UFRGS
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PPGBAN
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA ANIMAL

INSTITUTO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA ANIMAL

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Lepidópteros galhadores (Cecidosidae) da América do Sul: filogeografia de *Eucecidoses minutanus* Brèthes e descrição de dois gêneros e de três espécies novas

PORTO ALEGRE
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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.

Área de concentração: Biologia Comparada
Orientador(a): Prof. Dr. Gilson Rudinei Pires Moreira
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Dedico esta tese à minha mãe, Tânia, em agradecimento por todo amor e imenso apoio que sempre dedicou a mim, em especial durante a realização deste doutorado.

Agradecimentos

Agradeço primeiramente à minha família, Tânia, Carolina e Paula, por todo apoio e compreensão que sempre tiveram comigo e com minhas escolhas, e à meu pai Paulo (*in memoriam*) que de longe me guia e me dá forças para continuar nesta jornada.

Sou grata ao meu orientador Prof. Dr. Gilson Moreira, pela oportunidade de trabalhar com o que sempre sonhei, pela orientação e ensinamentos, e pela confiança em mim depositada, contribuindo imensamente no meu crescimento como pesquisadora.

À minha co-orientadora Dra. Gislene Gonçalves, pelo empenho, disponibilidade, e paciência que teve ao me orientar e inserir no "novo mundo" da molecular, contribuindo de maneira única na tese e na minha formação.

Ao professor Dr. Luis Parra, um grande mestre e agora amigo, que me recebeu de "braços abertos" em seu laboratório, sempre ajudando, mostrando seus conhecimentos e proporcionando oportunidades únicas tanto para a minha vida profissional quanto pessoal.

Ao Dr. Germán San Blas pela disposição em me auxiliar em campo, laboratório, no desenvolvimento de artigos, discussões por skype e amizade.

Aos colegas e funcionários do Laboratório de Citogenética e Evolução, em especial William e Luciano, pelo auxílio em laboratório. E à Camila Melo e Camila Zanella, do Núcleo de Genética e Conservação de Plantas, por toda a ajuda com a parte botânica molecular desse trabalho.

Aos atuais e ex colegas de laboratório do LMCI, Carol, Cristiano, Denis, Lucas, Luan, Júlia, Marcelo, Marina, Pedro, Ramoim, Rô, Sabrina, Sofia e Zé por todo auxílio, dentro e fora do laboratório. Em especial agradeço à Rosângela e Júlia, que estiveram ao meu lado nos melhores e piores momentos.

À todos os professores do Programa de Pós-Graduação em Biologia Animal, pelos ensinamentos e contribuições. Aos colegas do PPG, pela parceria dentro e fora da sala de aula.

À Capes, pela bolsa de estudos concedida para a realização deste doutorado.

Às minha amigas, Bia, Bruna, Mai, Paty e Thereza, sem as quais estes anos de doutorado não seriam os mesmos; obrigada pela força, paciência, esperança e a "luz" que cada uma de vocês sempre me proporcionou.

Agradeço também à Cássia, Jaque, Thê e Raquel, pela presença, sem vocês esses anos na cidade grande teriam sido muito menos coloridos! Vocês foram minha casa e caminho, obrigada de coração!

Ao meu parceiro canino Bacon, que está sempre ao meu lado, que escuta meus desabafos, que me ajuda a pôr a cabeça no lugar durante nossas caminhadas, e me transmite um amor incondicional!

E a todos que de alguma forma contribuíram para que esse sonho se tornasse realidade!

Muito obrigada!

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APRESENTAÇÃO

Esta tese é apresentada de acordo com as normas do Programa de Pós-Graduação em Biologia Animal, e está estruturada em quatro partes, Introdução geral, capítulos I, II, III e Considerações finais. Os capítulos estão sob a forma de artigos científicos já formatados para as revistas.

O capítulo I foi submetido à revista Plos One, em janeiro de 2018, e aborda a filogeografia de um gênero de microlepidópteros associado com uma formação orogênica da região Neotropical. O capítulo II foi publicado na revista ZooKeys em setembro de 2017, referente à descrição de uma nova espécie pertencente a um novo gênero de Cecidosidae para o sul do Brasil. O capítulo III foi submetido também para a revista Zookeys em junho de 2018, neste artigo um novo gênero e duas novas espécies do Chile são descritas.

