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GUILHERME WAGNER GUTIERREZ ATENCIO

STATUS DE CONSERVAÇÃO E ESTRUTURAÇÃO POPULACIONAL DE *EURYADES CORETHRUS* (LEPIDOPTERA, PAPILIONIDAE) UTILIZANDO MODELAGEM DE NICHO E MARCADORES MOLECULARES

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Tese apresentada ao Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do título de Doutor em Biologia Animal.
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Orientadora: Profª. Dra Helena Piccoli Romanowski
Coorientadora: Profª. Dra Maríndia Deprá

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BANCA EXAMINADORA

Dr. Eduardo Périco

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Dra. Vera Lúcia da Silva
Valente Gayeski

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Dra. Maria Ostília de
Oliveira Marchiori

_____

Dedico este trabalho à minha mãe, Nadja, por motivos que são numerosos demais para apenas uma folha de papel.

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"Disse a flor para o pequeno príncipe: é preciso que eu suporte duas ou três larvas se quiser conhecer as borboletas."
-Antoine de Saint-Exupéry, O Pequeno Príncipe

SUMÁRIO

RESUMO

E. corethrus é comum no sul do Brasil e em raros locais na Argentina e Uruguai, geralmente próximo da fronteira com o Brasil. Esta espécie de borboleta é comumente encontrada nos campos sulinos, porém, pouco se sabe de sua biologia. Os estudos que a incluem são na sua maioria inventários de fauna, com poucos dedicando-se a sua história de vida, relações com a planta hospedeira, filogeografia ou até mesmo requerimentos ambientais. Neste estudo foi buscada a borboleta partindo-se de registros prévios e de modelos de distribuição de espécie baseados nestes registros, que foram validados *in situ*. Neste processo, foram estimados os requerimentos ambientais para a presença da espécie e gerados dados inéditos, assim como foi investigado o estado de conservação das áreas onde é esperado que *E. corethrus* seja encontrada. Ainda, foi feita a revisão do *status* de ameaça para a espécie nos estados do Rio Grande do Sul, Santa Catarina e Paraná e também para o Brasil, sugerindo que sejam alterados para "Ameaçada" (EN) para os níveis estaduais e "Criticamente em Perigo" (CR) para o nível nacional. Dentre os animais coletados durante estas excursões em campo, 80 indivíduos de 8 localidades diferentes tiveram o marcador molecular Citocromo Oxidase I (COI) sequenciado. A análise dessas sequências mostrou que existe fluxo gênico entre as populações amostradas e não há indício de estruturação populacional entre elas. Os dados indicam que seja uma população panmítica e possivelmente um dos últimos refúgios da espécie no território Brasileiro. Este dado, juntamente com os dados ambientais coletados em campo, são essenciais para traçar estratégias de conservação e manejo desta espécie que apresenta evidências de estar sofrendo com a pressão antrópica, através de processos como perda de habitat e mortalidade devido à agrotóxicos.

Palavras-chave: conservação, perda de habitat, Lepidoptera, modelagem de nicho, agrotóxicos, COI, estruturação populacional

ABSTRACT

Papilionidae is one of the families that compose the insects commonly called butterflies, which together with moths make up the order Lepidoptera. *Euryades* [Felder, 1864] is a genus composed of two species: *Euryades corethrus* [Boisduval, 1836] and *Euryades duponchelii* [Lucas, 1839]*,* both found in southern South America, rarely coexisting. *E. corethrus* is common in southern Brazil but seldom found in some locations in Argentina and Uruguay, usually close to the Brazilian border. This butterfly species is commonly found in the southern grasslands, but little is known about its biology. Studies mostly deal with fauna inventories but few investigate its life history, host plant relationships, phylogeography or even environmental requirements. In this study we searched for the butterfly based on previous records and species distribution models based on these records, which were validated *in situ*. In this process, the environmental requirements for the presence of the species were estimated and previously unavailable data was generated. Furthermore, a review of the threat status for the species in the states of Rio Grande do Sul, Santa Catarina and Paraná and also Brazil, suggesting they be changes to "Endangered" (EN) for the state level and "Critically Endangered" (CR) for the national level. From the animals collected during this field work, 80 individuals of 8 different locations had the molecular marker Cytochrome Oxidase I (COI) sequenced. Analyses of those sequences showed that there is gene flow between those populations and therefore there is no population structure among them. There is evidence that this is a panmitic population and one of the last havens for the species in the Brazilian territory.. This data, together with the environmental data collected in the field, is essential to outline conservation and management strategies for this species which shows evidence of suffering from anthropogenic pressure, such as habitat loss and mortality due to pesticides.

Keywords: conservation, habitat loss, Lepidoptera, niche modeling, pesticides, COI, population structure

INTRODUÇÃO

Lepidoptera [Linnaeus, 1758] é uma ordem muito diversa, com aproximadamente 157.000 espécies descritas (Van Nieukerken et al. 2011), o que equivale à cerca de 10% das espécies de animais descritas até hoje. São animais que ocupam praticamente todo o planeta, com exceção da Antártica. Dentro da ordem, temos a divisão popular em Mariposas e Borboletas, com as primeiras tendo cerca de 139.000 espécies descritas, enquanto as últimas têm cerca de 18.000 espécies descritas (Van Nieukerken et al. 2011). Uma das famílias mais facilmente reconhecidas entre as borboletas é a Papilionidae (também chamada de *swallowtail,* ou "rabo-de-andorinha"), que possui em torno de 550 espécies (Scriber et al. 1995), sendo em sua maioria animais grandes e coloridos, tanto na fase larval quanto na fase adulta alada (Figura 1).

Figura 1: Exemplos de lagarta (*Papilio machaon,* Fonte: https://pxhere.com/en/photo/1248822 - domínio público) e adulto de Papilionidae (*Battus philenor*, Fonte: https://www.publicdomainpictures.net/en/view-image.php?image=105192&picture=swallowtail-butterfly - domínio público)

A família Papilionidae é dividida em 27 gêneros distribuídas em todos os continentes, sendo comuns em climas tropicais e temperados. A maior diversidade do grupo, assim como os maiores representantes em envergadura (Tribo Troidini [Talbot, 1939], gênero *Troides*), estão na Ásia. Dentre as tribos de borboletas, Troidini (Papilionidae) merece especial atenção. As espécies desta tribo são predominantemente tropicais, com as regiões da América do Sul e Indo-australianas como centros de distribuição (Condamine et al. 2013). São cerca de 135

espécies em 12 gêneros, ocupando a região Neotropical, Ásia e Oceania. Os Troidini do gênero *Euryades* são divididos em duas espécies: *Euryades corethrus* Boisduval [1863] e *Euryades duponchelii* Lucas [1839] (Lamas 2004), encontrados coletivamente nas regiões sul do Brasil e nos países vizinhos (Bustos 2010). Segundo Tyler e colaboradores (1994), a distribuição destas espécies seria muito similar (Figura 2).
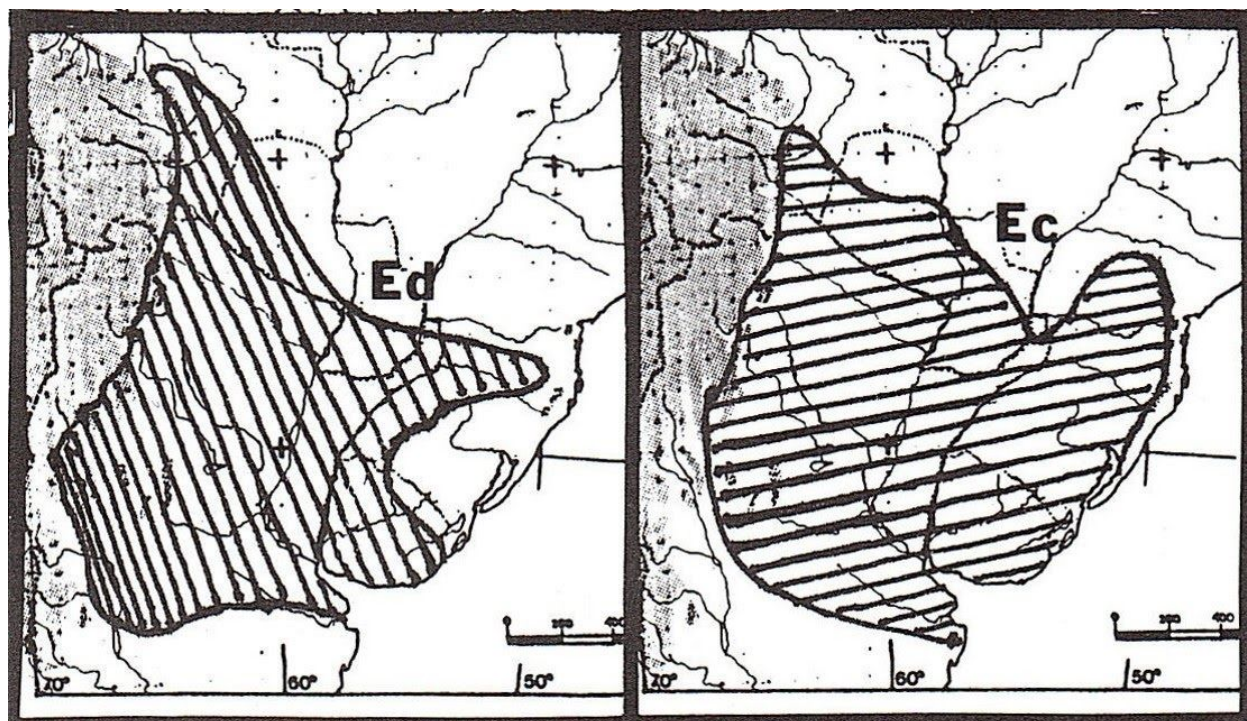

Figura 2: reprodução da figura de Tyler et al. (1994) com a distribuição proposta para *Euryades.* À esquerda, *Euryades duponchelii* (Ed) e à direita *Euryades corethrus* (Ec).

Para *E. corethrus*, abrangeria toda a região sul do Brasil e incluiria os países vizinhos (Argentina, Paraguai e Uruguai). No caso de *E. duponchelii,* não incluiria o Paraná e a área no Rio Grande do Sul seria um pouco menor, mas a sobreposição entre as duas é extensa. No entanto, Atencio (2014), partindo de dados já compilados e atualizados após uma ampla coleta dos dados disponíveis na literatura, em bancos de dados on-line e também com observações obtidas em campo, apontou para uma área de ocorrência que discorda dessa proposta, demonstrando que os pontos de ocorrência conhecidos para *E. corethrus* estão principalmente no Brasil, além de alguns pontos dispersos na fronteira entre Argentina e Uruguai (ao longo do rio Uruguai) e no sul do Paraguai, enquanto *E. duponchelii* não tem registros para o Brasil, sendo encontrada apenas no Uruguai Argentina, e Paraguai. (Figura 3).
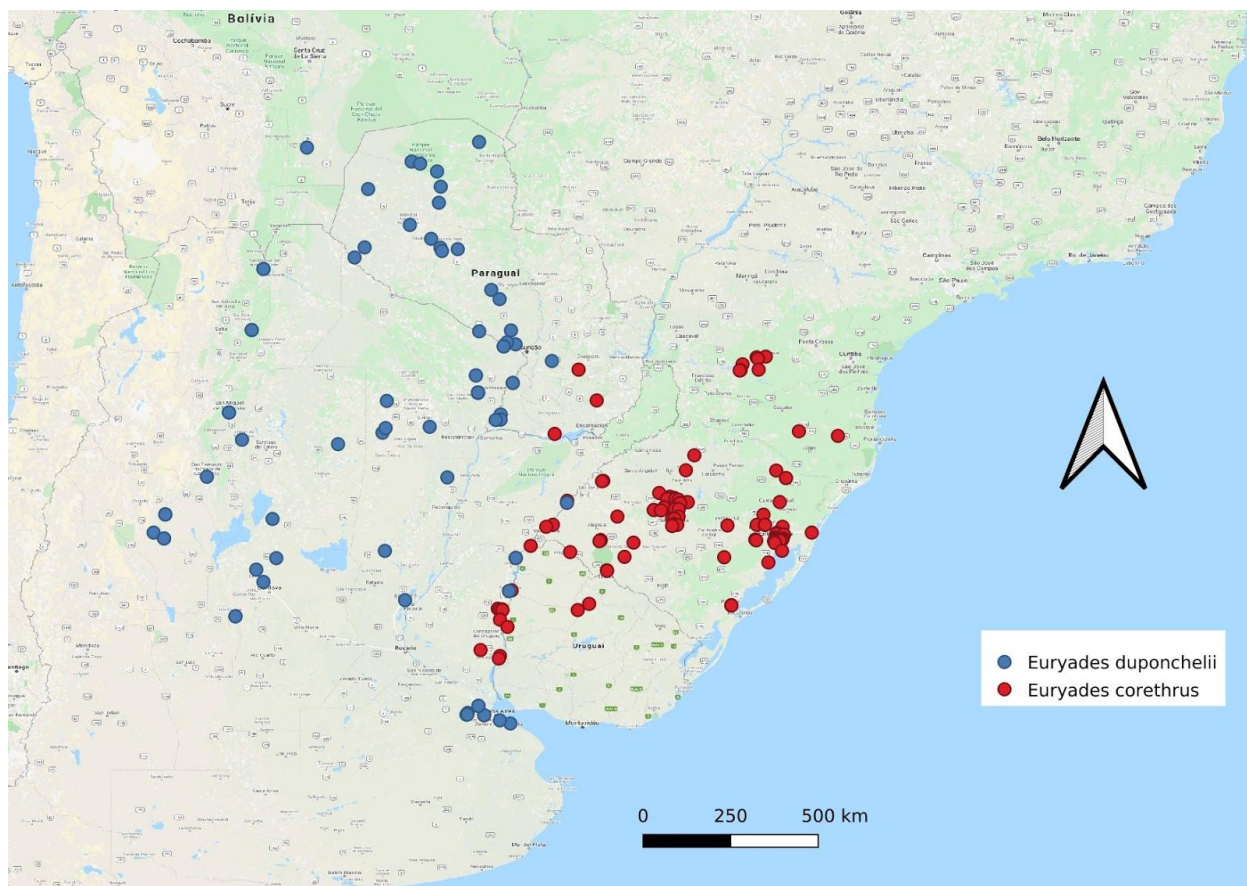
Figura 3: Pontos de ocorrência para *E. corethrus* (em vermelho) e para *E. duponchelii* (azul) demonstrando a baixa sobreposição entre as duas espécies..

