniscus dorsalis* (vulgarmente conhecido como

68   flamenguinho) ocorre na costa Sul do Brasil, entre o município de Imbituba, Santa Catarina, e o

69   município de Cidreira, Rio Grande do sul (Haddad, 2008). *Melanophryniscus montevidensis*

70   (vulgarmente conhecido como "sapito de Darwin") ocorre no limite da costa sul do Brasil,

71   entre o município de Santa Vitória do Palmar, RS, até Montevidéu no Uruguai (Maneyro e

72   Kwet, 2008) (Figura 2). A distribuição descrita por Haddad (2008) e Maneyro & Kwet (2008) é

73   a descrita, até então, para ambas às espécies.

74   A área de ocorrência das espécies está ao nível do mar e a poucos metros do oceano

75   Atlântico para a maioria dos registros, abrigando uma ampla e diversa fauna e contando com

76   várias espécies ameaçadas e endêmicas como, por exemplo, os lagartos *Liolaemus occipitalis*,

77   *L. arambarensis,* os tuco-tucos *Ctenomys flamarioni*, *C. minutus*, *C. lami* e o anfíbio

78   *Ceratophrys ornata* (Freitas 1995; Verrastro et al. 2006; Borges-Martins 2007; Fernandes *et al*.

79   2007; Canavero et al. 2010).

80

81
82 **Figura 2**: Distribuição de *Melanophryniscus dorsalis* e *Melanophryniscus montevidesnis*

83 (Adaptado IUCN 2011)

84

85 Grande parte dessa área de ocorrência tem se modificado através da ocupação e uso humano.

86 O habitat ocupado por ambas às espécies deste estudo consiste em ambientes de dunas com

87 pouca cobertura vegetal, sendo esta predominantemente de pequeno porte (Langone 1995,

88 Haddad 2008, Maneyro e Kwet 2008). As populações destas espécies sofrem por uma causa

89 comum e recorrente para espécies que ocorrem em ambientes litorâneos no sul do Brasil e

90 costa Uruguaia, que é o processo de urbanização, que gera, fragmentação, perda e

91 descaracterização de habitat e poluição (Haddad 2008, Maneyro e Kwet 2008). O mesmo

92 ocorre com outras espécies ameaçadas para a mesma área de ocorrência, como por exemplo,

12

os roedores do gênero *Ctenomys* no Brasil (Fernandes et al. 2007). Todas essas condições colocam ambas as espécies como vulneráveis na lista vermelha da International Union for Conservation of Nature (IUCN 2011), sendo que *M. dorsalis* consta também como vulnerável na Lista Vermelha das Espécies Ameaçadas do Rio Grande do Sul (Garcia e Vinciprova 2003) e no Livro Vermelho da Fauna Brasileira Ameaçada de Extinção (Haddad 2008).

No Uruguai, as populações de *M. montevidensis* registradas historicamente no extremo sul da ocorrência (Departamentos de Montevidéu e Canelones) não são mais encontradas e no departamento de Maldonado o número de registros diminuiu (Maneyro e Langone 2001). No Brasil o único registro documentado de *M. montevidensis* é para o extremo sul do município de Santa Vitória do Palmar, há mais de vinte anos atrás (Tedros et al. 2001). Não existem dados referentes ao *status* populacional de *M. dorsalis*, além de registros de ocorrências recentes. Nenhum trabalho mais detalhado sobre o status de conservação de *M. dorsalis* e *M. montevidensis* foi realizado até o momento além de dados de ocorrência e trabalhos com alcaloides (Mebs *et al*. 2005), morfológicos (Garrido-Yrigaray 1989), comportamentais (Prigioni e Garrido 1989) e de informações básicas sobre identificação e atividade reprodutiva (Kwet et al. 2005).

As espécies são consideradas irmãs no gênero *Melanophyniscus* e como tal possuem uma série de características em comum descritas por Kwet et al. (2005). As principais características morfológicas descritas para *M. dorsalis* são uma completa ou parcialmente interrompida linha vermelha médio-dorsal ou um traço dessa faixa entre e atrás dos olhos (Mertens 1933), porém indivíduos com nenhuma linha vermelha foram encontrados por Kwet et al. (2005), e no presente trabalho (Figura 3). Para a espécie *M. montevidensis* manchas laterais amarelas são características típicas e, por outro lado, nunca apresentam uma linha vermelha médio-dorsal (Kwet et al. 2005). No entanto, Kwet et al. (2005) coletou espécimes de *M. montevidensis* sem manchas laterais no corpo, embora o padrão uniformemente negro dorsal encontrado em

118 alguns indivíduos de *M. dorsalis* e *M. montevidensis* é considerado como sendo a principal

119 característica na diagnose de outra espécie do gênero, como *M. atroluteus* (Kwet et al. 2005).

120 Portanto, para essas espécies do gênero *Melanophryniscus*, somente características

121 morfológicas por si só não são "efetivas" para o diagnóstico de uma espécie. A variação

122 observada em nossas coletas é apresentada na figura 3.

123
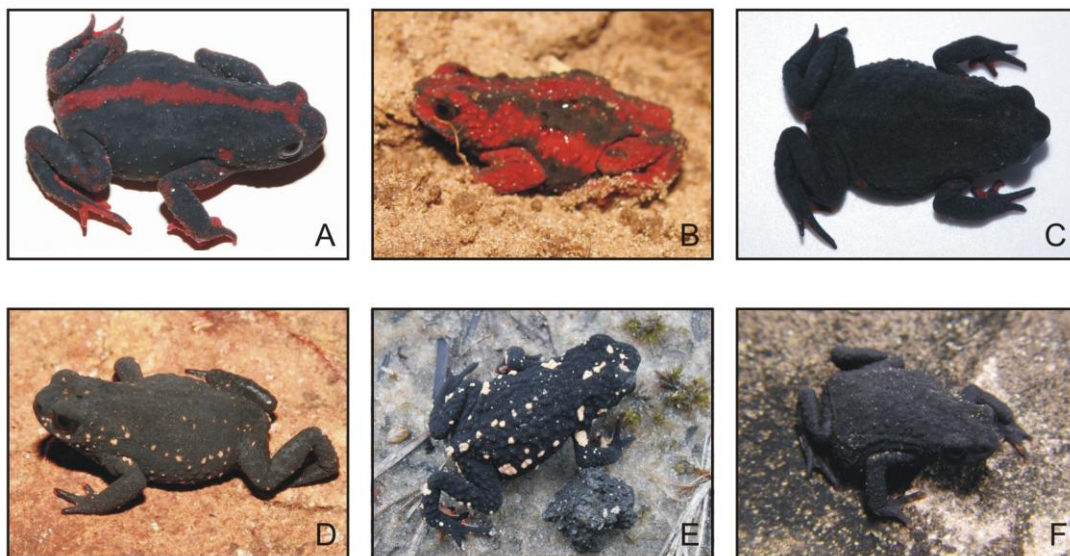
124
125 **Figura 3**: Variação morfológica observada em *Melanophryniscus dorsalis*, exemplar típico

126 conforme diagnose com linha vermelha médio-dorsal (A); com manchas vermelhas por todo o

127 corpo (B); e com ausência de linha média dorsal (C). Variação observada em *Melanophryniscus*

128 *montevidensis,* exemplar com alguns pontos amarelos na lateral do corpo (D); com diversos

129 pontos e manchas amarelas (E). Exemplar de *Melanophryniscus atroluteus* (F).

130

131 Kwet et al (2005) descrevem, através de sonogramas, os cantos de anúncio das três espécies

132 (*M. dorsalis, M. montevidensis* e *M. atroluteus*) salientando um pequena diferença, porém

133 significativa, entre o canto de *M. dorsalis* e *M. montevidensis.* No entanto, os autores não

134 observaram uma distinção clara entre os cantos de *M. montevidensis* e *M. atroluteus*. O status

135 de vulnerabilidade associado ao conjunto de fatores que claramente demonstram o

136 compartilhamento filogenético de nicho observado para ambas as espécies alvo deste estudo,

137 além das semelhanças morfológicas acrescidas à sua distribuição parapátrica, as torna um

138 ótimo modelo de especiação recente, ou não especiação, a ser estudado.

139

140 **1.3. Conservação e o uso de modelagem de nicho com espécies raras ou ameaçadas**

141 Ações conservacionistas baseadas em estudos científicos tem se tornado cada vez mais

142 aplicadas no Brasil (Bierregaard et al., 2001; Mittemeier et al. 1982), os maiores atos

143 conservacionistas estão ligados a criação de unidades de conservação entre o início da década

144 de 70 até a década de 90 (Mittermeier, et al 2005). A história da conservação no Brasil está

145 baseada em quatro áreas: a criação de unidades de conservação, o desenvolvimento de listas

146 nacionais e estaduais de espécies ameaçadas, o surgimento de organizações não

147 governamentais conservacionistas e, finalmente, o avanço da ciência da conservação no país e

148 o importante papel que ela tem exercido (Mittermeier et al., 2005). O conhecimento sobre a

149 distribuição, tanto quanto as características ambientais necessárias a cada espécie é

150 indispensável para qualquer ação conservacionista, tanto para organismos ameaçados quanto

151 para ecossistemas. Para isso uma ferramenta que está sendo amplamente utilizada para

152 identificar adequabilidade ambiental para espécies é a modelagem de distribuição de espécies

153 (SDM) (Philips, et al., 2006).

154 Essa técnica tem sido muito utilizada em biologia da conservação (Elith et al., 2006)

155 definindo estratégias para a escolha de áreas prioritárias para conservação (Nóbrega e De

156 Marco Junior, 2011) e direcionar buscas por novas populações (De Almeida et al., 2010). Os

157 modelos são uma excelente ferramenta para direcionar estratégias em relação a espécies

158   ameaçadas, entretanto com frequência é observado que a distribuição real de uma espécie

159   não corresponde à distribuição potencial apresentada por um modelo, e nesse sentido,

160   especialistas nos organismos são indispensáveis trazendo, de forma científica, informações

161   sobre história natural, ecologia e comportamento para a interpretação mais adequada dos

162   modelos. Alguns ótimos exemplos recentes sobre aplicação da técnica MNE em espécies raras

163   são conhecidos (De Almeida et al., 2010; De Siqueira et al., 2009).

164

165   1.4. Filogeografia e espécies ameaçadas

166   A filogeografia é uma disciplina relativamente nova que lida com arranjos de linhagens

167   genéticas, especialmente dentro e entre espécies relacionadas (Avise, 2009). Por volta de 1977

168   cientistas começaram a usar o DNA mitocondrial para direcionar precisamente o quão

169   coespecíficos indivíduos são genealogicamente associados a compartilhamento ancestral. A

170   filogeografia se tornou conveniente para refletir as dimensões espaciais e temporais sobre

171   microevolução. A rápida evolução do mtDNA também se reflete na presença, na maioria das

172   espécies animais, de alta variação da sequência de nucleotídeos, o que é um pré-requisito para

173   a análise filogeográfica (Avise, 2009). O tema é extremamente interessante e conveniente para

174   discussões sobre variações populacionais e deriva genética em populações de uma espécie

175   demonstrando em menor escala (microevolutiva dentro de uma espécie) o que ocorre em

176   maior escala (macroevolutiva) entres espécies e táxons superiores (Avise, et al., 1987; Avise,

177   2009).

178   A utilização do método de análise filogeográfica em espécies ameaçadas, desde que

179   possível à obtenção mínima de amostras, é uma ótima ferramenta para a avaliação do *status*

180   de variabilidade genética e assim entender o status de ameaça vinculada a este critério de

181   observação. Uma espécie pode ter baixa variabilidade genética em toda sua distribuição, em

182 parte dela, ou não estar com baixa variabilidade genética. Entretanto, apesar de não sofrer

183 com baixa variabilidade, uma série de outros fatores podem acarretar ameaças às populações

184 de uma espécie, principalmente associadas com ações antrópicas.

185

186 **1.5. Objetivos**

187

188 • **Analisar os padrões filogeográficos e inferir a história evolutiva de** *Melanophryniscus*

189 *dorsalis* **e** *M. montevidensis***, utilizando dois fragmentos de DNA mitocondrial.**

190 • **Avaliar o grau de divergência genética entre** *M. dorsalis* **e** *M. montevidensis***.**

191 • **Apresentar, através do uso de modelos de distribuição de espécies, áreas de**

192 **interesse para a preservação das duas espécies.**

193   **2. CAPÍTULO I**

194

195

196 **Identification of priority areas for conservation of two parapatric endangered species of red-**

197 **bellied toads using ecological niche models and hotspot analysis**

198

199   **ARTIGO SUBMETICO AO PERIÓDICO NATUREZA & CONSERVAÇÃO**

200

201 Jorge Bernardo-Silva*, Claiton Martins-Ferreira, Raul Maneyro, Thales R.O. de Freitas

202

203   **Research Letters**

204

205   **Identification of priority areas for conservation of two endangered parapatric species of red-**

206   **bellied toads, using ecological niche models and hotspot analysis**

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210

211   Jorge Bernardo-Silva[1]*, Claiton Martins-Ferreira[2], Raul Maneyro[3], Thales R.O. de Freitas[2]

212   [1] Programa de Pós Graduação em Ecologia, IB - Universidade Federal do Rio Grande do Sul, Av.

213   Bento Gonçalves 9500, Porto Alegre, RS, Brazil

214   [2] Departamento de Genética –IB- Universidade Federal do Rio Grande do Sul, C.P. 15053,

215   90501-970 Porto Alegre, RS, Brazil

216   [3] Laboratorio de Sistemática e Historia Natural de Vertebrados, Facultad de Ciencias, UDELAR,

217   Montevideo, Uruguay

218   Correspondência: [1] Programa de Pós Graduação em Ecologia, IB - Universidade Federal do Rio

219   Grande do Sul, Av. Bento Gonçalves 9500, Porto Alegre, RS, Brazil

220   E-mail: bernardo.silva@ufrgs.br,   bernardo.silvaj@gmail.com

221   **Keywords**: BIOCLIM, Consensus model, Conservation Biology, GARP, Maximum Entropy.