O trabalho foi desenvolvido no Laboratório de Morfologia e Comportamento de Insetos da Universidade Federal do Rio Grande do Sul, com bolsa de estudos concedida pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Além disso, um período de três meses de estágio foi realizado no Chile na Universidad de Concepción, sob a supervisão do Prof. Dr. Luis Eduardo Parra, com a finalidade de contribuir com dados para a tese.

RESUMO

O continente Sul Americano apresenta uma grande diversidade biológica, que pode ser explicada pelos resultados das interações entre plantas e animais, e de eventos climáticos e geológicos passados. Diferentes hipóteses sugerem como a biodiversidade da América do Sul pode ter evoluído; 1) através de um longo período de isolamento que, forneceu estabilidade e condições climáticas e biológicas; 2) com o soerguimento do Andes, por meio da vicariância geográfica e isolamento genético, aumentando a heterogeneidade e complexidade de habitats; e 3) pelas transgressões marinhas, que modificaram a paisagem, flora e fauna do continente. Por apresentar uma alta heterogeneidade de ecossistemas, a região Neotropical conta uma das maiores riquezas de insetos galhadores do mundo. Dentre esses, Lepidoptera, a segunda maior ordem de Insecta, conta com diversas famílias que são reconhecidas como indutoras de galhas e que, no entanto, não são devidamente exploradas/descritas. Cecidosidae são microlepidópteros indutores de galhas pouco conhecidos que apresentam uma estreita associação com plantas hospedeiras de Anacardiaceae. Na América do Sul, apenas quatro gêneros representam a família: *Cecidoses, Eucecidoses, Oliera* e *Dicranoses* (Davis 1998, Moreira 2012), sendo os três primeiros monotípicos. Neste contexto, o presente estudo explora a história evolutiva de *Eucecidoses* na região Neotropical, através de uma abordagem filogeográfica a partir de sua distribuição geográfica, associação com a planta hospedeira, e estrutura genética de populações deste táxon; e também descreve dois novos gêneros e espécies de Cecidosidae encontrados para o sul do Brasil e região central do Chile; os dados relacionados à história de vida, plantas hospedeiras e distribuição das novas espécies são fornecidos, assim como as relações filogenéticas destas novas espécies com as já descritas na literatura. Os resultados mostraram que o padrão de distribuição encontrado para *Eucecidoses minutanus* Brèthes (1916), não está relacionado diretamente com a distribuição de sua planta hospedeira, mas por eventos de dispersão e vicariância, que coincide com o arco orogênico Peripampásico. As análises revelaram seis linhagens distintas, geneticamente estruturadas e isoladas por distância em diferentes regiões biogeográficas. Um novo gênero e espécie de Cecidosidae, *Cecidonius pampeanus* Moreira e Gonçalves (2017), é descrito para o bioma Pampa no sul do Brasil; a galha induzida por esta espécie é severamente atacada por parasitoides e inquilinos, estes últimos modificando-a e tornando-a vistosa. Devido a isso, a espécie indutora permaneceu desconhecida por

muito tempo, sendo erroneamente atribuida a um hymenoptero, de fato um inquilino desta. *C. pampeanus* foi reconhecida através de filogenia molecular como uma linhagem nova e relacionada aos cecidosídeos Neotropicais. O novo gênero e espécie descrita para o Chile, *Andescecidium parrai* Moreira e Vargas (2018), aparentado com *Cecidonius*, passou por uma história de negligência semelhante à de *C. pampeanus*, sua galha foi por muito tempo estabelecida como induzida por um coleóptero, supostamente um cleptoparasita da galha do real indutor. Além de *A. parrai*, uma nova espécie adicional, *Oliera saizi* Moreira e Vargas (2018), é também descrita.

Palavras-chave: Microlepidoptera, cecidosídeos, arco Peripampásico, taxonomia.