    E. corethrus costumava ser abundante nos campos nativos contidos em sua área de distribuição (Tyler et al. 1994). No entanto, atualmente consta na lista vermelha de espécies ameaçadas de extinção para os estados do Rio Grande do Sul, Santa Catarina e Paraná, respectivamente nas categorias Vulnerável (VU), Ameaçada (EN) e Ameaçada (EN), segundo os critérios da IUCN. (Instituto Ambiental do Paraná 2007; Santa Catarina 2011; Rio Grande do Sul 2014).

    O gênero *Euryades* é geralmente associado a plantas do gênero *Aristolochia*. As plantas deste gênero possuem compostos tóxicos que são sequestrados pelas borboletas, tornando-as impalatáveis para os predadores (Pinheiro 1996). Atencio (2014) realizou modelagem preditiva da distribuição de *E. corethrus, E. duponchelii* e de cinco espécies de *Aristolochia* (*A. sessilifolia, A. lingua, A. angustifolia, A. labiata* e *A. fimbriata*), plantas

mencionadas como hospedeiras durante o estágio larval da borboleta (Biezanko et al. 1974; Klitzke e Brown 2000; Beccaloni et al. 2008; Volkmann e Núñez-Bustos 2010). Embora tenham sido encontradas sobreposições entre os modelos da borboleta e da planta hospedeira, a análise da sobreposição entre estes modelos não demonstrou relação entre a ocorrência da borboleta com uma espécie particular de *Aristolochia.* No entanto, os dados e modelos resultantes foram o ponto de partida deste projeto de doutorado, uma vez que os modelos não foram validados em campo e o conhecimento da genética da espécie era ainda incipiente. Considerando que nos últimos anos as áreas de campo sofreram degradação severa, a área de ocorrência real é provavelmente muito menor que a área sugerida pelos modelos, já que eles levam em conta apenas a adequabilidade climática. Dito isso, é esperado a perda de habitat inclua áreas onde *E. corethrus* já foi encontrada. Como exemplo, as áreas com formação de savana estépica (uma das regiões fitoecológicas com ocorrência de *E. corethrus*) foram praticamente eliminadas da região do Planalto das Missões, restando apenas alguns fragmentos (Cordeiro e Hasenack 2009), evidenciando o tipo de degradação sofrida pelos ambientes campestres. Embora seja uma espécie comum de ser encontrada, abundante em campos nativos e aparentemente resiliente às variações de temperatura e umidade, as pesquisas que tratam de *E. corethrus* são relativamente raras: principalmente inventários ou novas ocorrências (Dolibaina et al. 2011), com poucas abordando a biologia desta borboleta (Caporale et al. 2017 e Costa 2016).

Os campos estão entre os ecossistemas com maior riqueza de espécies de plantas do mundo, em algumas escalas até mais ricos que as áreas florestais (Wilson et al. 2012). Campos também estão envolvidos em vários serviços ecológicos, entre eles o gerenciamento de recursos hídricos e o sequestro de carbono do solo (Pillar e Vélez 2010). Além de sua notável relevância ecológica, os campos também são importantes para o bem-estar humano, pois, entre outras coisas, servem de base para a criação de gado e são facilmente convertidos em terras cultiváveis, além de sua beleza cênica (Pillar e Vélez 2010). Um projeto do PNUMA (Programa das Nações Unidas para o Meio Ambiente) chamado GLASOD (Avaliação Global da Degradação do Solo Induzida pelo Homem) mapeou a degradação de áreas com cobertura vegetal no mundo e apontou que 15% dessas áreas sofriam alguma forma de degradação antrópica. Se estivermos considerando áreas de cultivo, um relatório da FAO (Organização de Alimentos e Agricultura) aponta que, em escala global, 33% dessas áreas sofrem degradação entre moderada e grave. Nos três estados do sul do Brasil (Rio Grande do Sul, Santa Catarina e Paraná), que costumavam ter vastas áreas de pastagem (aproximadamente 218.000 km^2),

esse percentual hoje em dia é reduzido para cerca de 43% da área original (95.519 km^2) (Vélez-Martin et al. 2015). Esses remanescentes são distribuídos no bioma Pampa (Rio Grande do Sul) e em mosaicos com a Mata Atlântica (do planalto sul do Rio Grande do Sul ao Paraná) em suas partes de maior altitude. Apesar de uma clara distinção florística entre os campos do bioma Pampa e os campos do bioma da Mata Atlântica, a ocorrência de espécies simultaneamente nessas duas áreas (incluindo algumas das mais abundantes) nos permite chamar a formação campestre como um todo de "Campos do Sul" (Overbeck et al. 2015).

No Rio Grande do Sul, o estado mais ao sul do Brasil, as formações de campos e florestas têm algum grau de alteração em comparação com suas características originais (Cordeiro e Hasenack 2009). Usando imagens de satélite e posteriormente validação *in situ* para gerar mapas de cobertura e uso do solo, Cordeiro e Hasenack (2009) sugerem que a cobertura vegetal natural ou semi-natural do Rio Grande do Sul corresponde a 31,38% de sua área total. Desses, 62,21% correspondem a formações de campo. No entanto, para essa estimativa, os autores consideraram "campo" aquelas regiões cujo uso manteve os aspectos fisionômicos semelhantes às condições originais, incluindo campos nativos com extensas criações de gado (classificadas nesses casos como "cobertura semi-natural"). Por outro lado, campos intocados e bem preservados são escassos e estão diminuindo com o passar do tempo. Segundo Overbeck (2007), a taxa de conversão de área natural em área cultivada foi de aproximadamente 1.000 km^2 por ano entre 1976 e 2002. Entre 2002 e 2016, a área cultivada com soja no Brasil aumentou 79%, atingindo uma área de 33 milhões de hectares, aproximadamente 3.8% da área total do Brasil. Se adicionarmos a soja às áreas utilizadas para o cultivo de eucalipto e cana-de-açúcar, alcançamos uma área 48 milhões de hectares (5.6% do país), evidenciando as vastas áreas ocupadas por essas monoculturas (Bombardi 2017).

Analisando a localização e fitofisionomias das 79 unidades de conservação do Rio Grande do Sul, observa-se que apenas 25 estão oficialmente dentro da área considerada como Bioma Pampa e, em geral, protegendo áreas de mata. O favorecimento das áreas de mata em detrimento das áreas de campo fica ainda mais evidente quando consideramos que cerca de 0,5% dos remanescentes de pastagens estão dentro de áreas de proteção sem uso direto do solo e, principalmente, em mosaico (Overbeck et al. 2007). Essa falta de proteção legal deixa as áreas vulneráveis às pressões das atividades agrícolas e, portanto, as populações de plantas e animais dessas áreas também são deixadas em uma posição vulnerável.

Ainda, segundo dados de Bombardi (2017), o aumento da produção de grãos (principalmente soja) no período de 2002 e 2016, foi devido a um aumento na área, não na

produtividade. Em um período similar (2000-2015), o uso de agrotóxicos aumentou 135%, além dos 382 novos que foram liberados em 2019. Somados, esses dois fatores podem ter profundo impacto no meio ambiente, mesmo quando os remanescentes de campo não são convertidos em terras cultivadas, pois o efeito dos agrotóxicos não se limita à área de destino. Todas as comunidades vizinhas são afetadas, tanto animais quanto plantas.

Levando em conta a perda, fragmentação e contaminação de habitat, juntamente com a escassez de estudos envolvendo Lepidoptera, investigar estas populações é de suma importância. Além da importância de preservação da biodiversidade, estes animais têm também papéis cruciais: são polinizadores, além de servirem como indicadores de qualidade ambiental, uma vez que tem ciclo de vida relativamente curto, alguns com várias gerações ao longo do ano (Brown and Freitas 2000; Swaay et al. 2006; Fleishman and Murphy 2009) Desvendar os mecanismos, características e dinâmicas populacionais e de dispersão destes animais é indispensável para planejamento de estratégias de conservação e previsão de flutuações populacionais. As populações são governadas pela dispersão, que determina sua dinâmica e evolução. A coesão genética de uma espécie qualquer no espaço em que ela ocupa, sua persistência global apesar da extinção local e sua capacidade de acompanhar mudanças ambientais são dependentes de sua capacidade de dispersão (Ronce 2007). Se a população não é estruturada, a extinção local vai ter mitigado o efeito na população em geral, pois ela é mais homogênea e aqueles indivíduos podem ser repostos pela dispersão de indivíduos de outras áreas. Há muitas razões para a migração sazonal: evitar predação ou competição, expandir o habitat, explorar novos recursos, abandonar uma área degradada, procurar parceiros, etc. A migração não é um evento que ocorre sem consequências: deve haver uma vantagem reprodutiva em relação à permanência no habitat já ocupado (Southwood 1977). Seja qual for o motivo, entender esses processos e mecanismos é crucial para a conservação e para uma compreensão geral da biologia das espécies. Em uma revisão, Leibold et al. (2004) exemplificam todas as maneiras pelas quais a dispersão influencia a dinâmica e a evolução da comunidade e, portanto, como é essencial que os esforços de gerenciamento e restauração compreendam completamente esses processos. Exemplos disso podem ser encontrados para plantas, com a quase extinta *Centaurea corymbosa* (Colas et al. 1997), e em animais, com a borboleta *Euphydryas aurinia* (Junker e Schmitt 2010).

Portanto, os objetivos deste trabalho são: validar *in situ* os modelos previamente obtidos por modelagem de distribuição de espécies e usá-los como referência para a seleção de áreas de busca pelas borboletas. Essas áreas, por sua vez, serão utilizadas para melhorar a

modelagem de nicho usando os novos dados de ocorrência coletados durante as saídas de campo. O estado de conservação das áreas visitadas assim como de *E. corethrus* e de seus hábitats também será avaliado. Além disso, será avaliada a estruturação populacional e diversidade genética de populações de *E. corethrus* do sul do Brasil. A partir desses dados, esperamos estimar quais são os requisitos determinantes para a presença das espécies, sugerir estratégias de conservação e reavaliar o *status* de ameaça da espécie em nível regional e nacional.

Estrutura da tese

Nesta tese trazemos novos dados sobre a distribuição de *Euryades corethrus,* sobre as áreas de campo associadas à espécie e também novos dados moleculares da espécie, separados em dois capítulos. No primeiro capítulo atualizamos os modelos de distribuição para a espécie e avaliamos *in situ* os modelos gerados com os novos dados de ocorrência, verificando a presença da borboleta nas áreas com alta adequabilidade calculada. Além disso, avaliamos o estado de conservação das áreas de campo que têm alta adequabilidade prevista por estes mesmos modelos e estimamos quais os requerimentos ambientais para a presença da borboleta. O segundo capítulo faz um estudo molecular da espécie. Através da sequência parcial do marcador molecular Citocromo Oxidase I (COI), analisamos a estrutura populacional e história demográfica de populações de *E. corethrus.* Testes como a Análise de variância molecular (AMOVA) e Análise espacial da variância molecular (SAMOVA) foram empregados. A avaliação de estrutura populacional dessa espécie visa entender a dinâmica e os processos atuantes sobre os indivíduos e permite o planejamento de estratégias de preservação e recuperação.

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G.W.G. Atencio[1] (iD) **M. Deprá [2]** (iD) **H.P. Romanoswki[1]** (iD)

[1]Zoology Department, Federal University of Rio Grande do Sul - Av. Bento Gonçalves, 9500, Porto Alegre RS – Brasil

[2]Genetics Department, Federal University of Rio Grande do Sul - Av. Bento Gonçalves, 9500, Porto Alegre RS – Brasil

Corresponding author:
Guilherme Wagner Gutierrez Atencio, Department of
Zoology, Federal University of Rio Grande do
Sul, Av. Bento Gonçalves 9500/43435/218,
Porto Alegre, RS 91501-970, Brazil. ☐guilherme.atencio@outlook.com

Euryades corethrus: a grassland butterfly threatened by habitat loss and climate change

Abstract

Lepidoptera are highly dependent on their host plants in their life cycles, and therefore sensitive to habitat loss and pesticides. That is particularly true when we are dealing with specialist species like *Euryades corethrus*, that consume just a few species during their larval phase. Conservation and preservation efforts can not overlook climate chang, since climate is the main determining factor in vegetation distribution, but habitat loss due to conversion is still the highest impact factor for biodiversity loss. Grasslands are among the ecosystems with the highest species richness, in some cases highest that even forest areas. In the south of Brazil, grasslands are reduced to 43% of their original area, with only 0.5% of those remnants inside conservation units. Available data usually deals with the complete loss of biotypes, but subtle changes in structure, composition, and biological processes also cause degradation, possibly masking already degraded locales. Here, we validated *in situ* species distribution models for *E. corethrus,* searching for the butterfly in areas with high suitability, and evaluating the state of

conservation of the sampled areas and also the areas considered remnants. We found *E. cirethrus* in only 22.5% of the sampled areas, always in small patches, mosaics or road borders. With this new data, we updated the expected distribution for this species and suggest that the category should be changed to "Endangered" (EN) for the southern states of Brazil (Rio Grande do Sul, Santa Catarina and Paraná).

keywords: conservation, environmental requirements, habitat conversion, Lepidoptera, niche modeling, pesticides

Introduction

Discussion about species conservation and strategies for their preservation have been increasingly taking climate change into account. This is due to the fact that the natural vegetation distribution is mainly determined by climate (Eva et al. 2004). On the other hand, when we consider all biomes, habitat conversion is still the factor with the biggest impact on biodiversity until 2100, mainly due to habitat loss (Sala et al. 2000; Pardini et al. 2017). As Andrade (2015) points out, data on degradation is usually available only for complete biotope loss, but the process of degradation included much subtler changes in structure, composition in ecological processes within the ecosystem. At times, an area that is seemingly conserved might hide changes that hinder or even completely prevent the occurrence of ecological processes that are essential to the maintenance of biodiversity (Haddad et al. 2015; Pflüger et al. 2019).

Grassfields are among the ecosystems with the highest species richness in the world, on some scales even richer than forest areas (Wilson et al. 2012). Grassfields are also involved in various ecological services, among them being hydric resources management and soil carbon sequestration (Pillar and Vélez 2010). Besides their outstanding ecological relevance, grassfields are also very important to human well-being, for, among other things, serving as the basis for cattle grazing and ease of conversion to cropland, not to mention scenic beauty (Pillar and Vélez 2010). A UNEP (United Nations Environment Programme) project called GLASOD (Global Assessment of Human-induced Soil Degradation) mapped the degradation of areas with vegetation cover in the world and pointed out that 15% of those areas suffered some form of antropic degradation. If we are considering cultivation areas, an FAO (Food and Agriculture Organization) report points out that, on a global scale, 33% of those areas are suffering from moderate to severe degradation. On the three southern states of Brazil (Rio Grande do Sul,

Santa Catarina and Paraná), which used to have vast grassland areas, (approximately 218.000 km^2), this percentual is nowadays reduced to about 43% of the original area (95.519 km^2) (Vélez-Martin et al. 2015). Those remnants are distributed at the Pampa biome (Rio Grande do Sul) and in mosaics with the Atlantic forest (from the southern plateau of Rio Grande do Sul to Paraná) in its parts with higher altitudes. Despite a clear floristic distinction between the Pampa biome grassfields and the Atlantic forest biome fields, the occurrence of many species in both of those areas (including some of the most abundant ones) allows us to call the region as a whole as "Southern Fields" (Overbeck et al. 2015).