222   **Abstract**

223   The red-bellied toads *Melanophryniscus dorsalis* and *M. montevidensis* are listed as vulnerable

224   on the IUCN list. Uruguayan populations of *M. montevidensis* are no longer found near the

225   southern limit of its distribution, and information on the ecology and distribution of both

226   species is still needed. We are proposing consensus models and hotspot analysis to identify

227   priority conservation areas for these toads. Here, we present a consensus model based on

228   three algorithms that use presence-only data (BIOCLIM, GARP and Maximum Entropy).

229   Through consensus models, we used a new OpenModeller implementation called "hotspot",

230   which identifies areas of high environmental suitability. Our results indicated areas of high

231   environmental suitability for both species. Fieldwork validation also indicated their absence

232   from some high-suitability areas identified by the consensus and hotspot models. Five hotspot

233   areas of great interest to preserve both species are presented.

234

Introduction

Amphibians are one of the groups of organisms with the highest conservation concern

worldwide, because of their continuous and steady decline, mainly from climate change,

pollution, infectious diseases, and UV radiation (Crawford *et al*. 2010) as well as habitat loss

and competition from invasive alien species (Stuart *et al.* 2004). Although a variety of general

causes of decline are known, it is necessary to determine the reasons for specific declines of

each taxon so that remedial actions can be taken. In addition, the identification of an

endangered species can result in conservation efforts not only for the specific taxon, but also

for the habitat, or part of it, of the key species. However, any effort at conservation should be

based on reliable and detailed studies on occurrence and habitat occupation.

The use of ecological niche models (ENM) to infer species distributions has been growing

steadily (Franklin 2009), although different patterns of environmental suitability may be found

depending on the algorithms used. Thus, choosing the most appropriate model and algorithms

is still a challenge for researchers (Araújo & New 2007). Besides, based on the features intrinsic

to each algorithm, standard protocols to identify areas of potential occurrence are needed

(Rodríguez-Soto *et al*. 2011). In particular cases of restricted distribution or sparse populations

of rare or endangered species, using ENM may be necessary to identify a set of areas for

protection (De Siqueira *et al*. 2009).

The red-bellied toads *Melanophryniscus dorsalis* (Mertens 1933) and *M. montevidensis*

(Phillipi 1902) are small anurans inhabit southern Uruguay and the Brazilian states of Rio

Grande do Sul and Santa Catarina and are cited as vulnerable in the Red List of the

International Union for Conservation of Nature (IUCN 2011) see supplementary material S1.

Melanophryniscus dorsalis is also listed as vulnerable in the Red List of Threatened Species of

Rio Grande do Sul (Garcia & Vinciprova 2003) and in the Red Book of Brazilian Endangered

259   Fauna (Haddad 2008). In 2011, the "National Plan for Conservation of Herpetology Fauna from

260   Southern Brazil (*PAN*)" was published by the Chico Mendes Institute for Biodiversity

261   Conservation (ICMBio) in association with the Brazilian Ministry of the Environment. This *PAN*

262   was based on identification of priority actions to address threats to populations of endangered

263   species and their environment, with the main goal of developing actions to ensure the

264   preservation of the diverse amphibians and reptiles in southern Brazil. This initiative can be

265   greatly improved with the use of ENM tools to classify areas of high environmental suitability

266   for endangered species, which can be directly applied to reserve design and conservation

267   planning.

268   Here we applied ENM to predict the potential geographical distributions of *M. dorsalis* and *M.*

269   *montevidensis*, providing updated data on their occurrence and proposing new areas to be

270   considered for their management and conservation.

271

272   **Material and Methods**

273   *Study area*

274   The study was conducted on the southern coast of Brazil and on the Uruguay coast.

275   The environment consists of sand dunes and relict 'restinga' forests, bounded by the Cabo de

276   Santa Marta in Santa Catarina to the north and Cabo Polonio in Uruguay to the south (Hesp *et*

277   *al*. 2009). We mapped the existing protected areas along the species' distributions (Figure 1)

278   according to the SNAP (National System of Protected Areas) for Uruguay and the Federal Units

279   and environmental protection areas (APAS) of Brazil, as a basis for discussing conservation

280   implications of ENM modeling of the two species.

281

282

Preliminary models and occurrence data

284   Current knowledge about the distributions of *M. dorsalis* and *M. montevidensis* is

285   based on historical records from the literature and from the herpetological collection of the

286   Zoology Department of the Federal University of Rio Grande do Sul (UFRGS) and the collection

287   of Vertebrate Zoology, Facultad de Ciencias, Universidad de la República, Uruguay (ZVCB) (see

288   Table S1 in the Additional Supporting Information available at www.abeco.org.br). Distribution

289   estimates may therefore be subject to identification errors, resulting in biased estimations of

290   geographical distributions. To check these historical data, we initially generated a preliminary

291   distribution map using MAXENT (Phillips *et al*. 2006), which was used to identify areas for 12

292   field expeditions. Through these field expeditions, we confirmed the historical records and

293   searched for new occurrences in the most suitable areas as determined by the MAXENT

294   model. The coordinates were recorded using a GPS at the exact point of collection or

295   observation (the historical and new records, as well as the parameters of the initial MAXENT

296   models are described in supplementary material S1, Tables S1 and S2).

297   The thorough review of scientific collections and literature allowed us to include

298   records not previously included in the distribution maps from Brazilian red lists, such as points

299   6 and 8 for *M. dorsalis,* and 19, 20 and 25 for *M. montevidensis* (see Table S1). Considerable

300   new occurrence data were recorded for this study (Table S1), following the indicated areas in

301   Figure S1. We also included the new record published by Quintela et al. (2007), which extends

302   the known distribution of *M. dorsalis* 285 kilometers to the south and was reconfirmed by this

303   study (point 7) (Table S1). Field expeditions were conducted to confirm species occurrence in

304   points 6, 8 and 10 (*M. dorsalis*), with a successful rediscovery at point 6 and no success at

305   points 8 and 10. However, for some areas indicated as highly suitable, no records were found

306   (e.g., Florianopolis Island in Santa Catarina; see also Figure 4 and Figure S2).

307

308   ***Environmental data***

309   We started with 19 Worlclim's bioclimatic variables obtained through interpolated

310   data from derived rainfall and temperature, with a resolution of 2.5' arc-minutes (five

311   kilometers) and one variable of altitude (Hijimas *et al*. 2005), available at

312   http://www.worldclim.org/download. A first model was generated with the historical and new

313   occurrence data to generate a matrix with the values of each climate variable for each

314   occurrence record. We performed a Principal Components Analysis (PCA) on this matrix to

315   identify correlations between variables, selecting the axes that explain 95% of the correlation

316   structure. From this result we selected variables with the highest absolute coefficient in each

317   axis, allowing to select five bioclimate variables for *M*. *dorsalis* (minimum temperature in

318   coldest month, mean temperature in driest quarter, mean temperature in warmest quarter,

319   precipitation in driest month, and altitude) and four variables for *M. montevidensis* (annual

320   mean temperature, maximum temperature in warmest month, precipitation in wettest

321   quarter, and altitude). To avoid information loss, we included all variables represented in both

322   of the PCAs to generated models for the two species.

323

324   ***Ecological Niche Models***

325   The models were constructed on OpenModeller version 1.2.0 (Muñoz *et al*. 2009),

326   available at (http://openmodeller.sourceforge.net/). Five algorithms of presence-only data

327   were used for each species: Mahalanobis *Environmental Distances*, *BIOCLIM*, *GARP*, *Maximum*

25

328   *Entropy* and *SVM* (Support Vector Machine). We chose this set of methods because they cover

329   different categories of modeling algorithms, such as environmental envelopes, genetic

330   algorithms, and machine learning methods (Franklin 2009; Muñoz *et al*. 2009). We used the

331   three groups of algorithms to give the best optimization function consensus model, which has

332   clear advantages over the use of a simple model and in principle provides more robust

333   information for conservation plans (Araújo & New 2007).

334   From the five outputted models in OpenModeller, two of them (the SVM and Mahalanobis

335   Distances) were discarded because their predictions widely overestimated (although they had

336   acceptable AUC values) the known distributions of both species. These algorithms showed high

337   environmental suitability in all extension of maps, over predicting potential distribution areas

338   including areas of distribution of other species from this genus, such as *M. atroluteus* (open

339   areas in Brazil and Uruguayan Pampa) and, since our aim was to approximate the realized

340   distribution, and not potential directly by niche, our conservation strategies would be too

341   liberal and more prone to commission errors. All models were validated by calculating the area

342   under the curve (AUC) from a receiver operating characteristic curve (ROC).

343   For the consensus models, OpenModeller criteria of highest probability for areas that

344   have a higher frequency in the chosen models were used. To identify potential areas for

345   conservation, we used the hotspot function implemented in OpenModeller to obtain a

346   consensus model resulting from common areas of high environmental suitability for both

347   species (see Muñoz *et al*. 2009). The hotspot function provides a simple result for alpha

348   diversity for each cell, as demonstrated for other rare species of frogs (Giovanelli et al. 2008).

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350

351

Results

353   The consensus map constructed with the algorithms BIOCLIM, GARP and Maximum Entropy

354   (Figures 2 and 3) showed considerable variation in prediction for each species, which is

355   expected due to the algorithm variation. Even so, the high AUC values for both species for all

356   algorithms allowed us to attain high statistical reliability in their predictions (Table S2). The

357   map with the widest distribution area was generated by GARP, whereas BIOCLIM was the most

358   conservative for both species (Figures 2 and 3).

359   Our results clearly demonstrated that these species have important restrictions in their

360   habitats. Most of the suitable areas are restricted to the southern Brazilian coast for *M.*

361   *dorsalis* and to the Uruguayan coast for *M. montevidensis*. For *M. dorsalis*, only two records in

362   Brazil were in protected areas: Itapeva and Guarita state parks. These two areas are the only

363   ones with restinga forest with swamps in Rio Grande do Sul. The vast majority of new

364   occurrence records in Santa Catarina are within the APA Baleia Franca (protected area for

365   cetaceans). The consensus map for *M. montevidensis* showed moderately suitable areas

366   compared to *M. dorsalis*. The potential distribution of *M. montevidensis* extends from

367   Montevideo in Uruguay to Santa Vitória do Palmar in Brazil (Figure 3).

368   By integrating the distribution models and the new occurrence records, we identified five

369   areas of high importance using the hotspot function (Figure S2c), three in Brazil and two in

370   Uruguay. Based on specific criteria of environmental suitability of the consensus models and

371   the hotspot function, we found the highest probability for alpha diversity in these areas (Figure

372   4).

373

374

Discussion

New occurrence data

375

376

377　　　Ecological aspects of both species related to explosive reproductive behavior (Maneyro

378　& Kwet 2008; Haddad 2008) did not allow us to confirm absence when individuals were not

379　found in environmentally suitable locations during the fieldwork. We searched actively for

380　specimens of *M. dorsalis* at the southern end of its distribution on the "Ilha dos Marinheiros"

381　in Rio Grande. We made four field expeditions during the known reproductive explosion

382　conditions (after intensive rains in suitable areas), and found specimens only during the fourth

383　expedition, with no reproductive behavior recorded. This may indicate that specific aspects of

384　its activity should be observed at other potential sites of occurrence and reproduction. Points

385　6 and 8 (Table S1) are not only new species records, but also significantly extend the former

386　restricted distribution by 200 kilometers. Point 6 is the first record of *M. dorsalis* outside the

387　ocean-beach environment, on the west side of the Lagoa dos Patos, confirmed by this study.

388　Occurrence records 19, 20 and 25 for *M. montevidensis* updated the registry of the Red Book

389　of Endangered Fauna of Brazil (Haddad 2008) and the Red List of Threatened Species of Rio

390　Grande do Sul (Garcia & Vinciprova 2003). However, these records were, surprisingly, never

391　included in the red data books. These localities were probably excluded from previous

392　publications because of identification errors and/or because they were based only on

393　published records. Maneyro & Kwet (2008) have recommended the inclusion of *M.*

394　*montevidensis* in the list of endangered species for Rio Grande do Sul, because of its restricted

395　occurrence in Brazil.

396

397

398

399   *Consensus models and areas for conservation*

400   The final consensus models based on all available data for the two species showed important

401   commission errors, such as the areas of high environmental suitability on Florianópolis Island

402   (Santa Catarina), where neither species was recorded (Figure 4). These most likely occur due to

403   an inherent characteristic of the modeling process using ENMs, which does not consider the

404   presence of natural barriers to occupation and other factors, such as effects of biotic

405   interactions, so models only predict environmentally appropriate sites (even so assuming that

406   the correct environmental drivers of distributions were included in the modeling process).

407   However, it is also possible to argue the absence of some species in highly suitable areas may

408   indicate recent extinctions. For example, *M. montevidensis* was recorded previously in

409   Canelones Department, Uruguay, which shows high environmental suitability, but where it is

410   now considered extinct there (Maneyro & Langone 2001).

411   The alpha-diversity evaluation used to generate the hotspot map allowed us to demonstrate

412   conservative niche characteristics for a terminal phylogenetic branch, indicating recent

413   environmental history requirements common to both species. When applied to

414   phylogenetically terminal species with conservative niche characteristics, this function allows

415   one to qualitatively identify which environments are most suitable for both species.