ABSTRACT

The South American continent presents a great biological diversity that can be explained as results from plant and animal interactions, and from climatic and geological past events. Different hypothesis suggest how the South American biodiversity may have evolved; 1) through a long period of isolation, which provided stability and climatic and biological conditions; 2) the uplift of the Andes, through geographical vicariance and genetic isolation, raising the heterogeneity and habitat complex; 3) through marine transgressions, which modified the landscape, flora and fauna of the continent. Because of the high ecosystem heterogeneity, the Neotropical region counts with one of the greatest richness of galling insects in the world. Among them, Lepidoptera is the second largest order of Insecta and abridges several families that are recognized as gall inducing insects, however, they are not properly explored/described. Cecidosidae is a little known family of gall inducing microlepidoptera that presents strict relationship with Anacardiaceae host plants. In South America, only four genera represent the family: *Cecidoses, Eucecidoses, Oliera* and *Dicranoses* (Davis 1998, Moreira 2012), the first three being monotypic. In this context, the present study explores the evolutionary history of the genus *Eucecidoses* in the Neotropical region, through a phylogeograpical approach, association with host-plant, and genetic structure of populations of this taxa; and also describes two new genera and species of Cecidosidae found for south of Brazil and central region of Chile; the data related to life history, host-plants and distribution of the new species are provided, as well as the phylogenetic relations of these new species with the ones described in the literature. The results showed that the distributional pattern found for *Eucecidoses minutanus* Brèthes (1916), is not directly related to the host-plant distribution, but with events of dispersion and vicariance, which coincides with the Peripampasic orogenic arc. The analyzes revealed six distinct lineages, genetically structured and isolated by distance in different biogeographical regions. A new Cecidosidae genus and species, *Cecidonius pampeanus* Moreira e Gonçalves (2017) is described for the Pampa biome, south Brazil; the gall induced by this species is severely attacked by parasitoids and inquilines, the latter modifies the gall, promptly calling attention. Because of this, the true inducer remained unknown for a long period, being mistakenly attributed to a hymenoptera, in fact, its inquiline. *Cecidonius pampeanus*,

was recognized through molecular phylogeny as a new lineage related to the Neotropical cecidosids. The new genus and species described for Chile, *Andescecidium parrai*, related to *Cecidonius,* went through negligent history similarly to *C. pampeanus*, its gall was long established as induced by a coleoptera, supposedly a kleptoparasite of the true inducer. Besides to *A. parrai*, a new additional species, *Oliera saizi* Moreira e Vargas (2018), is also described.

Keywords: Microlepidoptera, cecidosids, Peripampasic arc, taxonomy.

INTRODUÇÃO GERAL

Biodiversidade na América do Sul

A América do Sul é um continente extenso que, além de apresentar uma grande variedade de climas, formações vegetacionais, ecorregiões e complexos padrões geomorfológicos, abriga uma parte expressiva da biodiversidade da Terra. A alta diversidade de biomas (e.g., tropical, costeiro, árido, montanhoso, deserto, etc.) encontrados atualmente no continente é resultado de ações bióticas e abióticas pregressas como, por exemplo, a interação entre plantas e animais e eventos geológicos (Ortiz e Cladera 2006). Para Antonelli e Sanmartin (2011), dentre os mecanismos abióticos que atuaram na evolução da biodiversidade Sul Americana, destacam-se o tempo, o clima, o soerguimento dos Andes e as transgressões marinhas.

Hipóteses biogeográficas contam a história evolutiva da região a partir de distúrbios climáticos e geológicos que atuaram na estruturação e diversidade de espécies; entretanto, ainda existem alguns conflitos em relação aos processos históricos e ecológicos responsáveis pela diversidade Neotropical (Matos-Maraví et al. 2012, Turchetto-Zolet et al. 2013). A primeira hipótese descreve que o continente após separar-se da África, há cerca de 100 milhões de anos, permaneceu isolado por muito tempo, favorecendo assim uma diversificação gradual de linhagens. Além disso, permaneceu sempre próximo ao Equador, condicionando a uma estabilidade climática e favorecendo baixas taxas de extinção (Mittelbach et al. 2007, Antonelli e Sanmartin 2011). Quanto ao clima, os altos níveis de precipitação e temperatura que propiciaram habitats heterogêneos, estão correlacionados com a alta diversidade de espécies; as flutuações climáticas do passado possivelmente promoveram a expansão de áreas abertas, desencadeando diversificação de vegetação adaptada e consequentemente fauna especializada (Kreft e Jetz 2007, Antolelli e Sanmartin 2011). O soerguimento dos Andes, a maior cadeia montanhosa próxima a uma floresta tropical, pode ter influenciado na diversificação de espécies de distintas maneiras, por exemplo: i) aumentando a heterogeneidade e favorecendo a radiação adaptativa de novos habitats, ii) criando corredores bióticos e favorecendo a especiação alopátrica para diversos taxa adaptados a ambientes montanhosos, e iii) produzindo vicariância geográfica e consequente isolamento genético (Antonelli e Sanmartin 2011). Adicionalmente, as

diversas transgressões e regressões marinhas que ocorreram durante a Era Cenozóica afetaram profundamente grande parte do continente, formando novos ecossistemas e alterando as condições na qual a flora e a fauna da região já haviam se adaptado (Ortiz e Cladera 2006).