In Rio Grande do Sul, the southernmost state of Brazil, both grassland formations and forest formations have some degree of alteration compared to their original characteristics (Cordeiro and Hasenack 2009). Using satellite images and later *in situ* validation to generate maps of soil coverage and usage, Cordeiro and Hasenack (2009) suggest that the natural or semi natural vegetation cover of Rio Grande do Sul corresponds to 31.38% of its total area. Of those, 62.21% correspond to grassland formations. However, for this estimate, the authors considered as "grassland" those whose usage has maintained the physiognomic aspects similar to the original condition, thus including native grasslands with extensive cattle raising (classified in those cases as "semi natural coverage"). On the other hand, actually well-preserved, pristine grasslands are scarce and are diminishing as time passes. According to Overbeck (2007), the conversion rate from natural area to cropland was approximately 1,000km2 per year between 1976 and 2002. Between 2002 and 2016 the area used to grow soy in Brazil increased 79%, reaching an area 11 times larger than Belgium. If we add to soy the areas used for growing eucalyptus and sugarcane, we reach an area 16 times larger than Belgium, highlighting the vast areas occupied by those monocultures (Bombardi 2017).

Adding to that, around 0.5% of the grassland remnants are inside protection areas without direct soil usage, and mostly in patches (Overbeck et al. 2007). This lack of legal protection leaves those areas vulnerable to the pressures of agricultural activities and, therefore, the plant and animal populations of those areas are also left in a vulnerable position. According to data from Bombardi (2017), the increase in grain production (mainly soybeans) is due to an increase in area, not productivity. This also may be implied in the increase in pesticides, which in a similar time frame (2000-2015) increased 135%. We can't ignore the impact the introduction of those products might cause in the environment, even when the remnants are not converted to cropland, since their effect is not limited to the target area. All surrounding communities are affected, animals as well as plants.

The butterflies from the tribe Troidini (Papilionidae) are oligophagous, feeding on just a few species of Aristolochiaceae (Scriber 1984; Weintraub 1995). The Lepidoptera taxocenoses (butterflies and moths), that rely on host plants during their life cycle, possibly suffer a huge impact from both habitat loss and pesticides. Furthermore, butterfly occurrence is related to host plant abundance (Brown Jr et al. 1981). Species in this tribe are predominantly tropical, with South America and Indo-Australian regions as the distribution centers (Condamine et al. 2013). The Troidini on the genus *Euryades* are divided into two species: *Euryades corethrus* Boisduval [1863] and *Euryades duponchelii* Lucas [1839] (Lamas 2004), collectively found in the southern regions of Brazil and neighbouring countries (Nuñez-Bustos 2010). According to Tyler (1994), the distribution of both of those two species would span the entire southern region of Brazil and include the neighboring countries (Argentina, Paraguay and Uruguay). However, Atencio (2014), in a widespread data collection from literature, online databases and entomological collection, points to an occurrence area that disagree with that proposal, demonstrating that the known occurrence points for *E. corethrus* are mainly in Brazil, plus a few scattered points on the border between Argentina and Uruguay (along the Uruguay river) and in the south of Paraguay (Figure 1). This indicates the occurrence area might be much smaller.

E. corethrus host plants species are common in the south of Brazil. During the larval stage, it feeds on plants of the genus *Aristolochia* and during the adult stage, it utilizes as a source of nectar, plants typically found in southern native grasslands, such as *Senecio* and *Eupatorium* (Costa 2016). Field observations indicate that *E. corethrus* has a strong association to *A. sessilifolia* during the larval stages. In fact, in every instance where a larvae was found, it was feeding on this particular species. This butterfly species used to be abundant on the native grassfields contained in its distribution area (Tyler et al. 1994), however, nowadays *E. corethrus* is listed in the IUCN Red list of endangered species for the states of Rio Grande do Sul, Santa Catarina and Paraná, in the categories of vulnerable (VU), Endangered (EN) and Endangered (EN), respectively. (Instituto Ambiental do Paraná 2007; Santa Catarina 2011; Rio Grande do Sul 2014). Although it is a species that used to be commonly found, abundant in native grassfields and apparently rugged, research dealing with *E. corethrus* is relatively rare: mainly inventories or new occurrences (Dolibaina et al. 2011), with just a few addressing the biology of this butterfly (Caporale et al. 2017).

Atencio (2014) performed predictive modelling of the distribution of *E. corethrus* and five species of *Aristolochia,* plants which are mentioned as host during the butterfly's larval stage (Biezanko et al. 1974; Klitzke and Brown 2000; Beccaloni et al. 2008; Volkmann and

Núñez-Bustos 2010). Although an overlap was found between the butterfly and the host plant, the analysis could not find a direct relation between the presence of a particular plant and the butterfly. Two constraints might have contributed to that result: (i) the occurrence records were not well-distributed throughout the species occurrence extent, and mostly very old and (ii) the predictive models were not validated in the field (*in situ)*. Considering that in recent years the grasslands have gone through severe degradation, the actual occurrence area is probably much smaller than the one in the models and areas that used to have *E. corethrus* populations possibly have suffered from habitat loss. As an example, areas with Steppic Savanna formation (one of the phytoecological regions with occurrences of *E. corethrus*) were practically eliminated from the Missions Plateau region, with only a few fragments left (Cordeiro and Hasenack 2009).

Therefore, the objectives of this work are: validate *in situ* the models previously obtained as well as use them as a guide for the areas to be evaluated, improve the niche modeling using the new occurrence data gathered during those field excursion e evaluate the conservation status of the surveyed areas. From this data we hope to estimate what are the determining requirements for the presence of the species, suggest conservation strategies and reevaluate the status in the IUCN Red list.

Materials and methods

Potential species distribution modeling

Aiming to identify remaining populations of the target species, we built models for the potential distribution of the species, using environmental information from the known occurrence points. Starting with the data gathered by Atencio (2014), we added new points from literature (Dolibaina et al. 2011; Viglione 2011) and online databases like PyBio (Drechsel 2014) and Ecoregistros (Ecoregistros), as well as checking if the previously used databases had anything new (GBIF and SpeciesLink (CRIA 2018). Climate variable data were obtained from BIOCLIM 2.0 (Fick and Hijmans 2017), while digital elevation data was SRTM 90 m versão 4.1 (Jarvis et

al. 2008), all with a spatial resolution of 2.5 minutes. As for variable selection, we are in agreement to De Marco and Nóbrega (2018): the best practice is to include all possibly relevant variables in a PCA and use a set of PCA-derived variables instead of selecting among the original variables. This was achieved using the software R (R Core Team 2018) through the function rasterPCA from the package *RSTools* and following two simple criteria: retain the axis with latent root higher than one (i.e. the Kaiser-Guttman criteria) and retain the set of components that explained at least 95% of the total variance (i.e. fixed cumulative eigenvalue criteria). The the PCA-derived variables were then written back to GeoTIFF (.tif) files using the function writeRaster. This allowed us to feed those variables to the package SDDM (Schmitt et al. 2017) in order to produce the ensemble models. The following algorithms were used, with default settings: general additive models (GAM), generalized linear models (GLM), multivariate adaptive regression splines (MARS), classification tree analysis (CTA), generalized boosted models (GBM), maximum entropy (Maxent), artificial neural networks (ANN), random forests (RF) and support vector machines (SVM). After *in situ* validation, the models were redone with the new data gathered on our surveys. For better visualization, we cropped the resulting ensemble model using the estimated native grassland remnants (Weber et al. 2016) as a mask.

Determining areas for *in situ* evaluation of the models

Our aim, besides model validation, was to increase the breadth of the occurrence data with locales with no previous data points and also verify if locales with previous record of the butterfly still had population present. We had the following criteria when selecting the areas in which we would look for *E. corethrus*: (i) areas with high habitat suitability predicted by the models generated with the updated data, (ii) spatial dispersion, (iii) previous record of the species, (iv) areas with native grassland remnants identified by satellite images (Cordeiro and Hasenack 2009; Weber et al. 2016), (v) when possible, locales inside or near a conservation area. Therefore, we established that six field trips would be done: 1. **West** (far West of RS), 2. **Northeast** (northeast and center-west of RS), 3. **North** (north of RS, SC and PR), 4. **Center** (center of RS), 5. **South** (south of RS) and 6.**Uruguay and Argentina** (northeast of Uruguay and northeast of Argentina) (Table A1 in the Supplementary Material).

Field work

The expeditions were always scheduled between November and April, months with higher activity and abundance of adults (Link et al. 1977; Costa 2016). Starting with the determination of the search area, **target locales** were chosen, following the criteria defined in the previous section. Also, a general route was established for each trip. Apart from that, we aimed to comprise the largest possible area within the calculated area of occurrence in order to sample individuals from different populations. Along the main route, we kept searching for locales that seemed minimally perturbed by anthropic activities and potentially a habitat for the butterfly. If that was the case, we would search for the butterfly as well as the host plant (opportunity locales) and we evaluated the conditions of the plant coverage, as well as the general condition of the surroundings. Locations were only sampled once, since multiple visits were not feasible given the timeframe and also the money and manpower needed to perform multiple visits to sites so far away from each other. Apart from the logistical hurdles, finding the butterflies was only part of the goal, and therefore we deemed that inspecting a wider area and evaluating more places considered "preserved" was more important.

The search for butterflies in the field was always performed between 8AM and 5PM, by at least 2 people with entomological nets, who would survey the potential area, not only looking for adults but also for the host plants that might be used by the larvae as well as plants that could be used by the adults. The team would walk all over the area, looking for adults either in flight or feeding, and whenever one was found, the coordinates were marked as a new occurrence point we considered the area had the presence of the butterfly. We focused on grassland areas, since this is the prefered habitat of the species. We also chose days when the weather forecast was favorable, since rainy days make the sighting and capture of lepidoptera much harder. If possible, we captured up to least 20 specimens in each area where *E. corethrus* was found, and we did that because of our plans of investigating the population structure of the butterfly. Therefore the collected specimens were deposited at the Lepidoptera Collection of the Zoology Department of the Federal University of Rio Grande do Sul (CLDZ).

Occurrence condition analysis

We evaluated the percentage of occurrences registered at the target locales in relation to their selection criteria (high habitat suitability, previous records or grassland remnants identified by satellite) and the situation of the area (presence or absence of grassland and habitat quality) and the surrounding area (vegetation, soil usage and level of anthropic perturbance, etc.).

Results

In total, we spent 29 days in the field and traveled more than 10.000km (Figure 2). The route included 34 target locales in 21 cities in three different Brazilian states along with two target locales in Uruguay and four in Argentina. To those, we added 29 opportunity locales, chosen along the route, due to the apparent quality of the habitat in regard to *E. corethrus*. Out of the six trips, the butterfly was found in only three, in 13 out of the 63 locales where it was sought (Figures 4 and 5 and Table A3 in the Supplementary Material). In 77.5% of the occasions, we could not find the butterfly in the selected target locales. In 15 cases, we found a grassland with apparent good quality, but the butterfly was not found. On 11 of those occasions, the areas were totally surrounded by monocultures of either soy or rice. Out of the 14 locales where we could find *E. corethrus,* six were small fragments of grassland where land use was not possible either due to terrain limitations (rocky, too steep, etc.) or legal limitations (road borders or conservation units). Most locales did not have a high abundance of specimens. In only two occasions it was a private property where the owner employed management techniques, such as fllowing or crop rotation. We observed that grasslands seemed to be better conserved in the Central and West regions. On the other hand, the North and South areas (especially Paraná) are practically devoid of areas that seem suitable as a habitat for *E. corethrus*. The update to our data added 89 new occurrence points, 75 of those from literature and 14 from our field work.

In total, the occurrence points went from 154 to 243.

The models generated have good predictive power, with the ensemble having an AUC of 0.94 and Kappa of 0.827 (see Figure A1 and Table A2 in Supplementary Material for individual model evaluations). The calculated distribution area for *E. corethrus* is in figure 2, along with the locales. In contrast to what we observed in the field, the area with high suitability is larger than the one in previous models (Atencio 2014). The area in green (high suitability) extended further West, towards Paraguay, near the border with Brazil and Argentina, while also filling the gaps in the northern parts of the area (Figure 4). We must remember this is in regard to potential environmental suitability. Not all of it is grassland, therefore, considering the available data from Pampa remnants (Weber et al. 2016), the main biome occupied by *E. corethrus*, the potential occurrence area shrinks to just 23% of the original area. Overlaying this with the points of occurrence, the area draws a polygon of approximately 45.000 km^2, about 16% of the area of the state of Rio Grande do Sul.

Discussion

Euryades corethrus is associated with savannas and grassland areas in Brazil, Uruguay and Argentina near Buenos Aires and the frontier with Uruguay. This particular butterfly used to be very common in Buenos Aires and the surrounding area (Nunẽz-Bustos, 2010), but sightings are getting rarer (EcoRegistros, 2019). They are butterflies associated with the Pampa and Chaco biomes. It seems to be abundant as long as the host plant is present, and the presence of humans does not seem to affect it too much, since it is found in small patches inside big city centers. On the other hand, the anthropic pressure through habitat loss seems to be the biggest threat to the species. The update to the records added well-distributed points of occurrence. The resulting models did not differ much from the ones obtained by Atencio (2014) in regard to latitude, with the Northern and Southern limits practically unchanged. An increase in the distribution area was expected, since the points cover a greater range, and that in fact was the case. The predictive models, however, only take into account climatic characteristics of those areas, since we chose not to include soil usage or conservation status when in those analises. This, the models are just an indication of the possibility of the presence of the butterfly, hence the need for *in situ* validation and ponderation before applying the results. In our experience, butterflies were not only found areas with natural grassland, but also botanical gardens, parks,

squares in urban centers, areas next to croplands, grazing areas and small strips of land right next to paved roads. Those strips of land, just 15 meters wide, are known to be important to species conservation, and have great potential for grassland restoration (Fernandes et al. 2018). It is extremely worrying that the species was found in only 22.5% of the surveyed locales, even after we drove for 10.000km. Those areas, beside having high suitability according to the models, should be native grassland. If those populations are shrinking or vanishing from locales inside the occurrence area, in areas that are supposed to be conserved or recovered to its natural state, then the host plants probably aren't there either. If we consider that those host plants are not particularly exigent in regard to habitat requirement, that is cause of concern about the conservation status of those areas. This data is worrying evidence of a trend, where areas that are officially "conserved" have shown to actually be fields devoid of fauna (particularly lepidoptera), demonstrating that the true status might be worse than previously thought. Most of the assumed range in Paraguay and Argentina has not been sampled and one could argue that the absence of the species in our data might likely be due to lacking samples rather than the absence of the species in these areas. However, those areas are regularly monitored by other researchers for the presence of the sister species *E. duponchelii*. Also,we went much further than the SMDs suggest as suitable, especifically to avoid undersampling. The larvae of *E. corethrus* are highly specialized, feeding on plants of the genus *Aristolochia* and the areas where this plant is commonly found happens to be one of the most impacted by human activity. Those plants used to be abundant in the native grasslands in their distribution area (Hoehne 1942) and, as well as the butterflies, they tolerate extremes of temperature and humidity throughout the year. The disconnection between the expected abundance and the scarcity found shows our lack of knowledge regarding the species and its environmental requirements for occurrence. The niche overlap between *E. corethrus* and *A. sessilifolia* (61,5%) suggests a reasonable commonality between the two species . Thus, the presence of the plant not only supplies the immature butterflies with food but might also indicate that the area is at least somewhat suitable for the adults. Those requirements certainly extend further than merely having the host plant present, as investigated by Atencio (2014).