416   Based on the hotspot map, we suggested five important areas for species conservation (Figure

417   4). These areas shelter a significant number of threatened species, such as the mammals

418   *Ctenomys flamarioni* and *C. minutus*, and the lizard *Liolaemus occipitalis* (IUCN 2011). We

419   found the highest probability for alpha diversity in these areas. In Santa Catarina, the "Baleia

420   Franca" Environmental Protection Area ("Baleia Franca" APA) contains the site with the

421   greatest potential for *M. dorsalis* conservation (area A in Figure 4). Some locations in this

422   region harbor endangered species and also meet the technical requirements for the

423   establishment of protected areas in Brazil, i.e., the presence of rare or endangered species,

424   ecosystems and processes.

425   Another area on the southern edge of the Baleia Franca APA, covering known populations of

426   *M. dorsalis*, is the Morro dos Conventos in Araranguá Municipality. In addition to meeting the

427   above requirements, this area also attracts tourists because of its coastal rock formations and

428   scenic beauty, increasing the potential for the creation of a protected area. However, the

429   purpose of the Baleia Franca APA is marine environmental conservation, and it is ineffective in

430   protecting threatened continental species. Thus, we suggest the creation of a new

431   conservation unit at hotspot *A* to ensure the preservation of *M. dorsalis* (Figure 4).

432   At hotspot *B* (Figure 4) we found two protected areas, Itapeva and Guarita state parks, which

433   are neighboring areas located in northeastern Rio Grande do Sul. The larger Itapeva Park

434   protects a remnant of Atlantic Forest, and is categorized as an Integral Protection Area to

435   protect ecosystems and rare or threatened species (SEMA 2006). The latest Itapeva Park

436   management plan was completed in October 2010, and the study still needs to be approved by

437   the State Secretariat of the Environment. This approval will legally establish Itapeva Park as an

438   actual protected area. Political problems have historically been an obstacle to the

439   establishment of protected areas, depending on Brazilian governmental actions to be

440   executed. Several technical studies have demonstrated the importance of this site in

441   protecting a unique ecosystem in southern Brazil (Wacchter 1986, Colombo *et al*. 2008). Our

442   study reinforces the effectiveness of the park as an important conservation area for *M.*

443   *dorsalis*.

444   Toward the south, two other areas were represented in our analysis, *C* and *D*. Site *C* not only

445   has the most suitable environmental characteristics for these amphibians, but also connects

446   the northern boundary of the distribution of *M. dorsalis*. Site *D* is the only known record of *M.*

447 *montevidensis* in Brazil. The region has also the only record of another threatened Brazilian

448 amphibian, *Ceratophrys ornata* (Vinciprova & Garcia, 2003), which occurs along the narrow

449 coastal strip between Rio Grande and Santa Vitória do Palmar municipalities.

450 Although today some protected areas do shelter *M. montevidensis* populations in Uruguay,

451 these areas are located in the northern part of the species' distribution and do not include the

452 recorded southern localities. In Montevideo and Canelones Departments, the species is now

453 considered extinct, with no recent records (Maneyro & Langone 2001). However, in the

454 Department of Maldonado there are still current records, but no conservation actions or

455 protected areas have been implemented. Thus, creating a protected area in this region is

456 extremely important for the preservation of *M. montevidensis* (area E in Figure 4).

457 Despite the indication of highly suitable areas in northeastern Santa Catarina, we found no

458 specimens of *M. dorsalis* on the north edge of Imbituba Municipality. By considering the

459 absence of historical records of *M. dorsalis* north of Imbituba Municipality, it is unlikely that

460 this species now occurs in these suitable areas.

461 Although the identification of highly suitable areas for both taxa provides important

462 information for their conservation, the size and genetic status of their populations still need to

463 be evaluated. Our results and conclusions can provide helpful information to the Brazilian

464 government, through the National Action Plan (*PAN*) for the conservation of threatened

465 amphibians and reptiles of southern Brazil managed by the Chico Mendes Institute for

466 Biodiversity Conservation (ICMbio).

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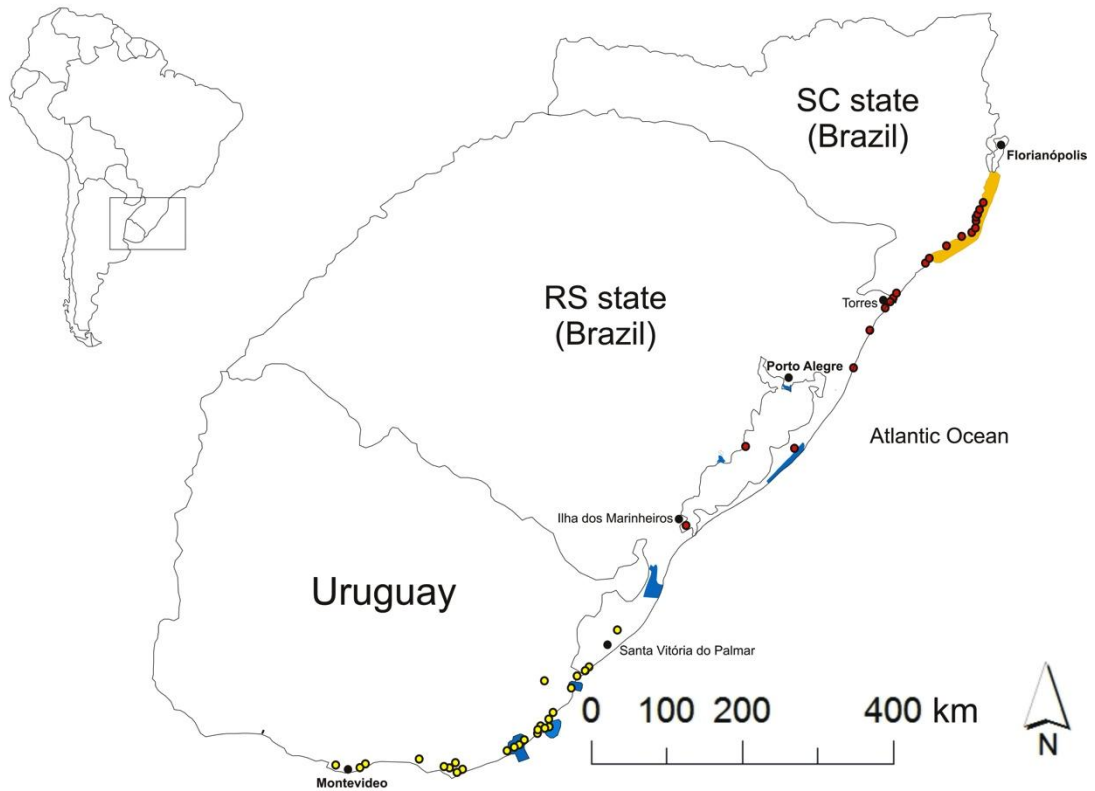
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Figure 1. Occurrence records for *Melanophryniscus montevidensis* (yellow circles) and *M. dorsalis* (red circles). Blue areas represent the Conservation units of Uruguay (according to SNAP) and the Federal Units of Brazil. Orange areas represent the Brazilian APAS.

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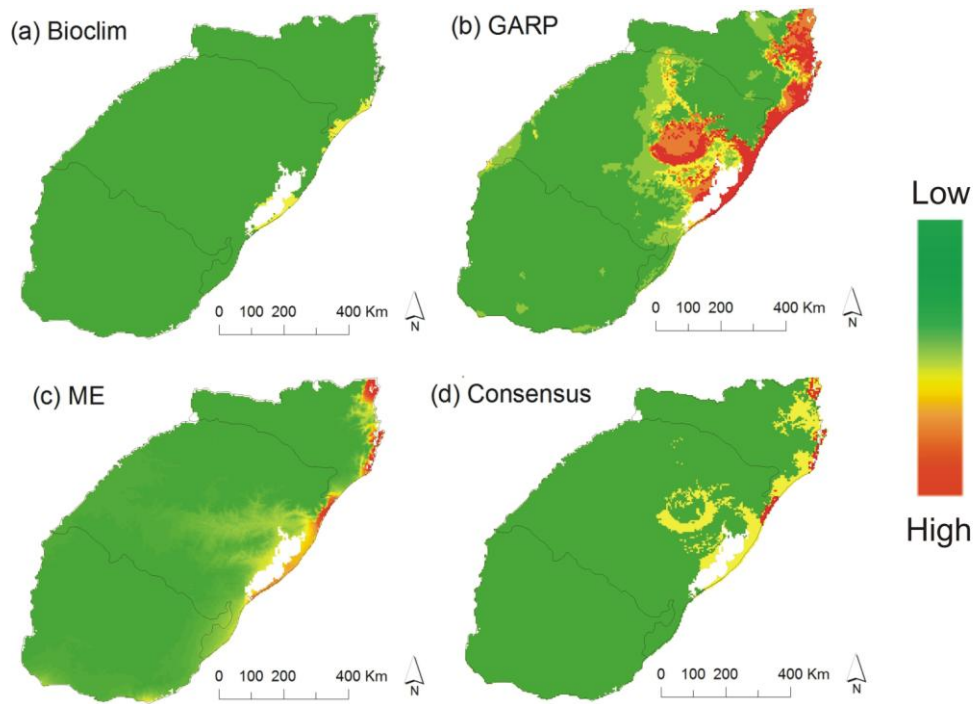
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Figure 2. Suitable areas for *Melanophryniscus dorsalis* according to: (a) BIOCLIM (b) GARP (c) ME and (d) Consensus Model.

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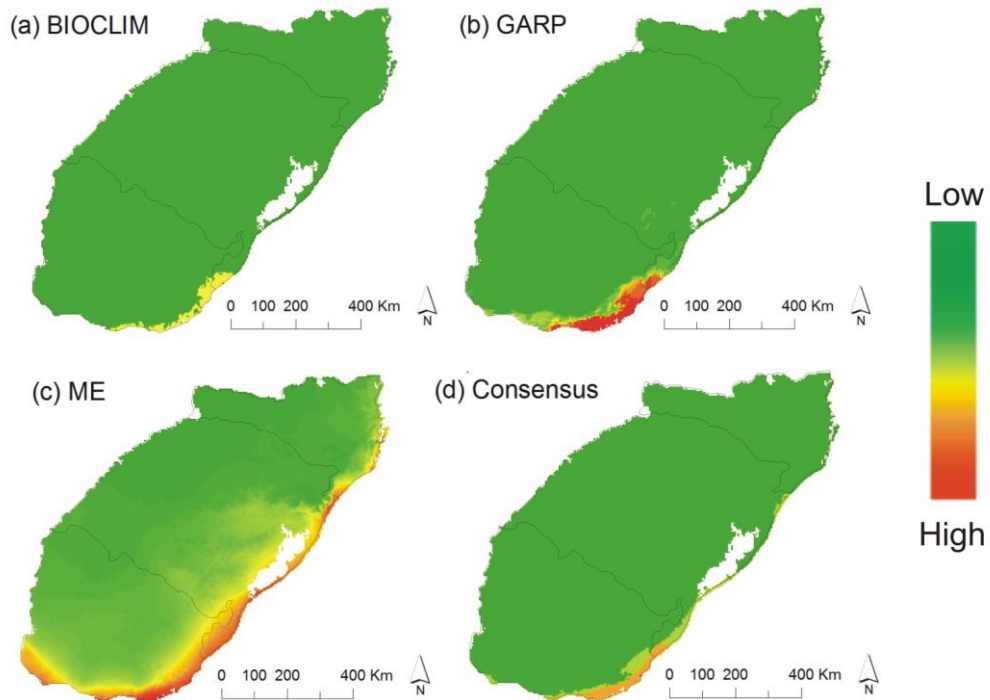
Figure 3. Suitable areas for *Melanophryniscus montevidensis* according to: (a) BIOCLIM, (b) GARP (c) ME and (d) Consensus Model.
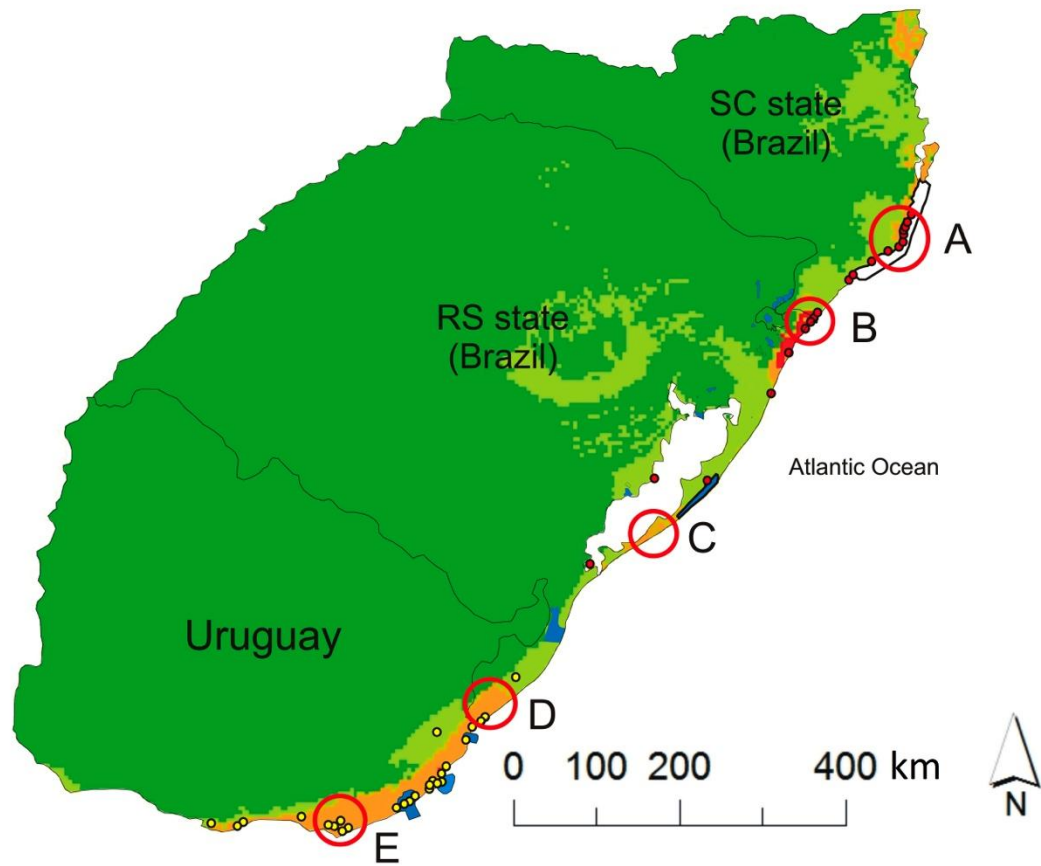
580
581 **Figure 4. Proposed areas for creation or activation of Conservation Units to preserve the red-**

582 **bellied toads *Melanophryniscus dorsalis* and *M. montevidensis*.**

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584

Supplementary material

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586

S1. Species status

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Melanophryniscus dorsalis, commonly known as "Flamenguinho", occurs in the south coast of Brazil, between Itapirubá beach, (Imbituba city, Santa Catarina State), and Cidreira city (Rio Grande do Sul State) (Haddad 2008). Historical records of *M. montevidensis*, commonly known as "Sapito de Darwin", cover from the edge of Brazil southern coast, in Santa Vitória do Palmar city (Rio Grande do Sul State) to Montevideo city, in Uruguay (Maneyro Kwet & 2008).

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The occurrence range for the most records of both species lies at sea level and a few meters from the Atlantic Ocean. This very similar environment houses a broad and diverse fauna with many endemic and endangered species listed at IUCN, such as the lizards *Liolaemus occipitalis*, *L. arambarensis*, Tuco-tucos *Ctenomys flamarioni*, *C. minutus*, *C. lami*, and amphibian *Ceratophrys ornata* (IUCN 2011). Most of the distribution area has been modified by human occupation and use. The habitat occupied by both species consists of sand dunes with small vegetation covering (Haddad 2008, Maneyro & Kwet 2008). The urbanization process, which drives to fragmentation, habitat loss and pollution, is a common problem not only for the red-bellied toads populations but also for all the species living in southern Brazil and Uruguayan coast (Haddad 2008; Maneyro & Kwet 2008). These conditions put both species as vulnerable on IUCN Red List of the International Union for Conservation of Nature (IUCN 2011). *Melanophryniscus dorsalis* is also listed as vulnerable on the Red List of Threatened Species of Rio Grande do Sul (Garcia & Vinciprova 2003) and in the Red Book of Brazilian Endangered Fauna (Haddad 2008). In Uruguay, the historically recorded *M. montevidensis* populations from the extreme south of Montevideo and Canelones departments are no longer found and

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609   decreased the number of records in the Maldonado department (Maneyro & Langone 2001).

610   In Brazil the only documented record of *M. montevidensis* is for the south extreme of Santa

611   Vitória do Palmar city, for over twenty years ago (Tedros *et al*. 2001). There are no data on the

612   population status of *M. dorsalis*, aside from new occurrence records. None specific work on

613   the conservation status of *M. dorsalis* and *M. montevidensis* was carried out yet besides

614   occurrence data and works with alkaloids (Mebs *et al*. 2005), behavioral and basic information

615   on identification and reproductive activity (Kwet *et al*. 2005).

616

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629   *montevidensis* (Philippi, 1902), two parapatric species from southern Brazil and Uruguay, with

630   comments on morphological variation in the *Melanophryniscus stelzneri* group (Anura:

631   Bufonidae). *Salamandra* 1/2/41:1–18.

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640   *Melanophryniscus montevidensis* (Anura, Bufonidae), from Uruguay. *Toxicon*, 6/46:641-650.

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642   Tedros M *et al*., 2001. *Melanophryniscus montevidensis* (Philippi, 1902) (Anura, Bufonidae).

643   *Cuadernos de Herpetología*, 15: 143

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645   **S2. Parameters on MAXENT preliminary models**

646   We generated preliminary models on MAXENT based on historical records to guide the field

647   expeditions. The MAXENT parameters for the models building were: number of iterations

648   established in 1000; replication type defined by cross-validation and delineation of threshold

649   rule on minimum training presence because they are endangered species (Pearson *et al*. 2007).

650   Due to the small number of occurrence data, manly for *M. dorsalis*, we use all bioclimatic

651   variables in these models.

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654 **REFERENCE**

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658 **S3**

Table S1. Occurrence data of *Melanophryniscus dorsalis* and *M. montevidensis*, state of occurrence and registration in conservation units (C.U.)

Point	Lat	Long	State	C.U.	Source data
1	-49.363	-28,931	SC (Brazil)		This paper
2	-49,332	-28,888	SC (Brazil)		This paper
3	-48,818	-28,595	SC (Brazil)		This paper
4	-48,776	-28,529	SC (Brazil)		This paper
5	-48,783	-28,449	SC (Brazil)		Red list*
6	-51,484	-31,073	RS (Brazil)		UFRGS collection*/This paper
7	-52,138	-31,994	RS (Brazil)		Quintela *et al*. 2007*
8	-50,889	-31,122	RS (Brazil)		UFRGS collection*
9	-48,669	-28,247	RS (Brazil)		Red list*
10	-49,986	-29,703	RS (Brazil)		Braun 1978*
11	-50,198	-30,132	RS (Brazil)		Braun 1978*
12	-49,780	-29,387	RS (Brazil)	X	Colombo *et al*., 2008*
13	-49,749	-29,345	RS (Brazil)	X	Braun 1978*

Melanophryniscus dorsalis

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14	-49,720	-29,303	SC (Brazil)	This paper
15	-49,847	-29,472	RS (Brazil)	This paper
16	-48,769	-28,384	SC (Brazil)	This paper
17	-48,753	-28,366	SC (Brazil)	This paper
18	-48,712	-28,337	SC (Brazil)	This paper
19	-49,123	-28,749	SC (Brazil)	This paper
20	-48,956	-28,642	SC (Brazil)	This paper

Melanophryniscus montevidensis				
1	-56,340	-34,850	MO (Uruguay)	ZVZB collection*
2	-56,058	-34,887	MO (Uruguay)	ZVZB collection*
3	-56,007	-34,845	CA (Uruguay)	ZVZB collection*
4	-55,041	-34,885	MA (Uruguay)	ZVZB collection*
5	-55,007	-34,890	MA (Uruguay)	ZVZB collection*
6	-54,867	-34,916	MA (Uruguay)	ZVZB collection*
7	-54,282	-34,675	RO (Uruguay)	ZVZB collection*
8	-54,218	-34,664	RO (Uruguay)	ZVZB collection*
9	-54,160	-34,649	RO (Uruguay)	ZVZB collection*
10	-54,121	-34,588	RO (Uruguay)	ZVZB collection*
11	-53,905	-34,453	RO (Uruguay)	ZVZB collection*
12	-53,905	-34,446	RO (Uruguay)	ZVZB collection*
13	-53,840	-34,412	RO (Uruguay)	ZVZB collection*
14	-53,795	-34,390	RO (Uruguay)	ZVZB collection*
15	-53,796	-34,345	RO (Uruguay)	ZVZB collection*
16	-53,778	-34,275	RO (Uruguay)	ZVZB collection*
17	-53,530	-33,945	RO (Uruguay)	ZVZB collection*

18	-53,460	-33,805	RO (Uruguay)	ZVZB collection*
19	-53,360	-33,722	RS (Brazil)	Nuñez *et al.*, 2004/This paper*
20	-52,976	-33,276	RS (Brazil)	UFRGS collection*
21	-55,360	-34,783	MA (Uruguay)	ZVZB collection*
22	-54,916	-34,930	MA (Uruguay)	ZVZB collection*
23	-54,926	-34,817	MA (Uruguay)	ZVZB collection*
24	-53,878	-33,737	RO (Uruguay)	ZVZB collection*
25	-53,369	-33,737	RS (Brazil)	Nuñez *et al.*, 2004/This paper*
26	-53,858	-34,392	RO (Uruguay)	ZVZB collection*

659 *RS* State of Rio Grande do Sul and *SC* State of Santa Catarina (Brazil). *MO* Department of
660 Montevideo, *CA* Department of Canelones, *MA* Department of Maldonado, *RO* Department of
661 Rocha (Uruguay). *Historical records.

662

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665 estado de Santa Catarina, Brasil (Anura: Bufonidae). *Iheringia Sér. Zool.*, Porto Alegre, 51: 39-

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678 **S4**

Table S2. AUC values for each algorithm (BIOCLIM, GARP, Maximum Entropy and MAXENT) for *M. dorsalis* and *M. montevidensis*.

Model	AUC values	
	M. dorsalis	*M. montevidensis*
BIOCLIM	1	0,99
GARP	0,98	0,99
Maximum Entropy (OM)	0,98	0,99
Maxent	Training (0,997) Test (0,993)	Training (0,998) Test (0,991)

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681 **S5**
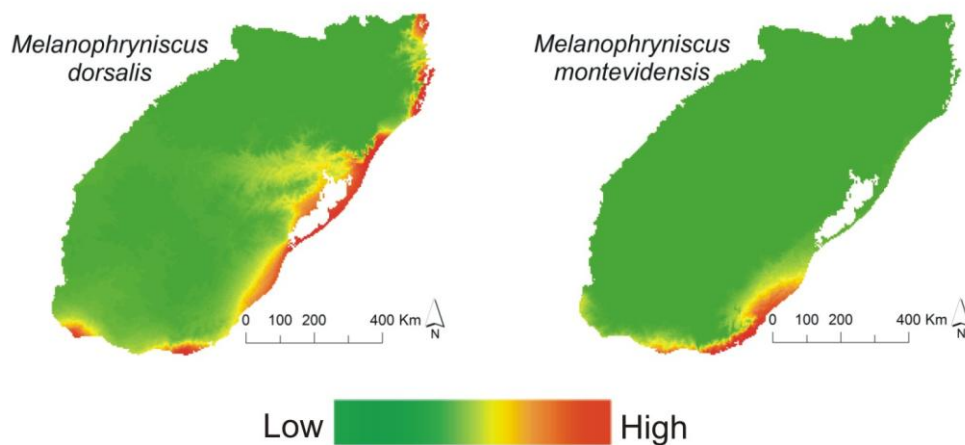
682

683 **Figure S1**. Distribution model with historical data of *Melanophryniscus dorsalis* and *M.*
684 *montevidensis* in MAXENT.

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691 **S6**
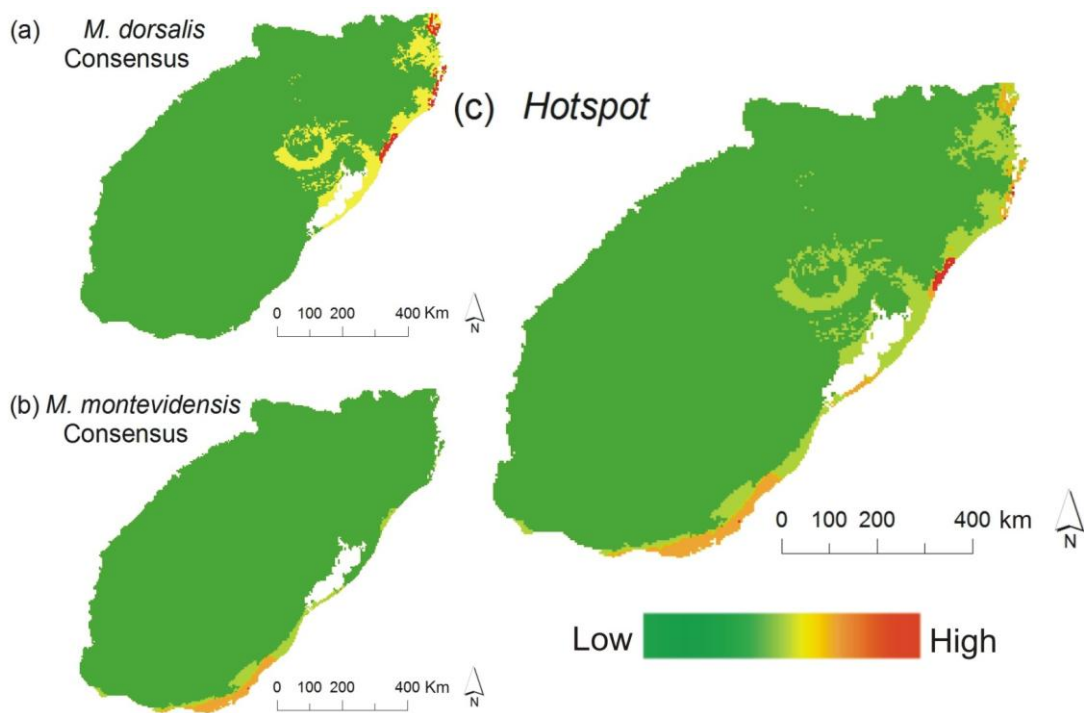
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694 **Figure S2**. Consensus model for *Melanophryniscus dorsalis* (a), *M. montevidensis* (b) and
695 hotspot map (c).

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697 **3. CAPÍTULO II**

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700 **Species-splitting: a recent speciation of two endangered red-belly toads in a young**

701 **environment of southern Brazil and Uruguay**

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703   **ARTIGO A SER SUBMETIDO AO PERIÓDICO CONSERVATION GENETICS**

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705 Jorge Bernardo-Silva*, Paula Angelica Rorato, Raul Maneyro, Thales R.O. de Freitas

706

707 **Species-splitting: a recent speciation of two endangered red-belly toads in a young**

708 **environment of southern Brazil and Uruguay**

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716 Jorge Bernardo-Silva[1]*, Paula Angélica Roratto[2], Raul Maneyro3, Thales R.O. de Freitas[2]

717 1 Programa de Pós Graduação em Ecologia, IB - Universidade Federal do Rio Grande do Sul, Av.