Dennis (2014) highlights in his concept of "resource based habitat" that "To describe a habitat for a species over a region, based on its resource use and suitable environmental conditions, requires an in depth knowledge of species not easily obtained. Even if a species' resources and conditions are well known, tracking them down in the field often presents immense logistical difficulties." Dennis (2014) advises the use of the term "biotope" (or

vegetation unit) in the place of habitat. The current knowledge on *E. corethrus* allows us to speculate that its "habitat" (*sensu* Dennis, 2014) includes heterogeneous grassland areas (not exclusively in the Pampa biome), with the presence of *Aristolochia* (almost certainly *A. sessilifolia*) and no extensive cattle raising. In our experience, the presence of cattle in those areas is not a big problem, if the area is big enough and/or if the plants are sheltered by either by the terrain or under thorny bushes. It's interesting to notice that, even with the new, evenly distributed occurrence points, the model area did not change much in regards to latitude, with the North and South limits remaining very similar to before.

We could hypothesize about the role of pesticides, since we found grasslands that apparently had high quality and suitability but were devoid of insects, actual island of grassland in a sea of cropland. A reduction in either quality or availability of the host plants could lead to a reduction in butterfly abundance, even if they don't suffer the direct effects of the herbicide toxicity (Smart et al. 2000). Without a doubt, pesticide use in this region is heavy, and they can disperse and hit areas that originally were not intended, which potentially affects the arthropod community, especially lepidoptera (Davis et al. 1991). Braak (2018) did an extensive revision on the effects of pesticides on butterflies, where he points out that their habitat usually lie between hedges and in the fragments between arable areas (Warren et al. 2001; Krauss et al. 2003), therefore susceptible to non-intentional application of pesticides, not to mention that the plants that are non targeted by this can still absorb those chemical compounds through the soil or water (Goulson 2013), exposing the butterflies even if they are not being targeted (Egan et al. 2014). In fact, recently a controversy was sparked due to pesticide drift, affecting non-targeted crops and causing economic losses to farmers growing other crops in the surrounding area. (Wenzel, 2019), with those properties located in the distribution area for *E. corethrus.*

Another important factor is the fact that insects are sensitive to temperature and desiccation, and therefore sensible to extreme climate events. Chown (2011) discusses the mechanisms and behavioural responses observed in insects in a drying environment. Apart from that, due to them being ectothermic, insects are directly affected by the rise in temperature, since they're tolerance is limited (Deutsch et al. 2008). Caporale (2017) found evidence that larvae of *E. corethrus*, in lab conditions, when reared at temperatures above 35°C, exhibit mortality rates much higher than those reared at 25ºC, indicating that the global rise in temperature might be another risk to the species. The selected environmental variables for the models were all related to pluviosity, which is one of the factors that is believed to be greatly affected by climate change, with a change not only in averages but also the frequency of

extreme weather events (Marengo 2015). Raises in average temperature cause more rainfall in rainy climates and on the other hand less rainfall in dry climates, exacerbating the difference between those two environments and affecting taxa that depend on rainfall, like Lepidoptera (Palmer 2010). Adding to that, we must consider that climate change and climate anomalies can cause a phenological asynchrony between the herbivorous insects and the host plants, a phenomenon that has already been observed in temperate climates by Singer and Parmesan (2010).

Dolibaina (2010) suggests that the status of the species should change from "Endangered" (EN) to "Critically Endangered" (CR) for the state of Paraná, since the monitoring done since the publication of the last Red List of Threatened Species for the state of Paraná, in 2004, shows that the populations are getting rarer, and the natural grasslands of the plateau are greatly reduced, probably extinct. The status in the state of Rio Grande do Sul, currently "Vulnerable" (VU) should be revised, due to the current situation. We suggest that the category should change to "Endangered" (EN), according to criteria from IUCN (IUCN, 2012) A2c (An inferred population size reduction of ≥50% over the last 10 years with a decline in area of occupancy, extent of occurrence and/or quality of habitat) and B2b(ii,iv) (Area of occupancy estimated to be less than 500 km^2 and continued decline in area of occupancy and number of subpopulations). Considering that the species only occurs in the Southern Fields and is also endangered in the state of Santa Catarina, we strongly suggest its inclusion in the Red List of Threatened Species for Brazil as well, with the status of "Endangered" (EN), according to criteria A2c. The scenario outlined here of the relationship between the potential area of distribution of the species and the remaining grasslands is extremely worrying. It is urgent that the goals for conservation established by the MMA (Brasil, 2013) be followed, before there is nothing else to save.
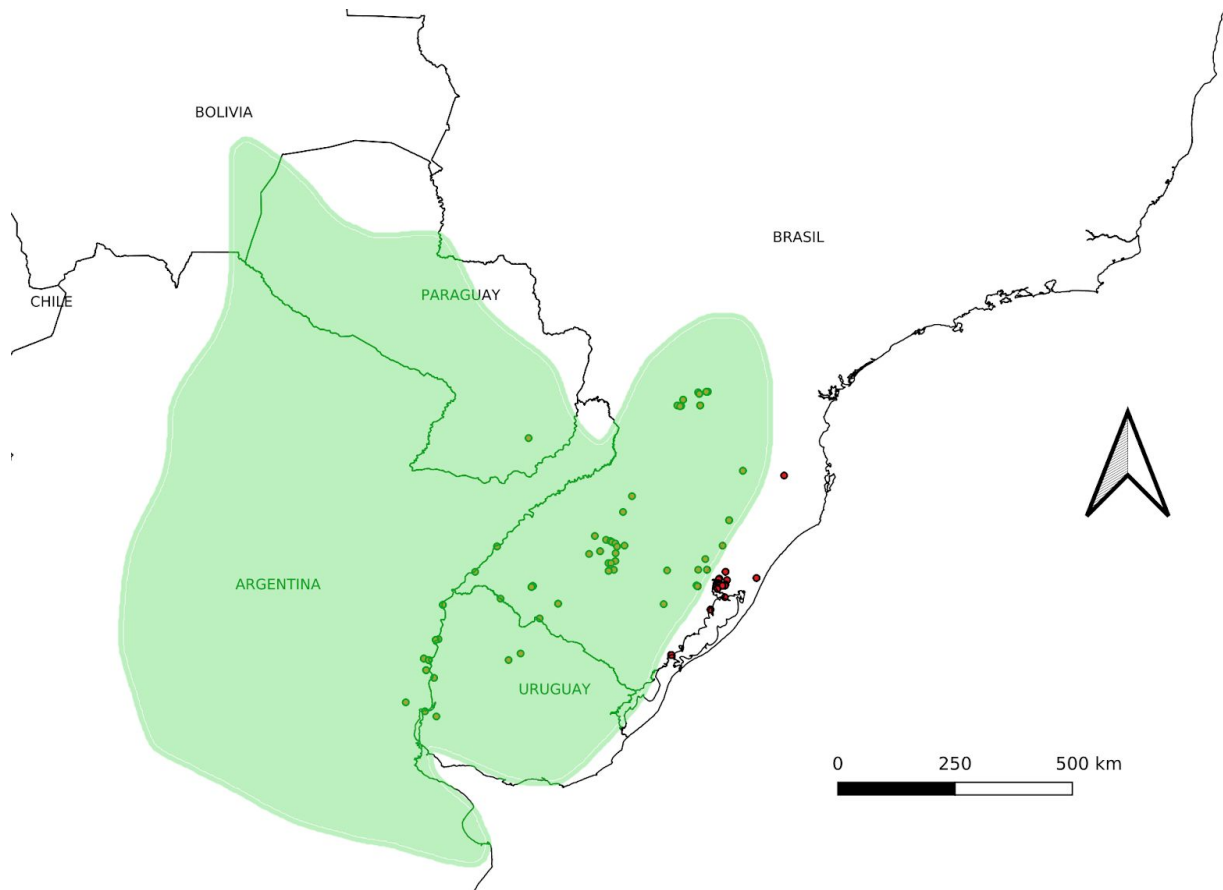
Figure 1: Occurrence points collected by Atencio (2014) overlayed by the occurrence area (light green) proposed by Tyler (1994).
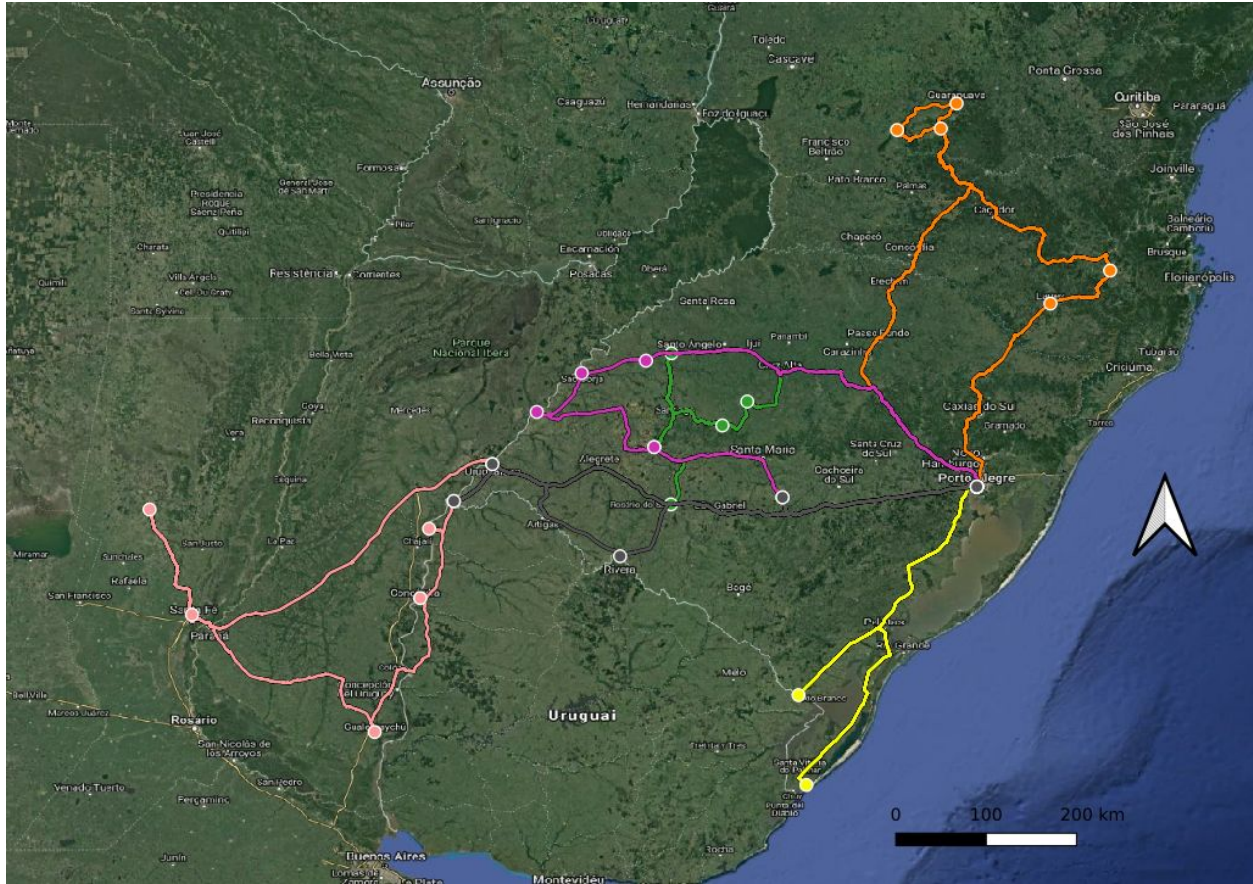
Figure 2: Routes for the six field excursions that occurred between March 2015 and February 2018, in search of *Euryades corethrus*:: 1(grey). West (far West of the state), 2 (purple). Northwest (northwest and center of the state), 3 (orange). North (north of the state, including the two other states of Brazil), 4 (green). Center (center of the state), 5 (yellow). Southl (south of the state) and 6 (pink).Uruguay and Argentina (northeast of Uruguay e northwest of Argentina).
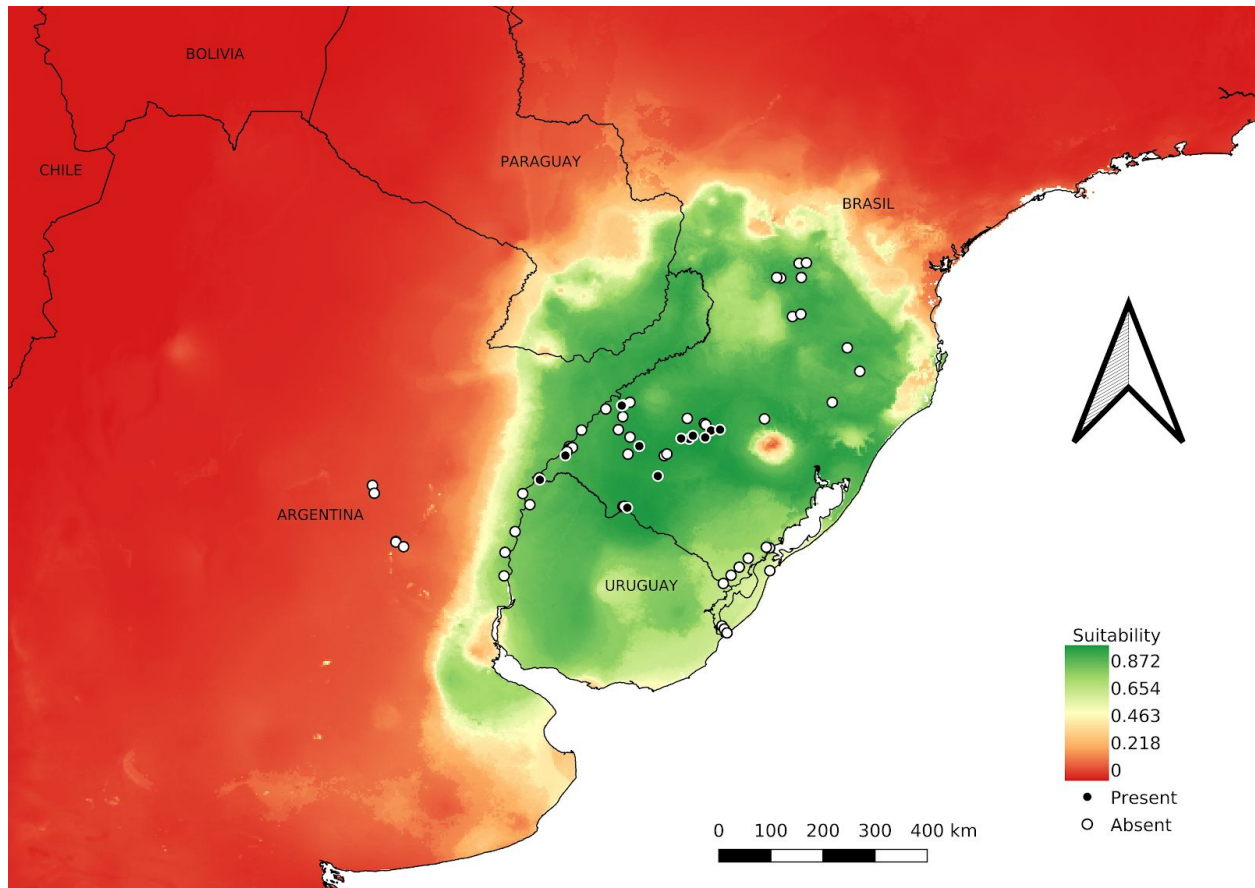
Figure 3: Locales overlayed to the final model for the suitability of *Euryades corethrus.* Points in black indicate locales where the butterfly was found while points in white indicate locales where the butterfly was not found. Green indicates highest suitability, while red indicates lowest suitability.

Figure 4 -Comparison between the model with data obtained by Atencio (2014) (A) and the model with the updated data, including the new points collected during the *in situ* validation (B). Green indicates higher suitability while red indicates lower suitability.

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Andrade BO, Koch C, Boldrini II, et al (2015) Grassland degradation and restoration: a conceptual framework of stages and 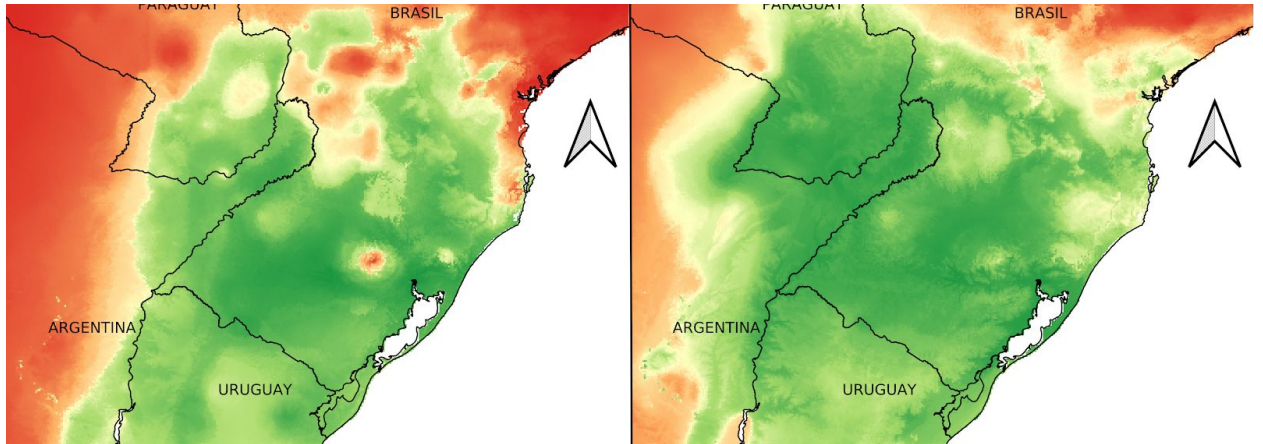thresholds illustrated by southern Brazilian grasslands. Nat Conserv 13:95–104. https://doi.org/10.1016/j.ncon.2015.08.002

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Supplementary Material
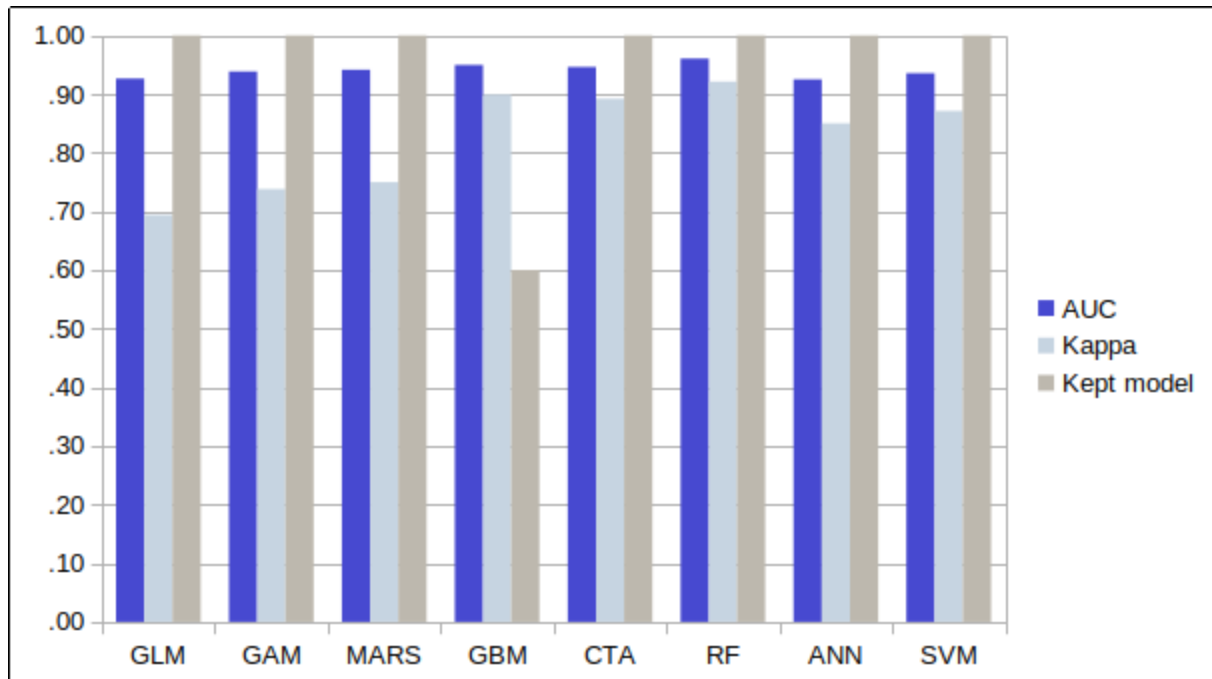
Figure A1: Model evaluation. Models evaluated with AUV >0.75 and then weighted with the previous metric means.

Table A1 – Description of the planned routes with biomes, municipality, conservation units, phyto fisionomy, previous record and target locales

Expedition	Biome	Municipality	Conservation Unit	Phyto Fisionomy	Previous record	Target locale
West	Pampa	Uruguaiana	Parque Municipal de Uruguaiana	Grassland	1991	1
		Barra do Quaraí	Espinilho State Park	Grassland	-	2
		Santana do Livramento		Grassland	2015	1
Northwest	Pampa	Sto. Antônio das Missões		Grassland	-	1
		São Francisco de Assis	RPPN Granja São Roque	Grassland	-	1
		São Vicente do Sul		Grassland	-	
		Manoel Viana		Grassland	-	
		Itaqui	São Donato Biological Reserve	Grassland	1942	
		Maçambara	São Donato Biological Reserve	Grassland	-	

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		São Borja		Grassland	-	
North	Atlantic Forest	Vacaria		Campos de altitude	2012	
		Lages		Altitude grassland	-	
		Curitibanos		Altitude grassland	2010	
		Pinhão		Altitude grassland	2010	
		Guarapuava		Altitude grassland	2010	
		Foz do Jordão		Altitude grassland	2010	
Center	Pampa	Júlio de Castilhos		Grassland	2012	1
		Tupanciretã		Grassland	2011	
		Rosário do Sul		Grassland	-	1
		Quevedos		Grassland	2011	1
		Jari		Grassland	2012	1
South	Pampa	Pelotas		Grassland	1995	1
		Rio Grande		Grassland	-	1
		Santa Vitória do Palmar		Grassland	-	1
		Jaguarão		Grassland	-	
Argentina and Uruguay	Pampa	San Gregório		Grassland	1959	
		Concepción de Uruguay		Grassland	-	
		Monte Caseros		Grassland	-	
		Concordia		Grassland	1940	
		Gualeguaychú		Grassland	2008	
		Santa Fé		Grassland	-	
		San Cristóbal		Grassland	-	

Table A2: Evaluation metrics for the resulting models

	threshold	AUC	omission.rate	sensitivity	specificity	prop.correct	Kappa	kept.model
GLM	.34	.93	.07	.93	.92	.92	.69	10
GAM	.36	.94	.06	.94	.94	.94	.74	10
MARS	.26	.94	.06	.94	.94	.94	.75	10
GBM	.51	.95	.02	.98	.92	.95	.90	6
CTA	.44	.95	.05	.95	.94	.95	.89	10
RF	.55	.96	.04	.96	.96	.96	.92	10
ANN	.74	.93	.07	.93	.92	.93	.85	10
SVM	.78	.94	.07	.93	.94	.94	.87	10

Table A3: Details of the expeditions, occurred between March 2016 and February 2018, with their distances, locales visited, soil usage and presence or not of the butterfly.

Expedition	Distance driven (in kilometers)	Locales visited	Observed soil usage	Days	Locales with *E. corethrus*	Captured individuals	Locales with grassland
Oeste	1477	10	Cattle raising	6	3	59	4
Noroeste	1351	13	Extensive agriculture	6	3	19	6
Norte	1708	10	Agriculture	5	-	-	2
Centro	1290	11	Agriculture/cattle raising	5	8	55	
Sul	1289	10	Agriculture	4	-	-	
Argentina/ Uruguai	2819	9	Agriculture	5	-	-	

CAPÍTULO 2

Population structure of the swallowtail butterfly *Euryades corethrus* (Lepidoptera, Papilionidae)

G.W.G. Atencio[1,2,5] (iD) **R. Zanini**[3] (iD) **M. Deprá** [1,3,4] (iD) **H.P. Romanoswki**[1,2] (iD)

[1]Zoology Department, Federal University of Rio Grande do Sul - Av. Bento Gonçalves, 9500, Porto Alegre RS – Brasil

1 Postgraduate Program in Animal Biology
2 Insect Ecology Lab, Zoology Department
3 Drosophila Lab, Genetics Department
4 Postgraduate Program in Genetics and Molecular Biology

Corresponding author:
Guilherme Wagner Gutierrez Atencio, Department of
Zoology, Federal University of Rio Grande do
Sul, Av. Bento Gonçalves 9500/43435/218,
Porto Alegre, RS 91501-970, Brazil. ⬜guilherme.atencio@outlook.com

Abstract

Euryades corethrus is a swallowtail butterfly of the Troidini tribe. This butterfly is endemic to grasslands in the south of Brazil, Uruguay, Argentina and Paraguay. It used to be an abundant species in these areas, but nowadays it is listed in the Red list of endangered species of the three southern states of Brazil. According to IUCN criteria, their status is: Vulnerable (VU) in Rio Grande do Sul and Endangered (EN) in Santa Catarina and Paraná. This species is reported to be oligophagous and feeds on *Aristolochia* spp during its larval stage, plants which are commonly found in southern grasslands. The areas of native grasslands have been diminishing, converted in crops and pastures, causing habitat loss for *Aristolochia* and, consequently, *E. corethrus.* The objective of this study is to assess the genetic diversity,

population structure of *E. corethrus* and its demographic history. We sampled eight populations from Rio Grande do Sul, Brazil and based on COI molecular marker, our results suggest a low genetic variability between populations, presence of gene flow and, consequently, lack of population structure. The populations from Rio Grande do Sul faced a bottleneck followed by a rapid expansion during the last glaciation and although the results suggest a population expansion, this is the result of events in the past. Habitat loss is a real threat, and might cause the isolation of populations, causing loss of genetic variability and, ultimately, the extinction of *E. corethrus* if no habitat conservation policy is adopted in the near future.

Keywords: Lepidoptera, phylogeography, population structure, butterfly, conservation, barcoding

Introduction

Euryades corethrus is a Neotropical swallowtail butterfly from the tribe Troidini (Papilionidae) that recently became a flagship species for the conservation of native grasslands (Silveira 2019a, Silveira 2019b). It is characteristic of this species the remarkable sexual dichromatism: males are black with red and yellow spots while females are translucent yellow with red spots in its posterior wings. This species is endemic to grasslands of southern regions of Brazil and neighbouring countries (Uruguay, Paraguay and Argentina) (Volkmann and Núñez-Bustos 2010) and they are mostly found from September to April.

The host plant species used by *E. corethrus* are commonly found in the south of Brazil. They feed on plants of the genus *Aristolochia* while on their larval stage and on plants such as *Senecio* and *Eupatorium* during the adult stage*,* plants which are common in southern native grasslands (Costa 2016). According to the available literature (Biezanko et al. 1974; Klitzke and Brown 2000; Beccaloni et al. 2008; Volkmann and Núñez-Bustos 2010), *E. corethrus* uses five *Aristolochia* species as host plants during its larval stage. However, we had always encountered the larval butterfly in the field feeding on *A. sessilifolia.* This could indicate that *E. corethrus* has a strong association to this particular species as a host plant.

As *E. corethrus,* many butterflies are habitat specialists with moderate long range dispersal abilities and, according to Todisco et al. (2012), they are highly sensitive to environmental change. Therefore their phylogeographical structure can be expected to closely reflect climate-driven range shifts.

The most important threat factor that appears to affect *E. corethrus* is the progressive shrinking of native grassland areas where the host plant can be found. According to Tyler et al. (1994), this used to be an abundant butterfly species in its distribution area; however, nowadays *E. corethrus* is listed in different categories in the Red list of endangered species for the Brazilian states of Rio Grande do Sul (vulnerable - VU) (Rio Grande do Sul 2014), Santa Catarina (endangered - EN) (Santa Catarina 2011) and Paraná (endangered - EN), (Instituto Ambiental do Paraná 2007).