718 Bento Gonçalves 9500, Porto Alegre, RS, Brasil

719 2 Departamento de Genética –IB- Universidade Federal do Rio Grande do Sul, C.P. 15053,

720 90501-970 Porto Alegre, RS, Brasil

721 3 Laboratorio de Sistemática e Historia Natural de Vertebrados, Facultad de Ciencias, UDELAR,

722 Montevideo, Uruguay

723 Correspondência: 1 Programa de Pós Graduação em Ecologia, IB - Universidade Federal do Rio

724 Grande do Sul, Av. Bento Gonçalves 9500, Porto Alegre, RS, Brasil

725 E-mail: bernardo.silva@ufrgs.br, bernardo.silvaj@gmail.com

726

727 **Abstract**

728

729 The red-bellied toads *Melanophryniscus dorsalis* and *M. montevidensis* are sister endangered

730 paratric species listed as vulnerable on the IUCN list. The restricted linear distribution across

731 southern Brazilian coastal plain and Uruguayan coast is the major threat for both species due

732 antropic occupation. We analyzed sequences of mitochondrial (Cyt*b* and COI) DNA markers

733 from 137 individuals representing all distribution of both species. We found a recent species-

734 splitting process with an estimated mean time to most recent common ancestor (tMRCA) in

735 3.14 mya for *M. dorsalis* and 3.53 mya for *M. montevidensis*. Based on our results we

736 hypothesized three colonization events of common ancestor to coast. The star-like haplotype

737 network, neutrality tests and Bayesian demographic reconstructions for both markers, in both

738 species, indicated demographic expansion. Molecular data also shows high variability. The

739 north population of *M. dorsalis* needs special attention because it is considered a Management

740 Unit (MU) since it's individuals present single frequency alleles, the strategy indicated for this

741 population is the delimitation of a specific area and population monitoring.

742 Our data mains help to provide important information set to a national database on

743 endangered species, we also aim provide important data source to the National Plan of Action

744 for the conservation and preservation of reptiles and amphibians threatened with extinction in

745 southern Brazil (PAN SUL) managed by the Chico Mendes Institute for Biodiversity

746 Conservation.

750   **Introduction**

751

752   The continuous and steady decline of Amphibians still attempts and concern researches.

753   Climate change, infectious diseases and UVB irradiation are the main causes (Crawford et al.

754   2010), however secondary causes such as habitat loss and introduction of exotic species have a

755   considerable effect (Diamond 1989). Human activities altering landscapes lead to habitat

756   fragmentation, clearly reducing connectivity among animals populations (Shepard et al. 2008;

757   Ford and Fahrig 2008; Pruett et al. 2009; Scherer et al. 2012). In Brazil several studies on

758   amphibian conservation status are listed by Verdade et al. (2012), but there is still a lack of

759   knowledge about this group. Taxonomic revisions and demographic range distributions are

760   primarily target to define species status and identify key priority in order to determinate

761   protected areas (Brooks et al 2004, Verdade et al 2012). Beyond the basic distribution data,

762   inference on genetic status shows more robust information about how endangered species

763   may be and the appropriated using of molecular markers to infer genetic population distance

764   may show dispersal process within species and a recent speciation process between close

765   species. Estimate genetic diversity among populations of endangered species provides

766   essential information to define appropriated conservations programs (Songmei and Zhang,

767   2012).

768   The red-belly toads of *Melanophryniscus* genus occur in half northern Argentina, interandean

769   valleys of southern Bolivia, central western and southern coastal lowlands of Brazil Paraguay

770   and Uruguay (Frost 2011, Kwet et al. 2005). The genus are probably monophyletic (Graybel

771   and Cannatella, 1995) and considered sister group of all the others genus of Bufonidae family

772   (Frost et al. 2006). The systematic classification is not the only to carry on the genus as out-

773   group in Bufonidae Family. Besides, reproductive behavior are described on explosive breeding

774   events in temporary ponds associated with heavy rainfall independently of the temperature

775   (Kwet et al. 2005), the toxic alkaloids contained in their skin (Mebs at al. 2005) and aposematic

776   behavior distinguishes them from most other anurans making an interesting evolutionary

777   group to understand.

778   *Melanophryniscus dorsalis* (Mertens 1933) and *M. montevidensis* (Phillipi 1902) are small

779   anurans, measuring between 20 and 27mm (Kwet et al. 2005), and occurring in Southeast

780   South America (Uruguay and Brazil). *Melanophryniscus dorsalis* occurs on the south coast of

781   Brazil, from the beach city Itapirubá (Santa Catarina State) to the Ilha dos Marinheiros in the

782   Rio Grande municipality, Rio Grande do Sul State (Quintela et al 2007) (Figure 1).

783   *Melanophryniscus montevidensis* occurs at the boundary of southern coast in Brazil, – in the

784   City of Santa Vitória do Palmar, and spread towards Montevideo in Uruguay (Maneyro and

785   Kwet 2008). Despite the restricted areas of occurrence, these species inhabit one of the largest

786   coastal plain in the world (Esteves et al. 2002). This formation consists of sandy fields derived

787   from the oldest depositional system of the southern coastal plain, formed by fluctuations in

788   the Atlantic Ocean sea level, approximately 400 000 years ago (Tomazelli et al. 2000). The

789   constant influence of ocean fluctuations over time made strong evolutionary pressures on

790   animal and plants populations.

791   The main morphological characteristics described of *M. dorsalis* are exhibited a complete or

792   partly interrupted red middorsal line or a trace of this stripe between and behind the eyes

793   (Mertens 1933), however individuals with no red line or partly red line were founded by Kwet

794   et al. (2005) and in the present study. In *M. montevidensis* lateral yellow blotches are typical

795   which, whilst never show a red middorsal line (Kwet et al. 2005). However, Kwet et al. (2005)

796   also collected many *M*. *montevidensis* with no lateral blotches at all, although the uniformly

797   black dorsal pattern found in some individuals of *M. montevidensis* and *M. dorsalis* is

798 considered to be the main character for the identification of another species, like

799 *Melanophryniscus atroluteus* (Kwet et al. 2005).

800 Both species are listed as vulnerable on International Union for Conservation of Nature (IUCN

801 2011) and *M. dorsalis* is also listed as vulnerable on the Red List of Brazilian Fauna Extinction

802 Threatened (Haddad 2008) and Red List of Endangered Species of Rio Grande do Sul (Garcia

803 and Vinciprova 2003). The occurrence area is restricted to dunes environment with little

804 vegetation cover of, predominantly small species (Langone 1995; Haddad 2008; Maneyro and

805 Kwet 2008). The urbanization process causing habitat loss and pollution is the main threatened

806 for both species (Haddad 2008; Maneyro and Kwet 2008).

807 In Brazil few authors discuss amphibian conservation status (see Verdade et al 2012), but a

808 large gap of information on species status information such as basic research, particularly

809 species inventory, taxonomic revisions and collection of field data still occurs (Verdade et al

810 2012), based on that Verdade et al (2012) suggest the creation of a national database of

811 amphibian species and indicated the harmonized these national database with other

812 information systems such as those provided by Sistema Nacional de Pesquisa em

813 Biodiversidade (SISBIOTA), International Union for Conservation of Nature (IUCN), Global

814 Biodiversity Information Facility (GBIF), Centro de Referência em Informação Ambiental (CRIA)

815 and VERTNET (a global museum database of vertebrate natural history collections). Another

816 interesting action was the National Plan for Conservation of Herpetology Fauna from Southern

817 Brazil (*PAN*), published in 2011 an initiative from Chico Mendes Institute for Biodiversity

818 Conservation (ICMbio) associated to the Brazilian Ministry of Environment. This *PAN* was based

819 upon identification and orientation of priority actions to address the threat of endangered

820 species populations and natural environments. The *PAN* main goal is the maintenance of

821 amphibians and reptiles diversity from Southern Brazil.

822 Based on the conservation status of both species and the main doubt about species validation,

823 the goals of our study were: (1) understand the recent evolutionary history of *M. dorsalis* and

824 *M. montevidensis* living in a young environment of southern Brazil and Uruguay; (2) Evaluate

825 the validation of these species as separated; (3) Provide genetic status information to support

826 conservation strategies.

827

828 **Materials and Methods**

829 Species samples

830 Specimens were collected from December 2009 to July 2011. Some field records were not

831 registered in literature or forgotten for some authors (Bernardo-Silva et al. in press) but

832 sampled in these study (Fig 1). In Uruguay some populations of *M. montevidensis* were

833 historically registered at the extremely south distribution at the Departments of Montevideo

834 and Canelones, but they were not found any more and in Maldonado department the

835 populations have been reduced (Maneyro and Langone 2001). In Brazil, the only historical

836 record of *M. montevidensis* is for Rio Grande do Sul state in Santa Vitoria do Palmar city,

837 which is deposited at numbers 1845-47 at Herpetology collection at the Universidade Federal

838 do Rio Grande do Sul (UFRGS) and was rediscovery in this study. There are only two Brazilian

839 historical records of *M. dorsalis* cited by Braun (1978) for the Cidreira and Capão da Canoa

840 beaches, besides the records 1755-1758 from (UFRGS collection), from Mostardas beach. All

841 these localities were searched out but no actual records of red-belly toads were found (Figure

842 1) and, except the Mostardas site, all of the other previous records with no more occurrences

843 of *M. dorsalis* nowadays showed great anthropogenic disturbance from coastal occupation.

844 A total of 137 specimens were sampled from 16 populations, 87 individuals of 10 populations

845 of *M. dorsalis* and 50 individuals of 6 populations of *M. montevidensis* (Fig. 1, Table 1). These

846   samples include all distribution range of both species and old collection records rediscovery in

847   our work and not included in the literature cited by Haddad (2008) and Garcia and Vinciprova

848   (2003). The occurrence area cited by Kwet et al. 2005 was extensively sampled and, even when

849   climatic conditions described on explosive breeding events in temporary ponds associated with

850   heavy rainfall happened, in some recognized samples sites, just after the fourth field

851   expedition specimens were sampled which expose the lack of basic ecological knowledge.

852

853   Molecular Methods, DNA extraction and PCR amplification

854   The total DNA was extracted following (Doyle and Doyle 1987). Phylogeographic analysis was

855   carried using two fragments of the mitochondrial DNA (mtDNA) amplified through polymerase

856   chain reaction (PCR) for 139 specimens of *M. dosralis* and *M. montevidensis* and 3 specimens

857   of *M. atroluteus*.

858   We sequenced two mitochondrial regions. Initially we used a fragment of the control region

859   (primers CytbA-L and H-ControlB; Goebel et al. 1999), however the control region sequences

860   showed saturated, been no informative to our questions. Amplifications of the cytochrome *b*

861   region using the primers MVZ15-L and ControlW-H (Goebel et al. 1999) were unsuccessful for

862   many   samples.   Then,   new   cytochrome   *b*   primers   were   designed   (foward   5'-

863   GTTATCTGCAGCCCATACG-3' (Cyt*b*M F and reverse 5'-ATCTGGGTCTCCAAGGAGGT-3' Cyt*b*MR),

864   which amplified a 280pb fragment and were applied in our study. The second region is the

865   cytochrome *c* oxidase I gene (COI) (primers LCO-1490 and HCO-2198; Folmer et al., 1994). PCR

866   amplifications for both Cyt*b* and COI were carried out in a reaction volume of 20 µl containing

867   20-80 ng of DNA, 0.2µM of each primer, 0.4 mM dNTP, 1x PCR buffer, 4 mM $MgCl_2$ and 1.0 unit

868   of Taq DNA polymerase (Invitrogen). Amplification conditions for cytochrome *b* start as one

869   cycle of 96ºC for 2 min, 50ºC for 45 sec and 72ºC for 2 min followed by 39 cycles of 30s at

870   94ºC, annealing (45sec at 48°C-52°C), 1 min and 30 sec at 72°C and a final extension at 72ºC for

871   10 min. For COI, cycling consist of 94ºC for 1 min, followed by 34 cycles of 30s at 94ºC,

872   annealing (1 min and 30 sec at 48°C-52°C), 1 min at 72°C and a final extension at 72ºC for 5

873   min. Sample size per population ranges from three to twenty individuals. All PCR products

874   were checked on agarose gel stained with ethidium bromide and purified using Exonuclease I

875   and Shrimp Alkaline Phosphatase (GE Healthcare) performed following the guidelines of the

876   suppliers and sent for sequencing at Macrogen Inc. (Korea).

877

878   Phylogeography analysis

879   Sequence electropherograms of the mitochondrial DNA (mtDNA) were visually inspected using

880   Chromas 2.33 (Technelysium Pty Ltd; http://www.technelysium.com.au/chromas_lite.html)

881   aligned and edited by eye using the Clustal W algorithm implemented in Mega 5.0 (Tamura et

882   al., 2011). All analyses described bellow were performed for cytochome *b* and COI sequence

883   data separately and for both concatenated (cytochome *b* and COI). The software DNASP 5.0

884   (Librado and Rozas, 2009) was used to define haplotypes (H) as well as to estimate nucleotide

885   (π) and haplotype diversity (Hh) following Nei (1987). A median-joining network (Bandelt et al.,

886   1999) was computed in the Network 4.6.0.0 program (http://www.fluxux-engineering.com).

887   The program ARLEQUIN 3.11 (Excoffier and Schneider 2005) was used to infer departures from

888   neutrality through Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997), and to calculated the F_{ST}

889   statistics for pairwise genetic differentiation between localities, with 1000 random

890   permutations to test the values for significance statistics.

891

892

893   Demographic analysis

894   Past changes in population effective size (Ne) of *M. dorsalis* and *M. montevidensis* were

895   characterized by generation Bayesian skyline (Drummond et al., 2005) and skyride plots (Minin

896   et al., 2008). These analyses follow a molecular clock to generate a posterior distribution of Ne

897   through time using Markov chain Monte Carlo (MCMC) sampling, and both are implemented in

898   the software BEAST v1.7.1 (Drummond et al., 2007).

899   In order to acquire an evolution molecular rate for mitochondrial data, to be applied to the

900   molecular clock analyses, we first construct a phylogeny of the Bufonidae family using a set of

901   species which cover the following fossil records: (i) the oldest fossil of Bufonidae (57 mya -

902   Baéz and Gasparini, 1979), (ii) the oldest fossil of *R. marina* (about 11 mya - Sanchíz, 1998), and

903   (iii) the *Bufo* genus splitting between Central and North America (20 mya - Tihen, 1951). The

904   mitochondrial molecular markers, the 12S, 16S ribosomal genes and the cytochrome *b* were

905   used, by the fact they are those with higher number of sequenced species available on

906   Genbank. Table S1 shows the list of Bufonidae species and theirs accession numbers used for

907   the molecular rate estimative.

908   The GTR+I+G model was selected by the AIC criterion implemented in MODELTEST 3.7 (Posada

909   and Crandall, 1998) for the concatenated 12S, 16S and cytochrome *b* sequences. We used the

910   Yule calibrated tree prior (Heled and Drummond, 2011), the relaxed molecular clock

911   (uncorrelated lognormal), and all the fossil records as normal priors, considering the

912   aforementioned ages as the upper limit of a 95% confidence interval. The computational

913   resources of Bioportal (https://www.bioportal.uio.no) was used to perform two runs of 50

914   million iterations sampling each 1,000 chains, and the first 10% iterations were discarded as

915   burn-in, for all BEAST analyses. Independent runs were summarized in TreeAnnotator 1.7.1

916   (Drummond   and   Rambaut   2007).   The   software   TRACER   1.5

917   (http://tree.bio.ed.ac.uk/software/tracer/) was used to check for convergence of the chains,

918   and to gather the estimated mean rate for the mitochondrial markers.

919   Finally, we performed the demographic analysis with the coalescent Bayesian skyline and

920   GMRF Bayesian skyride priors, on the basis of the estimated mean rate estimated for us that

921   was applied as a normal prior, to the concatenate cytochrome *b* and COI data sets for both

922   red-belly toads' species separately. The HKY+I and the TVM+I+G evolution models were

923   selected by the AIC criterion implemented in MODELTEST 3.7 (Posada and Crandall, 1998) for

924   *M. dorsalis* and *M. montevidensis*, respectively. Runs were carried on as described previously

925   and TRACER was also used for the skyline and skyride reconstructions.

926

927   **Results**

928   We obtained 616 bp for the COI and 281 for Cyt*b*m sequences. Sixty one haplotypes were

929   established for COI sequences and 38 for Cyt*b*m in a total of 140 individuals. Haplotype

930   diversity values were moderate to high, with an exception to *M. montevidensis* on Cyt*b*m

931   region, whereas nucleotide diversity values were low (Table 2), indicating that these species

932   are comprised of a relatively high number of closely related haplotypes.

933   We notice one sharing haplotype between *M. dorsalis* and *M. montevidensis* for Cyt*b*m marker

934   (see haplotype 19 Cyt*b*m figure 2) and one haplotype from *M. montevidensis* (H35 Cyt*b*m)

935   appears more related with *M. dorsalis*. On the other hand the same individuals are

936   represented at haplotypes 35 e 51 on COI region. Haplotype 35 from *M. montevidensis* is more

937   related with *M. dorsalis* populations and H51 from *M. dorsalis* are more related with *M.*

938   *montevidensis* populations (Figure 2). Due the sharing and closeness of *M. dorsalis* and *M.*

939   *montevidensis* haplotypes, we will present the networks of both species together (Figure 2).

940   Despite the difference in number of haplotypes, the general patterns were concordant for

941   both data sets. Due the high number of closely related haplotypes, the concatenated network

942   was too reticulated, then we will present and comment COI and Cyt*b*m haplotype network

943   separately (Fig. 2 and Fig. S1).

944   Despite the smallest geographical distance between ARBA, ILMA (*M. dorsalis* southernmost

945   distribution populations) and *M. montevidensis*, there is no sharing haplotypes between them.

946   Alternatively, *M. montevidensis* sharing and more closely haplotypes refers to the

947   northernmost *M. dorsalis* populations, indicating that the *M. dorsalis* south occupation

948   occurred far after the splitting of these two species. The three individuals of *M. atroluteus*

949   represented at MAVI populations appear more closely related with *M. montevidensis*

950   populations for COI, although the Cyt*b*m network shows an intermediary vector among *M.*

951   *atroluteus*, *M. montevidensis* and the *M. dorsalis* MARO populations (Fig. 2).

952   At an intraspecific level, few haplotypes were shared between several populations in both

953   markers of mtDNA from *M. dorsalis*. The central H2 haplotype from both markers and the

954   closely related unique or less shared haplotypes showed as a star-like complex network

955   formation indicates a demographic expansion.

956   The H2 haplotype, for both markers, are shared by three populations (MARO, MOCO and

957   ARBA). The general haplotypes network is very similar in both regions (Fig. 2). The network for

958   both mtDNA markers shows a similar pattern for north population of *M. dorsalis* (MARO). The

959   MARO population shows a different pattern comparing the remains populations of *M. dorsalis*,

960   these populations are equally related with all populations of *M. montevidensis* and the

961   remains populations of *M. dorsalis* (Fig. 2).

962   The H19 haplotype from Cyt*b*m was shared for both species. It was found for individuals form

963   *M. montevidensis* populations and only one individual of *M. dorsalis* from the ITSU population

964 (Figure 2, S1). Despite there were no haplotype sharing for COI region between both species,

965 some *M. dorsalis* individuals from ITSU showed the H35 haplotype, which is more related with

966 *M. montevidensis* populations. (Fig. 2 and Fig. S1). On the other hand, individual from the

967 CAPO population of *M. montevidensis* present the COI-H51 and the Cyt*b*m-H35 haplotypes

968 more related with *M. dorsalis* central haplotypes. Individuals from the extremely north

969 distribution of *M. dorsalis* (MARO population) showed the exclusive Cyt*b*m-H10, as well as the

970 COI haplotype set H12 to H17, which were detached from the remains populations of *M.*

971 *dorsalis* and *M. montevidensis*, although the *M. dorsalis* central COI-H2 and Cyt*b*m-H2

972 haplotypes were also represented in one individual from MARO.

973 From *M. montevidensis* the central H28 from COI and 24 from Cyt*b*m markers show similar

974 patterns of star-like complex network formation indicating a demographic expansion, with

975 more expressive number of unique or less shared haplotypes closely related from both

976 markers (Fig. 2).

977 For *M. dorsalis* geographically near locations show no significant values of F_{ST} such as ITSU,

978 GUAR and PATO. On the other hand, remote locations such as ILMA and ARBA show high and

979 significant values comprising the remains locations. The lowest F_{ST} values (0.12 between ARBA

980 and MOCO and between ARBA and ILHA) reflecting the sharing haplotipes Cyt*b*m-H2 and COI-

981 H2 (Table 3 and Figure S1). For *M. montevidensis* near locations like CHUI - CAPO, and CAPO -

982 ROCH show low and significant values for genetic F_{ST}. On the other hand distant locations such

983 as SAVI - PERO and SAVI - ROCH have high and significant values for genetic F_{ST} (Table 4 and

984 Figure S1).

985 All neutrality tests for *M. montevidensis* were negative and significant for the COI, Cyt*b*m and

986 the concatenated data set. For *M. dorsalis*, both Tajima's D and Fs's Fu were negative and

987 significant for Cyt*b*m, and the more sensitive Fu's FS test result in negative and significant

988   value for the concatenate dataset (Table 2). The negative and significant values for neutrality

989   tests suggest a recent history of population expansion for both species. Scenery of recent

990   population expansion was also indicated by the coalescent analysis using the molecular clock

991   (Fig. 3). All BEAST approaches results in good statistics (higher than 200) and stabilized MCMC

992   chains, as checked by TRACER, except for the GMRF Bayesian skyride prior. Then, only the

993   Bayesian skyline results are showed. The mean time to most recent common ancestor (tMRCA)

994   was 3.14 mya for *M. dorsalis* and 3.53 mya for *M. montevidensis*. The skyline plots indicate a

995   population expansion during the last 0.5 mya for both species (Fig. 3).

996

997   **Discussion**

998

999   Speciation

1000   Species determinations and classification are the main goal of systematic researchers, they

1001   work are extremely important and necessary to situated a species or a group of species on the

1002   phyilogenetic tree. However, considering a species as a discrete variable to recent evolution

1003   studies do not contemplate the true, species are a continuous variable (Butlin et al 2008) and

1004   for some refined studies we may not able to precisely define a species limited. The

1005   mitochondrial evidence obtained in our study, by the shared H19 Cyt*b*m haplotypes from *M.*

1006   *dorsalis* and *M. montevidenis* show a recent common ancestor (Fig 2). The south distribution of

1007   *M. dorsalis* show high haplotypes sharing with all the others localities from both species (H2

1008   from COI and Cyt*b*m plus ramifications with one or two mutations steps). The extremely north

1009   population of *M. dorsalis* are similarly related with the remains populations of *M. dorsalis* and

1010   all *M. montevidensis* populations. Moreover there are morphological, ecological, habitat

1011   similarity and the parapatric distribution from both species (Kwet and Maneyro 2005).

1012   *Melanophryniscus dorsalis* and *M. montevidensis* represent a typical example of a species-

1013   splitting occurring on time (Fig. 2). A similar situation was reported for many others taxa such

1014   as Lepidoptera (Mutanen 2005), birds (Uy et al 2009) and the interesting case of polar an

1015   brown bear (Webb et al 2012) that nuclear DNA markers reflect a species tree consistent and

1016   tuco-tuco that inhabits the same coastal plain (Lopes 2012). However, the brow bears native to

1017   Alaska's Alexander Archipelago are estimate that 5-10% of their nuclear genome is most

1018   closely related to polar bears, indicating ancient admixture between the two species (Webb et

1019   al 2012). Despite in our study we just analyzed mtDNA, we found a similar situations

1020   comprising the study of bears. The divergence of *M. dorsalis* and *M. montevidensis* presents

1021   through haplotype network from Cyt*b*m and COI, demonstrated evidence of recent expansion

1022   for both species. The MARO population presents exclusive haplotypes from genetic drift due

1023   the instability of the environment with introgression and regression events of ocean.

1024   These consistent results support a new hypothesis of at least three colonization events

1025   of common ancestor to coast. One colonization event to the extremely north distribution of *M.*

1026   *dorsalis* resulting MARO population (Fig. 2, Fig. S1), another colonization event to remains

1027   populations of *M. dorsalis* and a unique colonization event for *M. montevidensis* to Southern

1028   Rio Grande do Sul and Uruguay. Moreover, the out group population, *M. atroluteus*, was more

1029   associated with *M. montevidensis* than *M. dorsalis* from COI region and equally related with

1030   MARO population from *M. dorsalis*, and all populations of *M. montevidensis* from Cyt*b*m

1031   supporting our three colonization event hypothesis (Fig. 2).

1032   Kwet and Maneyro (2005) found a relative high dominant frequency, long pulse and

1033   repetition rate of the advertisement call of *M. dorsalis* differing from *M. montevidensis* and *M.*

1034   *atroluteus*. On another hand found no bioacoustically difference between *M. montevidensis*

1035   and *M. atroluteus*. Despite Kwet and Maneyro (2005) used only the advertisement calls of *M.*

1036   *dorsalis* from ITSU location and from Laguna SC (with no discriminated location) and calls of La

1037 Paloma in Uruguay from *M. montevidensis*, the acoustic results from that study was not

1038 surprise comparing our data. The advertisement calls and the closely relationship from mtDNA

1039 haplotypes network give a nice a picture of these speciation on time processes and

1040 nevertheless, do not lead questioning the species validation as separated mainly because their

1041 parapatric distribution.

1042

1043 Young environment

1044 The southern Brazilian and Uruguayan coastal plain are extremely dynamics ecosystems

1045 creating several water channels among all extension (Tomazelli and Villwock, 2000;

1046 Weschenfelder et al., 2008; Dillenburg et al., 2009; Hesp et al., 2009). According Villwock et al.

1047 (1986) and Tomazelli et al. (2000) the fluctuation in the Atlantic Ocean produced great lateral

1048 displacements of the shoreline, during at least four transgressive-introgressive cycles. Even we

1049 not proposed the evaluation of natural barriers on these work, we do not found any

1050 association between population's divergence and water channels or another potential barrier.

1051 The disassociation with any natural barriers is the indication of a continuous species

1052 movement and colonization of the coastal plain fallows the Atlantic Ocean fluctuations.

1053 Observing the linear distribution of both species, the first idea of genetic distance associated

1054 with geographic distribution may come by but, the haplotype network presented for Cyt*b*m

1055 and COI show no geographic association and no evidence to any possible barriers among

1056 populations (Fig. 2). The central H2 haplotype from COI and Cyt*b*m show the two extremes of

1057 *M. dorsalis* distribution sharing haplotypes.

1058

1059

Variability and conservation

1061   Understand the population's dynamics is extremely important to define conservation

1062   strategies. The isolated populations are more prone to extinctions than populations with

1063   recognized gene flow. We do not found evidences of populations decline in *M. dorsalis* and *M.*

1064   *montevidensis* witch contrasting the three populations of *Ctenomys flamarioni* occurring in

1065   southern cost of Barzil (Fernández-Stolz 2007). On the contrary, molecular mtDNA evidences

1066   show high variability for both species and the negative and significant values for neutrality

1067   tests (Table 2) and the coalescent analysis using the molecular clock (Fig. 3) strongly suggest

1068   population expansion for both species. Moreover the high number of unique haplotypes that

1069   differ in one or very few nucleotide changes from a central and widespread haplotypes from

1070   both species also indicated expansion. This high variability is surprisingly unexpected because

1071   the ecological observations on reproductive behavior on explosive breeding events in

1072   temporary ponds associated with heavy rainfall may indicate a possible genetic population's

1073   monomorphism due the high related specimens at the same locations. However these

1074   consistent results represent a recent history expansion by a young environment,

1075   complementary studies with other markers, mainly microsatellite markers, are needed to

1076   identify the existence of bottlenecks and threats to both species.