Despite being a flagship species, very little research has been carried on this butterfly: records in the literature are mainly inventories or new occurrences (Dolibaina et al. 2011), with a few studies dealing with the biology of the species (Caporale et al. 2017), only recently focusing on its ecology (Atencio unpublished and Costa 2016). Related to evolutionary history, *E. corethrus* have been included in studies regarding the origin of butterflies (de Jong 2003) . The origin and evolution of the tribe Troidini have been analysed (Braby et al. 2005), even as phylogenetic relationships within Troidini (Silva Brandão et al. 2005) and biogeography and diversification patterns of Troidini (Condamine et al. 2012). Other studies involve phylogenetic relationships and divergence time of Papilionidae (Condamine et al. 2012, Allio et al. 2019). However, there is none analyzing the genetic diversity and population structure of *E. corethrus.*

A structured population has less gene flow, which can eventually lead to isolation and speciation. On the other hand, gene flow is a "creative force", preventing speciation and natural selection and therefore inhibiting genetic evolution (Slatkin 1987). Knowing the extinction risks and mapping priority areas needed to preserve not only individuals but also the species' genetic diversity are invaluable tools for defining such strategies.

Because there is no information on how the populations of *E. corethrus* are structured and the conservation actions are urgently needed, this work was aimed at obtaining data regarding the genetic diversity, population structure, gene flow and demographic history of *E. corethrus* from southern Brazil. Our results provide resources for future management plans and conservation strategies.

Material and methods

Specimen collection

The specimens used in this study were collected in the state of Rio Grande do Sul, southern Brazil, as shown in Figure 1 (also in Table 1) and were all captured in their adult stage. The only exceptions were specimens from Eldorado do Sul, Rio Grande do Sul (CE), which were provided by a collaborator after being collected as eggs and reared in the lab. All other butterflies were collected in the field, between March of 2016 and January of 2017. A total of 80 individuals from 8 different localities were used. The collection sites were chosen based on previous knowledge about their distribution and remnants of preserved grassland (Atencio et al., unpublished data). We could not find the butterfly outside of the state, even though the expected area of occurrence was broader than that. The criteria for DNA sequencing was to have at least 10 specimens for each location and locales as widespread as possible within the known area of occurence in order to test if the butterflies form just a single population or several smaller populations within the area considered. Captured specimens were frozen as soon as possible and kept in a freezer until DNA extraction.

DNA extraction and amplification

Total genomic DNA was extracted from thorax following Mega and Ravers (2011). DNA quality and concentration was measured using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher). COI gene fragment was amplified by PCR using the universal primers LCO-1490 (forward) and HCO-2198 (reverse) from Herbert et al. (2003). PCR was conducted in 10 μL volume reactions, as follows: 6.75 μL ultrapure water, 1.0 μL 10x PCR buffer, 0.5 μL MgCl$_2$, 0.25 μL dNTP mix (10 mM), 0.2 μL of each primer (20 mM), 0.1 μL Platinum Taq DNA polymerase (5 U/μL), 1 μL DNA (approximately 50 ng). Thermal cycling conditions followed Doorenweerd et al. (2015), with modifications: 94ºC for 3 minutes; 30 cycles of 94º for 30s, 50ºC for 30s and 57ºC for 40s; final extension at 72ºC for 5 minutes. PCR products were purified with ExoSAP-IT™ (Applied Biosystems). Sequencing reactions were performed both forward and reverse at Macrogen (Seoul, South Korea). Electropherograms were assembled, checked and sequences were edited and aligned using both strands for confirmation with Geneious Prime 2019.2.1 (https://www.geneious.com). All sequences were aligned with MAFFT (Rozewicki et al. 2019), implemented in Geneious Prime 2019.2.1. Sequences were compared to available sequences in GenBank to confirm identity and submitted to GenBank (Table 1).

Table 1. Localities, collection sites, coordinates, sample codes and accession numbers (GenBank) of *Euryades corethrus* from Southern Brazil.

Locality	Collection site	Coordinates	Sample	Accession number
Eldorado do Sul	Estação Experimental Agronômica de Eldorado do Sul	-30.108977, -51.675674	CE02	MN510336
			CE04	MN510337
			CE05	MN510338
			CE06	MN510339
			CE08	MN510340
			CE09	MN510341
			CE11	MN510342
			CE12	MN510343
			CE16	MN510344
			CE22	MN510345
Barra do Quaraí	Parque Nacional do Espinilho	-30.273140, -57.476846	E01	MN510346
			E02	MN510347

			E03	MN510348
			E04	MN510349
			E06	MN510350
			E07	MN510351
			E08	MN510352
			E09	MN510353
			E10	MN510354
			E11	MN510355
Júlio de Castilhos	Fazenda Bom Retiro	-29.13961, -53.65226	FBR01	MN510356
			FBR02	MN510357
			FBR03	MN510358
			FBR04	MN510359
			FBR08	MN510360
			FBR10	MN510361
			FBR12	MN510362
			FBR13	MN510363
			FBR15	MN510364
			FBR17	MN510365
	Fazenda Mauro Silva	-29.11981, -53.45107	FMS01	MN510366
			FMS02	MN510367
			FMS03	MN510368

			FMS04	MN510369
			FMS08	MN510370
			FMS09	MN510371
			FMS10	MN510372
			FMS11	MN510373
			FMS12	MN510374
			FMS13	MN510375
Santo Antônio das Missões	Fazenda Iviretã	-28.58186, -55.64873	FZI01	MN510376
			FZI02	MN510377
			FZI03	MN510378
			FZI04	MN510379
			FZI05	MN510380
			FZI06	MN510381
			FZI07	MN510382
			FZI08	MN510383
			FZI09	MN510384
			FZI10	MN510385
Santana do Livramento	Lago do Batuva	-30.86711, -55.52462	S01	MN510386
			S02	MN510387
			S03	MN510388
			S04	MN510389

			S05	MN510390
			S06	MN510391
			S07	MN510392
			S08	MN510393
			S11	MN510394
			S12	MN510395
Santo Antônio das Missões	Fazenda Querência	-28.5705, -55.61827	SA02	MN510396
			SA03	MN510397
			SA05	MN510398
			SA07	MN510399
			SA09	MN510400
			SA11	MN510401
			SA12	MN510402
			SA14	MN510403
			SA16	MN510404
			SA17	MN510405
Uruguaiana	Fazenda São Felipe	-29.69835, -56.90919	U01	MN510406
			U12	MN510407
			U13	MN510408
			U14	MN510409
			U15	MN510410

			U16	MN510411
			U17	MN510412
			U18	MN510413
			U19	MN510414
			U20	MN510415

Genetic diversity and population structure

Maximum parsimony haplotype networks were reconstructed to illustrate relationships among haplotypes of *E. corethrus* using Median joining algorithm in PopArt (Bandelt et al. 1999; Leigh and Bryant 2015). The pairwise genetic difference was estimated for all populations by calculating Wright's F-statistics (Fst) in Arlequin 3.5.2.2 (Excoffier and Lischer 2010). The variation among and within populations was estimated by Analysis of Molecular Variance (AMOVA) in Arlequin 3.5.2.2 using 1,000 permutations.

The population structure was evaluated by spatial analysis of molecular variance (SAMOVA) implemented in SAMOVA 2.0 (Excoffier et al. 1992; Dupanloup et al. 2002; Excoffier and Lischer 2010), without *a priori* information about population structure and also by Bayesian Analysis of Population Structure (BAPS) ver. 6.0 with the 'spatial clustering of groups' models followed by admixture analysis (Corander and Marttinen 2006, Corander et al. 2008a, Corander et al. 2008b, Cheng et al. 2013). BAPS uses both approaches to determine the most appropriate clustering (default model) and best groups in a fixed number of clusters (fixed-K mode).

Tests of demographic equilibrium and population expansion

Haplotype and nucleotide diversity were calculated using DnaSP 6.0 (Rozas et al. 2017). Demographic equilibrium was tested in each operational population unit by calculating Fu's Fs (Fu 1997) and Tajima's D (Tajima 1989) statistics. P-values for the two statistics were obtained as the proportion of simulated values smaller than or equal to the observed values ($\alpha = 0.02$ for Fu's Fs, and $\alpha = 0.05$ for Tajima's D). Expected mismatch distributions and parameters of sudden expansion s = 2lT were calculated using Arlequin 3.5.2.2 by a generalized least-squares

approach (Schneider and Excoffier 1999), under models of pure demographic expansion and spatial expansion (Ray et al. 2003; Excoffier 2004). The probability of the data under the given model was assessed by the goodness-of-fit test implemented in Arlequin 3.5.2.2. Parameter confidence limits were calculated in Arlequin 3.5.2.2 through a parametric bootstrap (10,000 simulated random samples).

Estimation of time to most recent common ancestor and reconstruction of demographic histories

We used the software BEAST 2.6.1 (Bouckaert et al. 2019) to estimate time to most recent common ancestor of South Brazilian populations of *E. corethrus*. The GTR (General Time Reversible) (Nei and Kumar 2000) model for nucleotide substitution was estimated in JModelTest (Guindon and Gascuel 2003; Darriba et al. 2012). Our dataset was combined with published sequences of New World Papilionidae (Supplementary material 1) for this analysis. We assumed a relaxed lognormal molecular clock for this analysis. The tree prior was the Calibrated Yule process model. Our tree was calibrated based on previous studies (Condamine et al. 2012, Allio et al. 2019). Three independent analyses were run for 20,000,000 generations, with Markov chains sampled every 2,000 generations. Tracer 1.7.1 (Rambaut et al. 2018) was used to determine convergence, measure the effective sample size of each parameter (ESS), and calculate the mean and 95% highest posterior density (HPD) intervals for divergence times. The consensus tree was compiled with TreeAnnotator 2.6.0 and displayed and edited in iTOL v4 (https://itol.embl.de/) (Letunic and Bork 2019).

The demographic history of population was estimated by running the MCMC analysis under the Bayesian skyline tree prior in BEAST 2.6.1. The HKY (Hasegawa, Kishino and Yano) model for nucleotide substitution (Hasegawa M, Kishino H and Yano T 1985) was estimated in JModelTest. We assumed a relaxed lognormal molecular clock with a mean rate of 0.0075 (corresponding to the divergence rate of 1.5% per million years). This rate is commonly used in lepidoptera studies (DeChaine and Martin 2005; Canfield et al. 2008; Kawakita and Kato 2009; Oliver et al. 2012; Pfeiler et al. 2012; Wang et al. 2014). Tracer 1.7.1 was used to reconstruct Bayesian skyline plot (BSP) (Drummond et al. 2005).

Results

Genetic diversity

The dataset of COI alignment contains 657 bp, of which 16 were variable (13 synonymous and 3 non-synonymous substitutions) and 6 parsimony informative. These polymorphic sites defined 17 haplotypes (Figure 1).
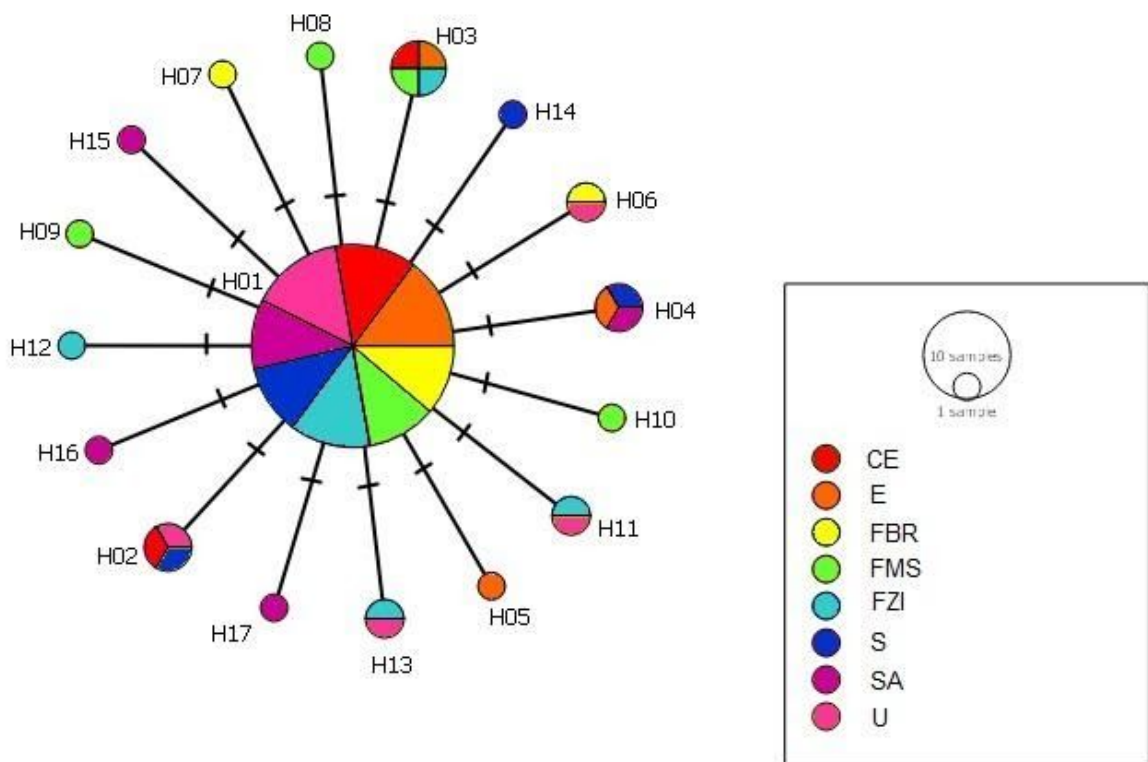

Figure 1. Haplotype network obtained from COI haplotypes of *Euryades corethrus* sampled in RS, Southern Brazil. Each circle represents a different haplotype, with the size of each circle proportional to the number of individuals displaying that particular haplotype. The haplotypes found in more than one individual are identified and represented by pie charts; Different colours represent the locations where that haplotype was found. Dashes indicate the number of mutational steps between each haplotype.

The haplotype diversity (Hd) ranged from 0.378 in CE to 0.667 in FMS, FZI, SA and U. The nucleotide diversity (π) ranged from 0.00061 in CE to, 0.00122 in FMS, FZI, SA and U. The summary of genetic diversity indices and neutrality tests are presented in Table 2.

Table 2. Summary of genetic diversity indices and neutrality tests for *Euryades corethrus*.