1077   However we found more than one haplotype for all populations in COI region and, excepted

1078   the one population (MARO), all remains populations in Cyt*b*m region also has more than one

1079   haplotype, which also indicated high variability. These results show that both species are not

1080   impacted by low genetic variability based on mtDNA region. Once again the data remains

1081   further studies with nuclear markers such microsatellites are needed to understand the high

1082   population dynamic of these species. According Moritz (1994) the MARO population is a

1083   typical example of significant divergence group of individuals identifies by distinguish allelic

1084   frequency of neutral mtDNA defining an MU (Management Unit). MARO population is very

1085   indicated to be focus of a more conservation strategies such as delimitation areas and

1086   population monitoring strategies.

1087   According the proposal made by Verdade et al (2012) our study can provide an interesting data

1088   information about two global threatened amphibians' species. Despite we not found any

1089   evidence of genetic populations declines on these species, they clearly are in a patch

1090   distribution found in surroundings villages and cities close to the shore are considered to be a

1091   potential threat for both species (Kolenc, 1987, Maneyro et al. 1995, Langone 2002, Garcia and

1092   Vinciprova 2003, Kwet and Maneyro 2005, Bernardo-Silva et al. in press). Therefore, human

1093   causes associated with the mischaracterization of habitat through uncontrolled occupation of

1094   the southern coast of Brazil and Uruguay is clearly the main threats to these species.

1095   Our results and conclusions can provide helpful information to national database of amphibian

1096   species proposal by Verdade et al (2012) and the Brazilian government, through the National

1097   Action Plan (*PAN*) for the conservation of threatened amphibians and reptiles of southern

1098   Brazil managed by the Chico Mendes Institute for Biodiversity Conservation (ICMbio).

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Table 1 Sampled populations of *Melanophryniscus dorsalis* and *M. montevidensis*

Population code	Location name	Species	Historical data
MARO	Mar Grosso	*M. dorsalis*	Braun 1978/ Bernardo-Silva et al. in press
IPOA	Ipoã	*M. dorsalis*	Bernardo-Silva et al. in press
SAMA	Farol de Santa Marta	*M. dorsalis*	Bernardo-Silva et al. in press
ILHA	Ilhas	*M. dorsalis*	Bernardo-Silva et al. in press
MOCO	Morro dos Conventus	*M. dorsalis*	Bernardo-Silva et al. in press
PATO	Passo de Torres	*M. dorsalis*	Bernardo-Silva et al. in press
GUAR	Praia da Guarita	*M. dorsalis*	Braun 1978/ Bernardo-Silva et al. in press
ITSU	Itapeva Sul	*M. dorsalis*	Colombo et al., 2008
ARBA	Arambaré	*M. dorsalis*	UFRGS collection/ Bernardo-Silva et al. in press
ILMA	Ilha dos Marinheiros	*M. dorsalis*	Quintela et al. 2006

SAVI	Santa Vitória	*M. montevidensis*	PUCRS collection
CHUI	Chuí	*M. montevidensis*	UFRGS collection/Bernardo-Silva et al. in press
LACO	La Coronilla	*M. montevidensis*	ZVZB collection
ROCH	Rocha	*M. montevidensis*	ZVZB collection
CAPO	Cabo Polônio	*M. montevidensis*	ZVZB collection
PERO	Perla de Rocha	*M. montevidensis*	ZVZB collection

1321

1322

1323 **Table 2** Genetic diversity estimatives cytochome *b* and cytochrome *c* oxidase I (COI) and

1324 concatenated data set (Cyt*b*m + COI). Number of segregating sites (S), nucleotide diversity (π),

1325 number of haplotypes (H), haplotype diversity (Hd), Tajima's D, and Fu's Fs neutrality tests and

1326 graphic distribution of the mismatch analysis.

Data set	S	π	H	Hd	Tajima's D*	Fu's Fs**
Melanophryniscus dorsalis						
Cyt*b*	20	0.01240	20	0.882	NS	NS
COI	35	0.00728	25	0.86	-1.63	-12.97
Cytb+COI	55	0.00902	39	0.96	-1.28 NS	-24.73
Melanophryniscus montevidensis						
Cytb	13	0.00280	9	0.447	-1.61	-7.85
COI	34	0.00664	26	0.95	-1.74	-16.37
Cytb+COI	47	0.00545	29	0.96	-1.80	-25.41

1327 *Significant values for P<0.05; **Significant values for P<0.02; NS non significant value

1328

1329 **Table 3** Genetic differentiation results of concatenated data set (Cyt*b*m + COI) for *M. dorsalis*

1330 (F_{ST} values).

	MOCO	ILHA	SAMA	IPOA	MARO	ARBA	ILMA	GUAR	ITSU	PATO
MOCO										
ILHA	0.02									
SAMA	**0.30**	**0.41**								
IPOA	**0.37**	**0.46**	**0.25**							
MARO	**0.75**	**0.72**	**0.73**	**0.77**						
ARBA	**0.12**	**0.17**	**0.37**	**0.27**	**0.73**					
ILMA	**0.26**	**0.29**	**0.30**	**0.37**	**0.69**	**0.22**				
GUAR	**0.25**	0.37	0.07	**0.49**	**0.67**	0.29	0.19			
ITSU	**0.32**	0.34	**0.20**	**0.53**	**0.63**	**0.41**	**0.27**	0.05		
PATO	0.19	0.05	0.31	**0.36**	**0.67**	0.02	0.20	0.23	0.21	

1331 Significant estimates are in bold, after Bonferroni adjustments (α = 0.00111 for COI+Cyt*b*m).

1332 Refer to Appendix S1 for population code definitions.

1333

1334   **Table 4** Genetic differentiation results of concatenated data set (Cyt*b*m + COI) for *M.*

1335   *montevidensis* (F_{ST} values).

	CHUI	PERO	CAPO	LACO	ROCHA	SAVI
CHUI						
PERO	0.13					
CAPO	0.04	0.07				
LACO	**0.13**	**0.22**	0.03			
ROCHA	**0.21**	-0.05	**0.17**	**0.31**		
SAVI	0.02	**0.36**	0.01	0.05	**0.44**	

1336   Significant estimates are in bold, after Bonferroni adjustments ($\alpha = 0.00333$ for COI+Cyt*b*m).

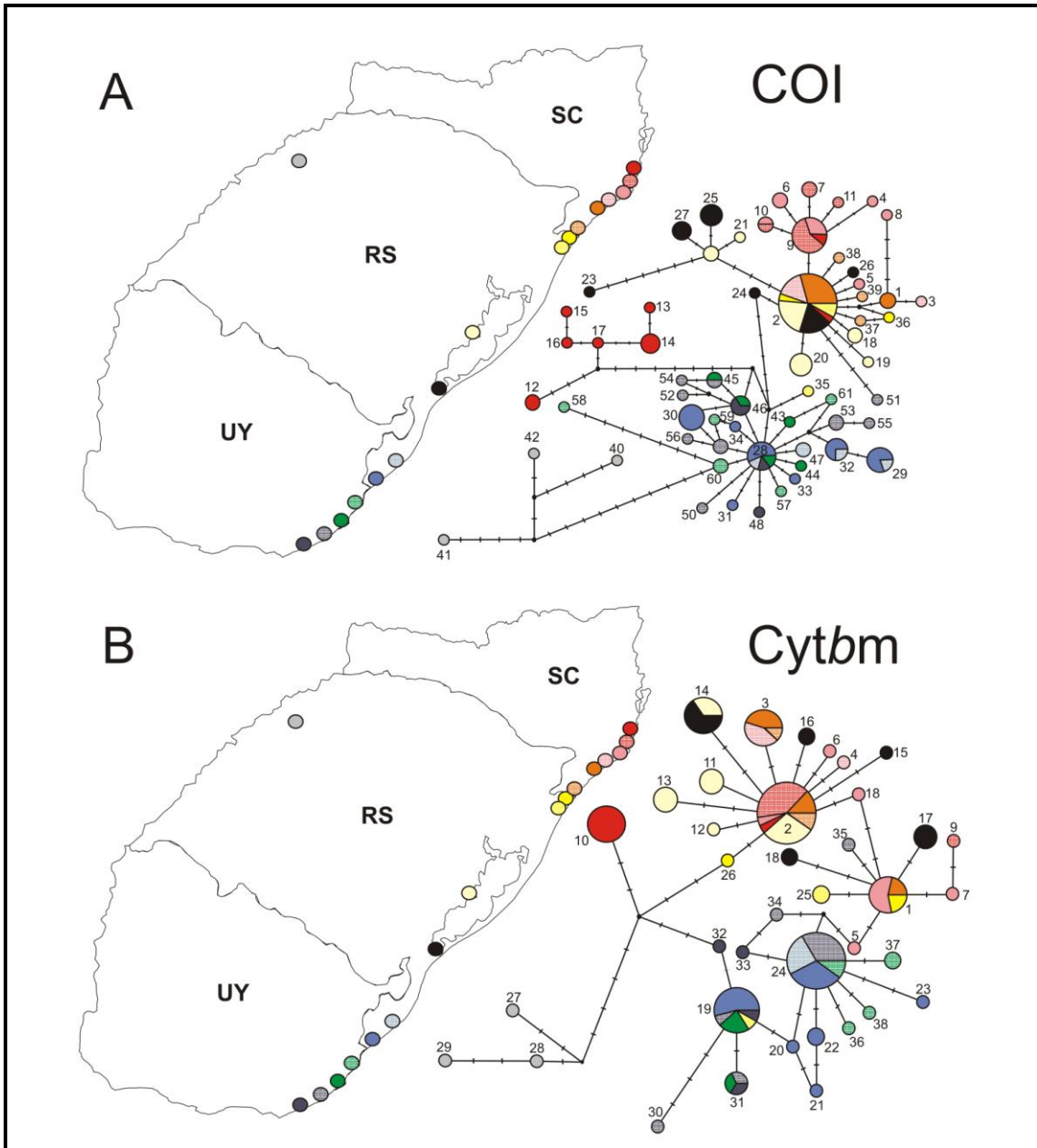1337   Refer to Appendix S1 for population code definitions.

1338
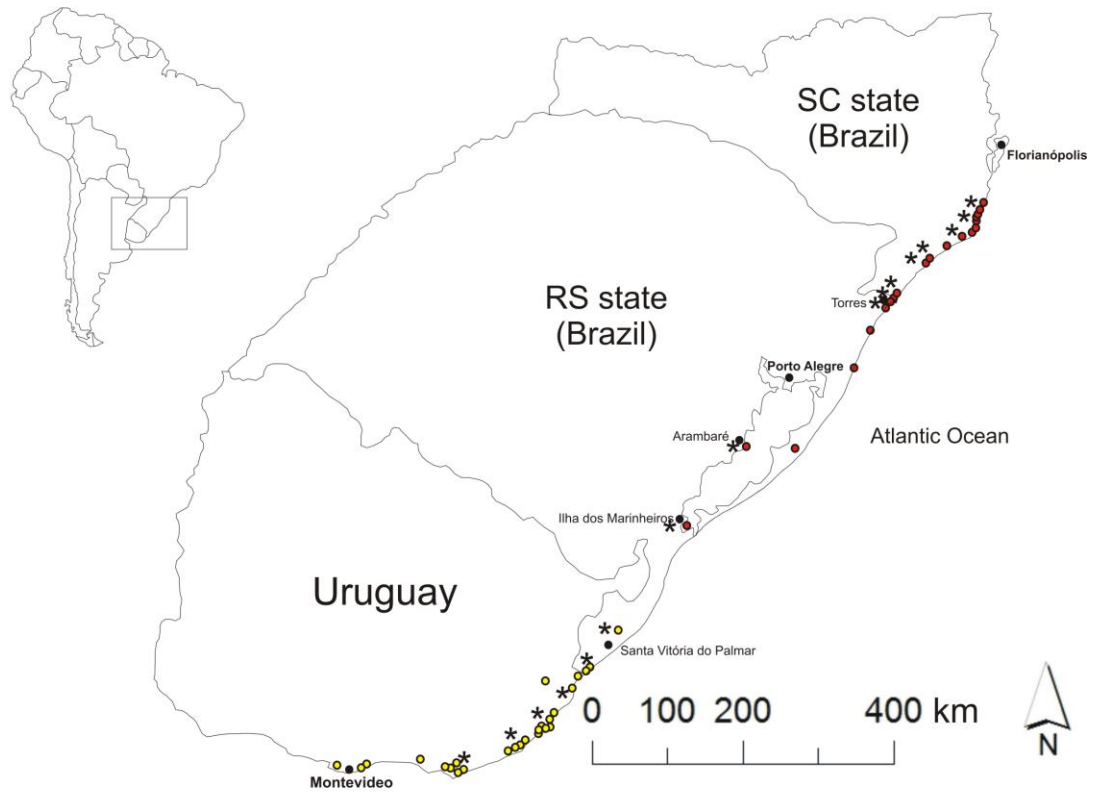
1339

1340 **Fig. 1** Historical and recent occurrence records for *Melanophryniscus montevidensis* (yellow

1341 circles) and *M. dorsalis* (red circles) Asterisk indicates sampled populations.

1342

1343

Fig. 2 Haplotype network of COI and Cyt*b*m populations of red-belly toads *Melanophryniscus*

montevidensis and *M. dorsalis*. Haplotypes are depicted according to sampled sites (localities

names for code on Table 1 and Figure S1) and areas are proportional to frequencies, cross

hatches represent mutation steps and black points represent medium vectors.