Locale	N	S	h	Haplotypes	Hd±SD	π±SD	Tajima's D	Fu's Fs
CE	10	2	3	H1, H2, H3	0.378±0.181	0.00061±0.00031	-1.40085 p=0.096	-1.164 p=0.039
E	10	3	4	H1, H3, H4, H5	0.533±0.180	0.00091±0.00036	-1.56222 p=0.03*	-1.964 p=0.008*
FBR	10	2	3	H1,H6, H7	0.417±0.191	0.00068±0.00034	-1.40085 p=0.085	-1.164 p=0.032
FMS	10	4	5	H1, H3,H8,H9,H10	0.667±0.163	0.00122±0.00039	-1.66706 p=0.037*	-2.847 p=0.003*
FZI	10	4	5	H1, H3, H11, H12, H13	0.667±0.163	0.00122±0.00039	-1.66706 p=0.032*	-2.847 p=0.001*
S	10	3	4	H1, H2, H4, H14	0.533±0.180	0.00091±0.00036	-1.56222 p=0.055	-1.964 p=0.013*
SA	10	4	5	H1, H4, H15, H16, H17	0.667±0.163	0.00122±0.00039	-1.66706 p=0.003*	--2.8472 p=0.003*
U	10	4	5	H1, H2, H6, H11, H13	0.667±0.163	0.00122±0.00039	-1.66706 p=0.034*	-2.847 p=0.002*
Total	80	16	17	H1-H17	0.542± 0.068	0.00097± 0.00015	-2.31193 p=0.000*	--20.832 p=0.000*

(N) Number of individuals sampled; (S) number of polymorphic sites; (h) number of haplotypes; (Hd) haplotype diversity; (π) nucleotidic diversity with standard deviation (SD); (D) and (F) statistics for neutrality test. Significance level for D is 0.05 and for F is 0.02.

From the 17 haplotypes recovered, 10 haplotypes were unique to a single locality and one haplotype was found in all localities. The remaining haplotypes range from 2 to 4 localities

(Figure 2). The most common haplotype was present in all population samples and was the most abundant haplotype across all populations, identified in 70% of all the sampled individuals across the entire study region. This main haplotype was the core of a star-like cluster topography, central to all other haplotypes, most of them represented by a single individual and divergent by a single mutation (Figure 1).
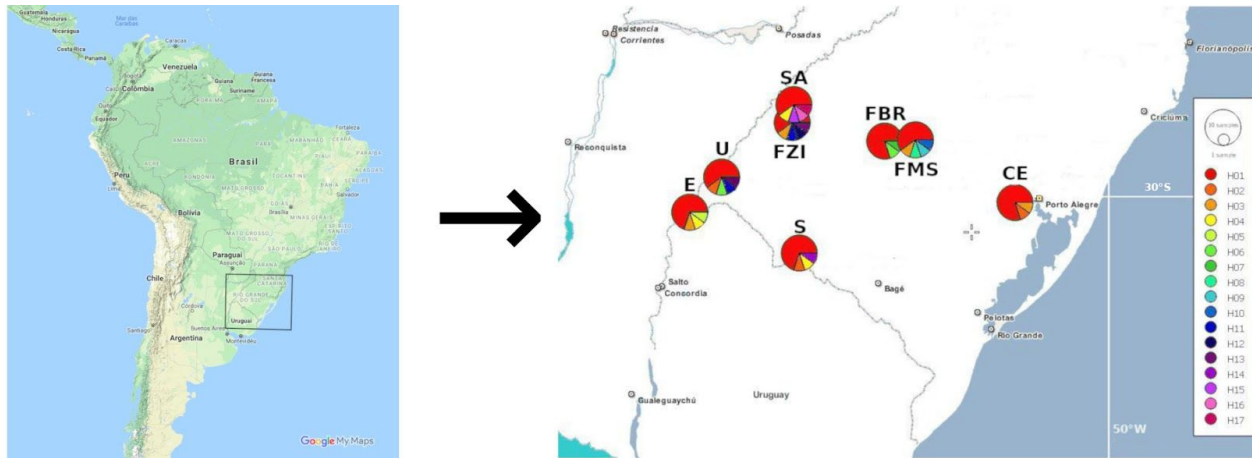

Figure 2. Geographical distribution of the haplotypes of *Euryades corethrus* from Southern Brazil. The size of the pie charts is proportional to the number of individuals sampled from each population, and each colour in the pie charts represents a different haplotype; The same haplotype found in different populations is represented by the same colour in the corresponding pie charts.

Fu's Fs and Tajima's D statistics were calculated for all operational population units defined a priori and for a unique population approach. Values of Fs and D were negative for all populations, and significant for most of them (Table 2). In stable populations, over time, it is expected for those values to be close to zero. Since our values were negative and significant for most of them, the null hypothesis of constant population size should be rejected. Just as is the case with Tajima's D, the value calculated for Fu's Fs in an indication of population expansion.

Mismatch analysis corroborates the sudden expansion model (Table 3). The theta value (0.05 ma) indicates the true time since population expansion. The observed mismatch distributions corresponded to the expected frequency distributions of pairwise nucleotide site differences in an exponentially growing population (Figure 3). Also, the mismatch distribution was unimodal indicating demographic expansion.

Table 3. Mismatch analysis of the *Euryades corethus* population from Southern Brazil under sudden expansion model and spatial expansion model.

	Sudden expansion model	**Spatial expansion model[1]**
Tau	0.7	0.6
Tau 5%	0.49	0.37
Tau 95%	1.54	2.12
Theta (Ma)	0.05	0.20
Theta 5%	0.00	0.00072
Theta 95%	0.35	0.47
Corrected p-value	0.04*	0.29

[1] Parameters of the spatial expansion assuming constant demographic size

Theta= true time since population expansion

*Significant p-value
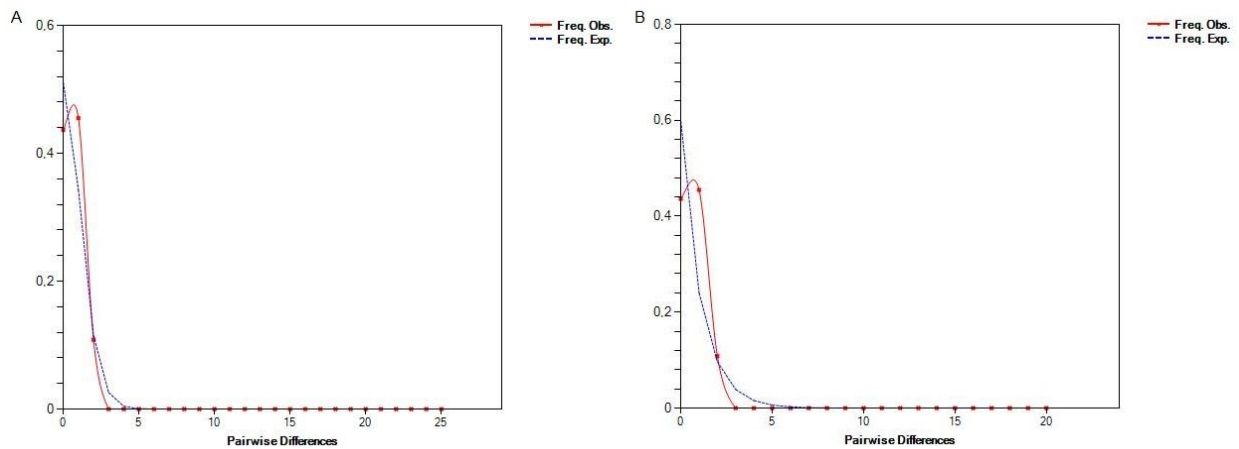
Figure 3. Frequency distributions of the observed pairwise nucleotide site differences, or mismatch distributions for the *Euryades corethrus* population from Southern Brazil, compared to expected frequency distributions for a population under demographic expansion (A) and for constant demographic size (B).

Population structure

The population structure was evaluated by spatial analysis of molecular variance (SAMOVA) without a priori information about population structure and also by Bayesian Analysis of Population Structure (BAPS). Both analyses presented similar results, indicating a lack of population structure and therefore suggesting the populations defined a priori based on the geographic location are a unique panmictic population. The analysis of molecular variance (AMOVA) calculated the source of variation among and within populations (Sum of squares was 1.900 and 23.400, respectively); the variation within populations corresponded to 100% of total variation. Also, the average Fst value was -0.01676 (p value = 1) indicating no genetic differentiation between populations. Fst values are supposed to range from 0 to 1, but small samples with low differentiation levels can produce negative values close to zero. Pairwise Fst values for all populations are represented in Figure 4.
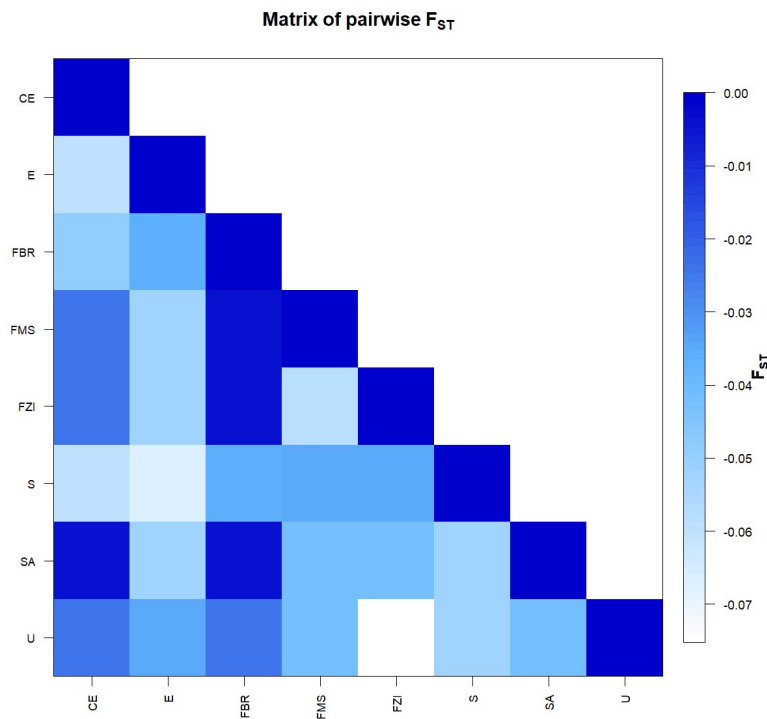

Figure 4. Matrix of pairwise Fst for *Euryades corethrus* populations from Southern Brazil.

Demographic history

The effective sample size (ESS) for the Bayesian Skyline Plot was larger than 200, suggesting that the MCMC (Markov chain Monte Carlo) mixed properly and that the number of generations was sufficient to infer size changes.

Bayesian skyline plot indicated an overall pattern of population stability throughout the Pleistocene. A fluctuation near the present (around 100,000 ya), suggests a population bottleneck followed by rapid population expansion (~50,000 ya). (Figure 5).
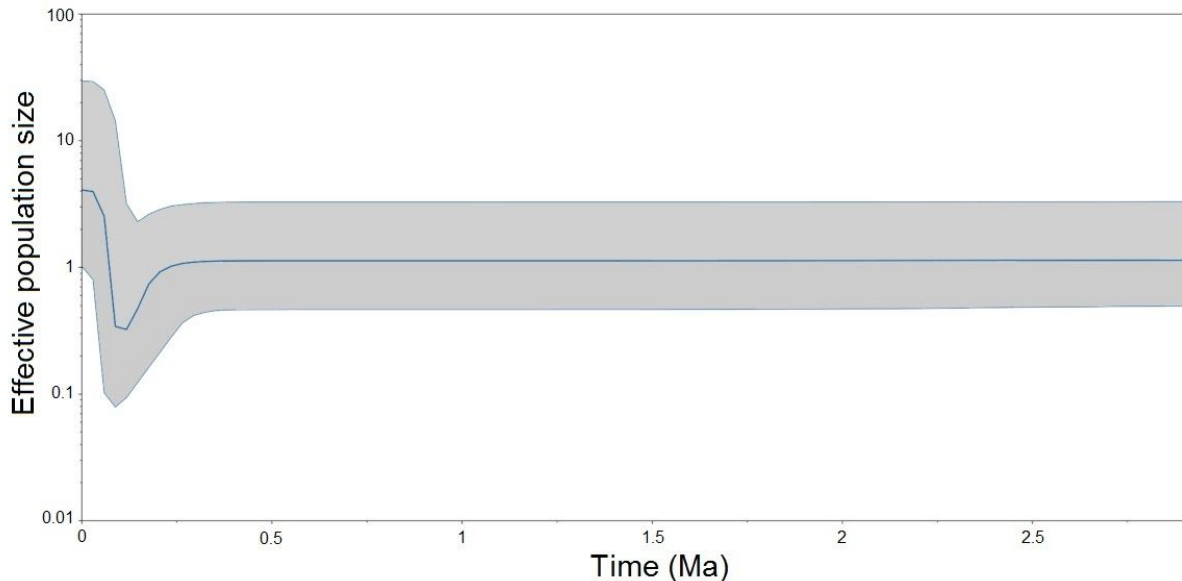

Figure 5. Demographic history of *Euryades corethrus* population from Southern Brazil reconstructed using Bayesian skyline plot based on *COI* gene with divergence rate of 1.5% per million years. X-axis is the timescale before present, and Y-axis is the estimated effective population size. Solid curve indicates median effective population size; the shaded area indicates 95% highest posterior density (HPD) intervals. Time is represented in million years (Ma).

Three independent runs show high convergence in the estimation of time to most recent common ancestor, with average (ESS) value above 1,800. A Bayesian topology is shown in Figure 6, with the divergence times estimated under the relaxed lognormal clock. The split between the two species of the genus *Euryades* was around 9.6 Ma (5.6393-13.7073), during the Pliocene (Figure 6). The most recent common ancestor of the Southern Brazilian population of *E. corethrus* was in the Late Pliocene - Early Pleistocene, around 2.5 Ma (1.045-3.1975). The samples of *E. corethrus* from the same locality were not recovered together in the calibrated bayesian analysis, corroborating the results suggesting a lack of population structure and the existence of gene flow between them.
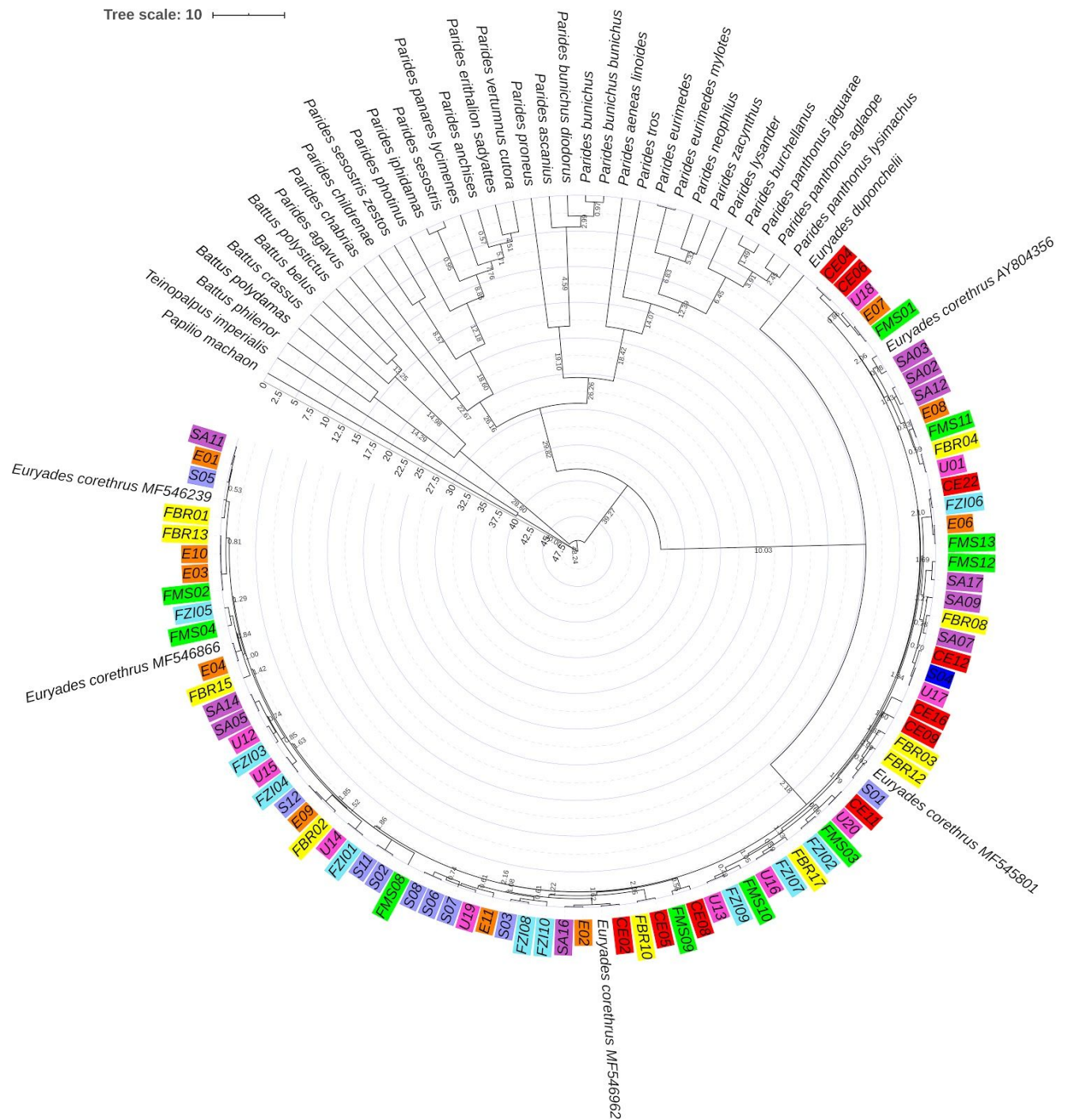
Figure 6. Divergence time estimation generated from a 120 taxa COI dataset, including 85 sequences of *Euryades corethrus*, 33 taxa from Troidini tribe and 2 outgroups (*Teinopalpus imperialis* and *Papilio machaon)*. The collection sites of *Euryades corethrus* from Southern Brazil are represented by different colors: CE (red), E (orange), FBR (yellow), FMS (green), FZI (cyan), S (blue), SA (purple), U (magenta).

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Discussion

Although typically not sufficient when unraveling population-level questions, mitocondrial (mt) *COI (Cytochrome Oxidase I)* barcoding is a useful tool during the early stages of investigation, highlighting patterns of genomic diversity within the target species (Hajibabaei et al. 2007). Some preliminary phylogenetic tests were considered using the available molecular data for eight specimens captured in Argentina (available in BOLD) but no further analysis was performed because the number of individuals per locale (4) was too low to meet our criteria of at least 10 specimens.

The COI sequences of *E. corethrus* revealed very low nucleotide diversity (0.00097± 0.00015) and a relatively high haplotype diversity (0.542± 0.068). Despite the haplotype diversity, most haplotypes occur at a very low frequency and are differentiated from a dominant (probably ancestral) haplotype by a single mutational step. The haplotype network shows a "star-like" pattern (Figure 2), which is indicative of a bottleneck event followed by population expansion or founder effect (Slatkin and Hudson 1991; Avise 2000).

Tajima's *D* and Fu's *Fs* values were negative for all populations and significant for most of them, indicating an excess of rare nucleotide site variants compared to the expectation under a neutral model of evolution. Fu's Fs test, also shows negative values for all populations, indicating an excess of rare haplotypes over what would be expected under neutrality. These results lead us to reject the neutrality hypothesis. A negative Tajima's D indicates population size expansion after a bottleneck or a selective sweep and/or purifying selection (Tajima 1989). A negative value of Fs is evidence for an excess number of alleles, as would be expected from a recent population expansion or from genetic hitchhiking (Fu 1997).

Schmitt and Seitz (2002) investigated the genetic structure of *Polyommatus coridon* (Lepidoptera: Lycaenidae) and also verified very low Fst values, and they found no hierarchical structure between and within the study areas and also no correlation between genetic and geographical distances. They attribute this lack of congruence between geographical and genetic pattern to the high dispersal power of some species, corroborating Hastings and Harrison (1994).

Our results suggest that populations from Southern Brazil probably represent one metapopulation sufficiently interconnected for the maintenance of genetic diversity, indicated by the lack of genetic differentiation and, therefore, population structure. The sufficient gene flow

between populations reduces or restrain the process of geographic differentiation (de Jong et al. 2011). This phenomenon is common in flying insects, especially in migratory and/or good dispersers, such as Monarch butterflies (Brower and Boyce 1991), bumble bees *Bombus terrestris* (Estoup et al. 1996), and dragonflies *Anax junius* (Freeland et al. 2003) (review in de Jong et al. 2011). According to Lv et al. (2019), species with high dispersal ability and with a high number of habitat patches have a better chance of maintaining gene flow and panmixia. Papilionidae are known as strong fliers, so this might be a factor. Also, the most recent common ancestor of *E. corethrus* from Southern Brazil population is around 2.5 ma, which could explain the low genetic diversity and also the free gene flow between populations, since there was not enough time for the rise of a barrier that ought to cause the isolation of these populations.

The BPS pattern suggests a long period of population stability, with a decrease in population size around 100,000 ya. This population decrease was followed by a rapid population growth around 50,000 ya. A similar pattern was found in *Atlapetes albinucha* (Aves, Passerellidae) from Mexico and Colombia, where there was a decrease in population size around 100,000 ya, followed by rapid population growth and range expansion during the Late Pleistocene (Rocha-Méndez et al. 2018).

The recent demographic expansion is in accordance with broadly observed patterns of population expansion in different taxa, following the last glacial period, which ended around 12,500 years ago (de Jong et al. 2011). The glacial maximum period was characterized by cooler and drier conditions, which caused the contraction of forest areas into small refugia, causing a decrease of the available habitat for many species (Prentice and Jolly 2000).

 Contrasting with this, the grassland ecosystems and open vegetation landscapes had expanded through South America, creating new suitable areas during the glaciation (Behling 2002) . Species from open areas, belonging to different taxa, such as mammals (Mapelli et al. 2012, Mora et al. 2013, Peçanha et al. 2017)*,* reptiles (Felappi et al. 2015)*,* insects (Cristiano et al. 2016) and plants (Fregonezi et al. 2013) respond to Pleistocene climate changes in a different way than florestal species. According to Turchetto-Zolet et al. (2012), 68% of the studies in populations associated with open fields showed population expansion during glacial cycles and/or fragmentation during interglacial periods.

Apart from the populations of Estação Agronômica de Eldorado do Sul (CE) , (which is an area under permanent management), the remaining populations are distributed in small grassland patches amidst a matrix of soy monoculture and livestock areas. Historically, the *E. corethrus* was abundant and the extension of occurrence spanned through the three southern

states of Brazil. Recent records, however, are rare (Atencio et al. unpublished data) and, probably, the largest populations are the ones reported in this study.

Although our results are consistent with population expansion, this reflects the expansion of open areas during the last glaciation. The habitat degradation is a relatively recent event and might cause population isolation, genetic depletion and risk of extinction for *E. corethrus* if no habitat conservation policy is adopted in the near future. The habitat fragmentation can erode neutral and adaptive genetic diversity of populations caused by decreases in the effective population size and inter-populational gene flow (Johansson et al. 2007; Dixo et al. 2009; Wang et al. 2011; Liu et al. 2013; review in Lv et al. 2019). Thus, habitat fragmentation threatens not only local, but regional, and ultimately global biodiversity (Tilman et al. 1994, Dobson 1997).

It is possible that Rio Grande do Sul has become the last refuge of the species in Brazil. A recent work by Atencio et. al (2019, unpublished data) surveyed the areas where the butterfly was expected to be found but very few places actually had either the butterfly or suitable grassland. Argentina and Uruguay have only very few scattered records in recent years, but the conservation status of native grasslands in these countries seems to be as dire as the one in Brazil, according to Atencio et. al (2019, unpublished data) and the relationship between these populations and the ones in Brazil remains to be investigated.

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Supplementary material

New world Troidini species and accession number.

Species	Accession number
Battus belus	AY804350
Battus crassus	AY804351
Battus philenor	AF170875
Battus polydamas	AY804352
Battus polystictus	AY804354
Euryades corethrus	AY804356
Euryades corethrus	MF545801
Euryades corethrus	MF546239
Euryades corethrus	MF546866
Euryades corethrus	MF546962
Euryades duponchelii	AY919290
Parides ascanius	AY804364
Parides bunichus	AY804366
Parides bunichus bunichus	EF102900
Parides bunichus diodorus	EF102902
Parides burchellanus	EF102903
Parides chabrias	AY804368
Parides childrenae	AY804369
Parides erithalion sadyattes	GU163965
Parides eurimedes	AY804370
Parides eurimedes mylotes	GU164015
Parides iphidamas	GU163969
Parides lysander	AY804372
Parides neophilus	AY804373
Parides panares lycimenes	GU163992
Parides panthonus aglaope	EF102906
Parides panthonus jaguarae	AY804376
Parides panthonus lysimachus	AY804377
Parides photinus	AF170877
Parides proneus	AY804378
Parides sesostris	AY804381
Parides sesostris zestos	GU164026
Parides tros	AY804382
Parides vertumnus cutora	AY804383
Parides zacynthus	AY804384
Teinopalpus imperialis	GQ268351

CONCLUSÃO

Como apontado no Capítulo 1, depois de 29 dias em campo, percorrendo mais de 10.000 km em território Brasileiro, Argentino e Uruguaio, podemos perceber que a situação é alarmante. Locais que há poucos anos atrás eram áreas com abundância de indivíduos estão rapidamente se tornando vazios de fauna, sendo convertidos em lavoura, pasto ou loteamentos urbanos. Buscando os locais com registros mais antigos, de 10, 20 ou 30 anos atrás, observamos que a situação é ainda mais grave. Em que pese a presença de campos com qualidade adequada para abrigar as borboletas, permitimo-nos seguir esperançosos ao notar que elas são muito robustas, sendo encontradas em beiras de estrada, pequenas manchas em meio às fazendas e até mesmo em refúgios dentro das cidades, em praças e jardins botânicos. No entanto, a resiliência de *E. corethrus* não é ilimitada, e se a conversão de habitat continuar neste ritmo, eventualmente não restarão campos e tampouco borboletas desta espécie.

Em apenas 22,5% dos locais avaliados a borboleta foi encontrada, o que mostra que os modelos de predição de distribuição de espécies são uma ferramenta importante para conservação, mas que precisam ser usada em conjunto com outras, como imagens de satélite e - fundamentalmente - em conjunto com a validação em campo. Não basta assumir que as distribuições das espécies vão seguir fielmente a adequabilidade calculada, pois esta refere-se apenas às condições climáticas, que são apenas uma parte dos requerimentos das espécies. O habitat de uma espécie é muito mais do que isto. Por exemplo, se não houver disponibilidade de recursos alimentares para que a lagarta e/ou o adulto se alimente, não há como a borboleta completar seu ciclo de vida.

No capítulo 2, dados moleculares inéditos foram obtidos para *E. corethrus*. A análise do marcador COI revelou que há baixa diversidade nucleotídica e alta diversidade haplotípica, com 17 haplótipos nesta amostra. Destes, 10 eram exclusivos de apenas uma localidade e um está presente nas 8 localidades amostradas. A análise espacial de variância molecular (SAMOVA) e a análise Bayesiana de Estrutura de População (BAPS) indicam que esta população não está estruturada. *Bayesian skyline plot* indica um padrão geral de estabilidade da população durante o Pleistoceno. Uma flutuação próxima do presente (em torno de 100.000 m.a.) sugere um gargalo seguido por rápida expansão (~50.000 m.a.). Tudo isso aponta para uma metapopulação suficientemente interconectada para manter a diversidade genética, indicado

pela falta de diferenciação genética. A diferenciação geográfica é impedida pelo fluxo gênico entre as populações.

Diante dos resultados obtidos, sugerimos a possibilidade de que o Rio Grande do Sul seja o último refúgio de *E. corethrus* no Brasil. Dolibaina (2010) sugere que o status da espécie seja alterado de "Ameaçada" (EN) para "Criticamente Ameaçada" (CR) para o estado do Paraná, já que o monitoramento desde a publicação do Livro Vermelho de Fauna Ameaçada em 2004 mostra que as populações estão se tornando mais raras e as áreas de campo natural naquela região estão drasticamente reduzidas. O status no estado do Rio Grande do Sul, devido à situação atual, também deve ser revisto. Atualmente "Vulnerável" (VU), sugerimos que a categoria mude para "Em perigo" (EN), de acordo com os critérios da IUCN. Considerando que a espécie ocorre apenas nos Campos do Sul e também está ameaçada de extinção no estado de Santa Catarina, sugerimos fortemente sua inclusão na Lista Vermelha de Espécies Ameaçadas para o Brasil também, com o status de "Ameaçada" (EN). Fora do país a situação também é preocupante: pesquisadores Argentinos que colaboram com nosso laboratório relataram que não tem mais encontrado a espécie na cidade de Buenos Aires, e até mesmo áreas afastadas da área urbana tem registros recentes escassos, o que sugere que sua inclusão na lista internacional de espécies ameaçadas seria uma providência importante. Todos estes dados e modelos são cada vez mais importantes para reverter esta tendência e frear a erosão da biodiversidade nos nossos campos, mas sem políticas claras e eficientes de preservação é como se estivéssemos modelando as cadeiras do salão de jantar do Titanic.