1348
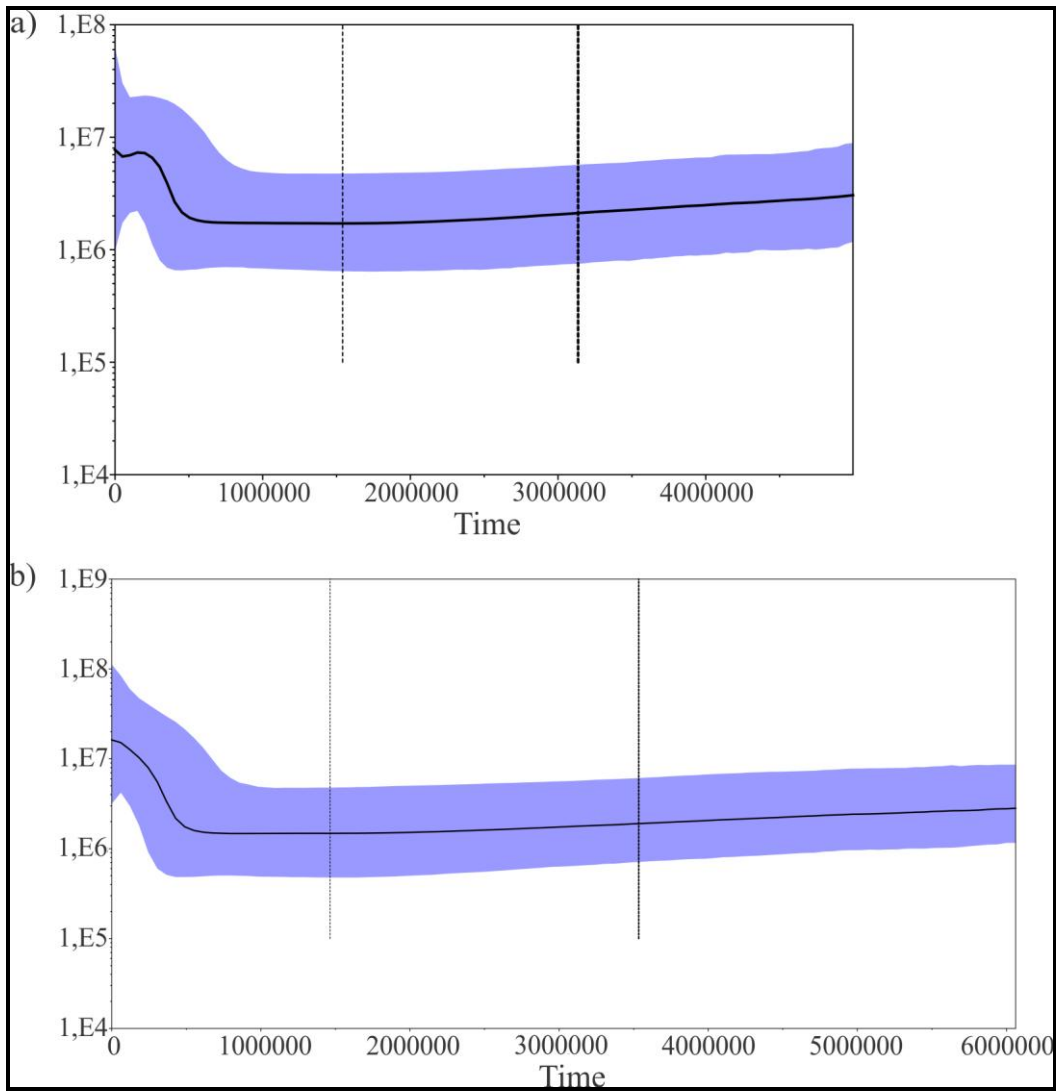
Fig. 3 Bayesian skyline reconstructions for the concatenate Cyt*b*m and COI sequences of *Melanophryniscus dorsalis* (a) and *M. montevidensis* (b). The thin and the thick vertical dotted lines are the lower and the mean estimated tMRCA, projected on the time line, and the blue areas overlay show the 95% highest posterior density (HPD) limits for the effective population size.

APPENDIX

Table S1. List of species and the Genbank accession number for the 12S-16S and cytochrome b

sequence applied to molecular clock analysis. Species groups included in the calibration points

are detached as i, ii and iii (see text).

Calibration point	Taxon	Genbank accession numbers	
		12S-16S	**Cytochrome *b***
	Melanophryniscus	DQ158421	HM159223
iii	*Cranopsis alvarius*	DQ158425	L10956
	Anaxyrus americanus	DQ158426	L10957
	Rhinella margaritifera	DQ158312	HM159224
	Rhinella granulosa	GU178788	HM159225
i	*Rhinella crucifer*	DQ158447	HM159226
	Rhinella achavali	GU178787	HM159227
	Rhinella arenarum	DQ158429	HM159228
ii	*Rhinella rubescens*	DQ158486	HM159229
	Rhinella icterica	DQ158462	HM159230
	Rhinella poeppigii	GU178779	HM159233

Rhinella jimi	GU178784	HM159234
Rhinella schneideri	DQ158480	HM159235
Rhinella marina	DQ158474	HM159236

1364

1365
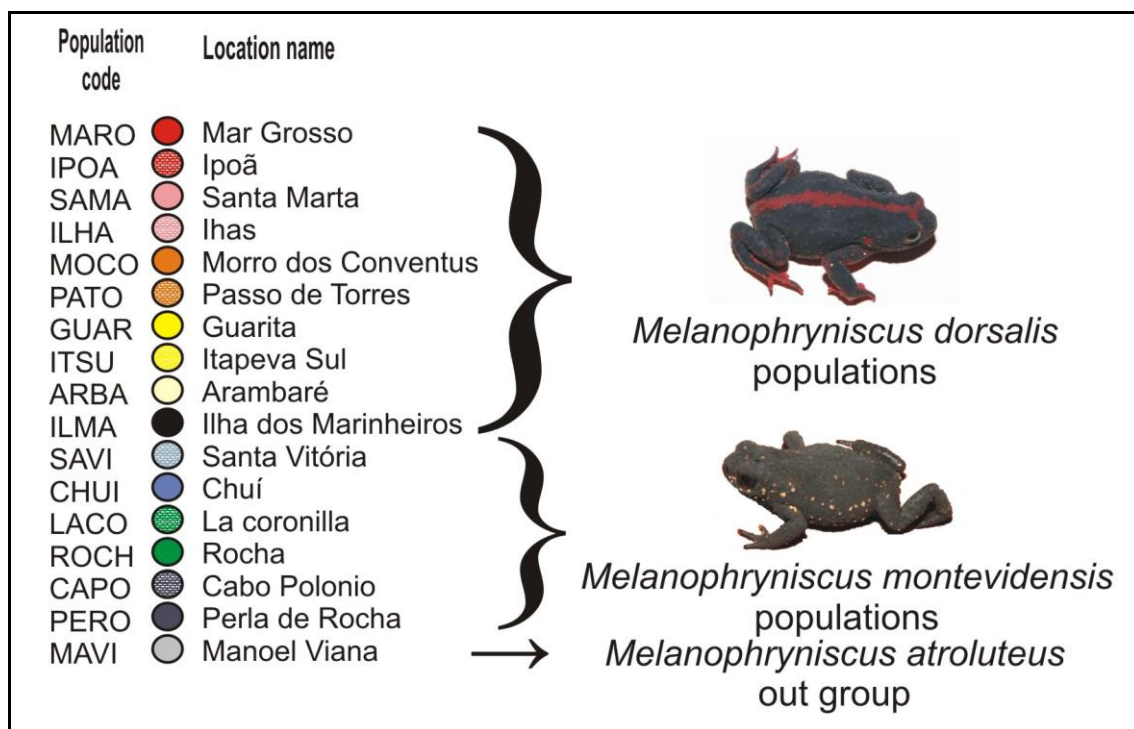
Figure S1. Species populations codes of *Melanophryniscus dorsalis*, *M. montevidensis* and *M.*

atroluteus as out group.

1369

1370

1371

4. CONSIDERAÇÕES FINAIS

Temas como especiação e até mesmo evolução ainda são tabus tanto na sociedade quando para alguns membros do meio acadêmico que se propõe a trabalhar com biodiversidade. De forma generalizada muitos de nós ainda não somos capazes de observar e compreender a natureza como um processo, como algo em constante transição. A história da obra e vida de Darwin até nosso contemporâneo Dawkins nos demonstra o quão complexo o tema é visto por muitos de nós e isso vai desde pessoas com nenhum grau de instrução até doutores em alguma área das ciências biologicas. Esse estudo, assim como diversos outros, demonstram claramente que, seja qual for à definição para espécie que adotarmos, essa definição necessariamente deverá estabelecer esse grupo de organismos como uma variável continua e não discreta assim como já observada por Butlin e colaboradores (2008).

A atuação de sistematas e taxonomistas é extremamente importante para que novos trabalhos sejam desenvolvidos a partir de informações basais quanto a táxons, entretanto o avanço do conhecimento aponta e apontará uma série de erros cometidos por especialistas que não tinham ferramentas adequadas no seu tempo para analises mais refinadas. O avanço do conhecimento e dos métodos de observarmos a natureza não desmerece trabalhos clássicos e antigos, pelo contrário, é um grande incentivo para compreender os processos de especiação e evolução em nível microevolutivo.

Nesse estudo foi abordado um caso de recente especiação com duas espécies parapátricas de sapinhos da barriga-vermelha (*Melanophryniscus dorsalis* e *M. montevidensis)*, um ótimo exemplo de especiação ocorrendo agora. A validade das espécies, como entidades biológicas separadas, não foi refutada, entretanto alguns profissionais observariam que havendo algum compartilhamento genético entre diferentes espécies, então estes organismos seriam da mesma espécie. Claramente um momento evolutivo é observado para essas duas espécies do

gênero *Melanophryniscus* com uma separação das duas espécies de um ancestral comum em torno de 3.5 mya. Ambas as espécies claramente possuem sinais de expansão populacional do tipo "*star-like*" que consiste em uma formação que demonstra expansão demográfica com um haplótipo central e vários outros proximamente associados com uma ou poucas mutações. Também foi observado que não existem evidências de baixa variabilidade genética ou gargalo de garrafa, tornando claro que a falta de variabilidade genética não é um problema para ambas as espécies.

Apesar de aspectos importantes sobre os padrões filogeográficos e de especiação ocorrendo com *M. dorslais* e *M. montevidensis* terem sido esclarecidos neste estudo, muitas questões ainda podem ser exploradas, como por exemplo, a aplicação de marcadores de microssatelites para elucidar questões de herdabilidade entre populações. Assim como um estudo mais amplo com populações de *M. atroluteus* e outras espécies do gênero para entender os padrões evolutivos do grupo.

Baseado somente nos dados moleculares uma atenção especial deve ser dada para a população denominada Mar Grosso (localidade no município de Laguna). Essa população apresentou haplótipos únicos e igualmente relacionados a ambas as espécies e segundo os critérios propostos por Moritz e colaboradores (1994) essa população deve ser classificada como uma UM (Unidade de manejo). As UM são unidades indicadas para o monitoramento e atenção especial visando a conservação.

Foram utilizados modelos de distribuição de espécies para observar a potencial área de ocorrência das espécies com dados históricos. Os modelos gerados com dados históricos nos permitiram direcionar as buscas por novos locais de ocorrência e aumentar o número de registros, principalmente para *M. dorsalis*. Os modelos se demonstraram muito úteis revelando uma série de novos pontos e confirmando outros com registros históricos.

1420 Posteriormente realizamos uma série de análises para apresentar áreas com real potencial

1421 para a conservação de ambas as espécies.

1422 O mapa final, que apresenta áreas de interesse para ambas as espécies, indica uma série de

1423 áreas de alto interesse para a conservação. Uma série de novos pontos de ocorrência é

1424 apresentada, assim como pontos de ocorrência historicamente omitidos em listas estaduais,

1425 nacional e mundial para ambas as espécies. Para este caso em específico a utilização dos

1426 modelos de nicho ecológico para tomada de decisões quanto a medidas de conservação é

1427 extremamente importante, pois nenhuma das espécies sofre com baixa variabilidade genética,

1428 sendo sua principal ameaça associada a ações antrópicas desenfreadas da ocupação e uso da

1429 faixa litorânea do sul do Brasil e Uruguai.

1430 Assim como alguns estudos sobre a dinâmica da planície costeira do Sul do Brasil sobre a biota,

1431 como os descritos para tuco-tucos por Lopes e colaboradores 2012 e para plantas por Lorenz-

1432 Lemke e colaboradores (2010), este trabalho visa aumentar o conhecimento e compreensão

1433 sobre os processos associados às espécies ocorrentes no mesmo ambiente costeiro do Sul do

1434 Brasil. A interessante e triste coincidência nessa série de estudos é observar que a maior

1435 ameaça aos organismos está associada a ações antrópicas recentes, através da especulação

1436 imobiliária na costa sul do Brasil, e especificamente neste estudo, na costa Uruguaia. Espécies

1437 de fauna e flora não sofrem de baixa variabilidade genética nessas regiões e sim de

1438 fragmentação de habitat, causado exclusivamente por ação humana.

1439 Medidas severas visando à manutenção das espécies e ecossistemas ameaçados ocorrentes

1440 nestes ambientes são necessárias. Através de estudos para diversos grupos de organismos a

1441 ação mais importante a ser feita é a preservação de áreas apontadas como de alta

1442 adequabilidade ambiental para estas espécies como apresentadas no primeiro artigo desta

1443 tese.

1444 89

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1600 **6. ANEXOS E APÊNDICES**

1601 6.1 Author Guidelines *Natureza & Conservação* (submissão do artigo I)

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