Além da complexidade nos processos responsáveis pela alta diversidade observada na região Neotropical, os respectivos padrões são amplamente desconhecidos (Daza et al. 2009). Em especial, devido aos estudos serem realizados apenas para biomas *hotspots* de diversidade, como por exemplo, Amazônia e Mata Atlântica; assim, pouca atenção tem sido dada ao conjunto de biomas, principalmente de forma a comparar a existência de uma história faunística comum para a região.

Embora reconhecida como uma área de "hibridização biótica" entre as regiões Neotropical e Andina, a zona de transição da América do Sul (sensu Morrone 2004) ainda é pouco explorada, em particular em relação às cadeias montanhosas na porção leste da Cordilheira dos Andes (correspondente às províncias biogeográficas de Prepuna e Monte, sensu Morrone [2014]), na Argentina. Essas áreas, e aquelas montanhosas localizadas na costa atlântica do Brasil aparentemente compartilham linhagens existentes para diversas espécies endêmicas (Ringuelet 1961, Mattoni e Acosta 1997, Crisci et al. 2001), um padrão supostamente determinado pela tectônica do Terciário (Ringuelet 1961, Mattoni e Acosta 1997, Crisci et al. 2001). Porém, a influência de eventos orogênicos na diversidade de fauna das regiões mencionadas acima ainda não foi estudada em insetos, e permanece inexplorada do ponto de vista filogeográfico em nível específico para qualquer táxon em nível específico. Um grupo de organismos com ampla relevância em estudos ecológico-evolutivos, e, portanto, modelo dentro desse contexto, são os insetos galhadores, os quais possuem características peculiares de história de vida que potencialmente se refletem nos processos de diversificação.

Insetos galhadores

Formam um grupo de organismos ecologicamente definidos, os quais possuem uma complexa associação com as plantas hospedeiras (Shorthouse et al. 2005, Carneiro et al. 2009). As galhas representam uma reação de crescimento das plantas em resposta ao ataque de organismos parasitas, relacionados principalmente à atividade de alimentação (Mani 1964). Podem ser induzidas por diversos grupos de artrópodes, entretanto, aquelas induzidas pelos insetos são as mais diversas em termos fenotípicos

(Coelho et al. 2009). Estimativas sugerem que a riqueza destes organismos pode chegar até cerca de 210 mil espécies de artrópodes indutores no mundo, no entanto, apenas uma pequena fração já foi reconhecida e descrita de maneira sistemática (Coelho et al. 2009, Fernandes et al. 2014). A indução de galhas por lepidópteros é reconhecida para várias famílias (Miller 2005, Luz et al. 2014), porém a grande maioria é referida apenas supra genericamente, e associada com os respectivos morfotipos e planta hospedeira (Maia et al. 2014, Hanson et al. 2014), ou seja, sem descrever as espécies indutoras.

Uma linhagem de insetos galhadores altamente diversa constituem os microlepidópteros, um grupo ancestral dentro de Lepidoptera (Walhberg et al. 2013). Os microlepidópteros ocorrem em quase todas as regiões biogeográficas, embora para o Neotrópico poucas espécies tenham sido descritas (Krinstensen et al. 2007, Nieukerken et al. 2011, Brito et al. 2016). Em geral se destacam pela fidelidade a uma determinada linhagem de planta hospedeira, como por exemplo, a família Cecidosidae, de exclusivo hábito galhador, associada somente a espécies de *Schinus* (Anacardiaceae) na região Neotropical.

Cecidosidae

É uma pequena família de micromariposas da infraordem Heteroneura, clado monotrisia (sensu Davis 1998) que surgiu há cerca de 125 milhões de anos atrás (van Nieukerken et al. 2011, Regier et al. 2015) (Fig 1) e possui distribuição restrita ao hemisfério Sul. Apesar dos hábitos alimentares altamente especializados nessa família (e consequentes adaptações morfológicas), ela ainda é pouco estudada em termos ecológico-evolutivos comparativamente a outras micromariposas da superfamília Adeloidea, como por exemplo, o grupo irmão Prodoxidae ocorrente no Neártico, que representa um modelo para estudos de co-evolução (Althoff et al. 2012). Um dos principais impedimentos é a questão taxonômica, uma vez que poucas espécies são formalmente conhecidas para o Neotrópico, visto que o hábito noturno, o tamanho reduzido (envergadura da asa não ultrapassando 14 mm de comprimento), e pequena variação em estruturas morfológicas do estágio adulto tornam o trabalho de prospecção e descrição mais difícil do que usual.

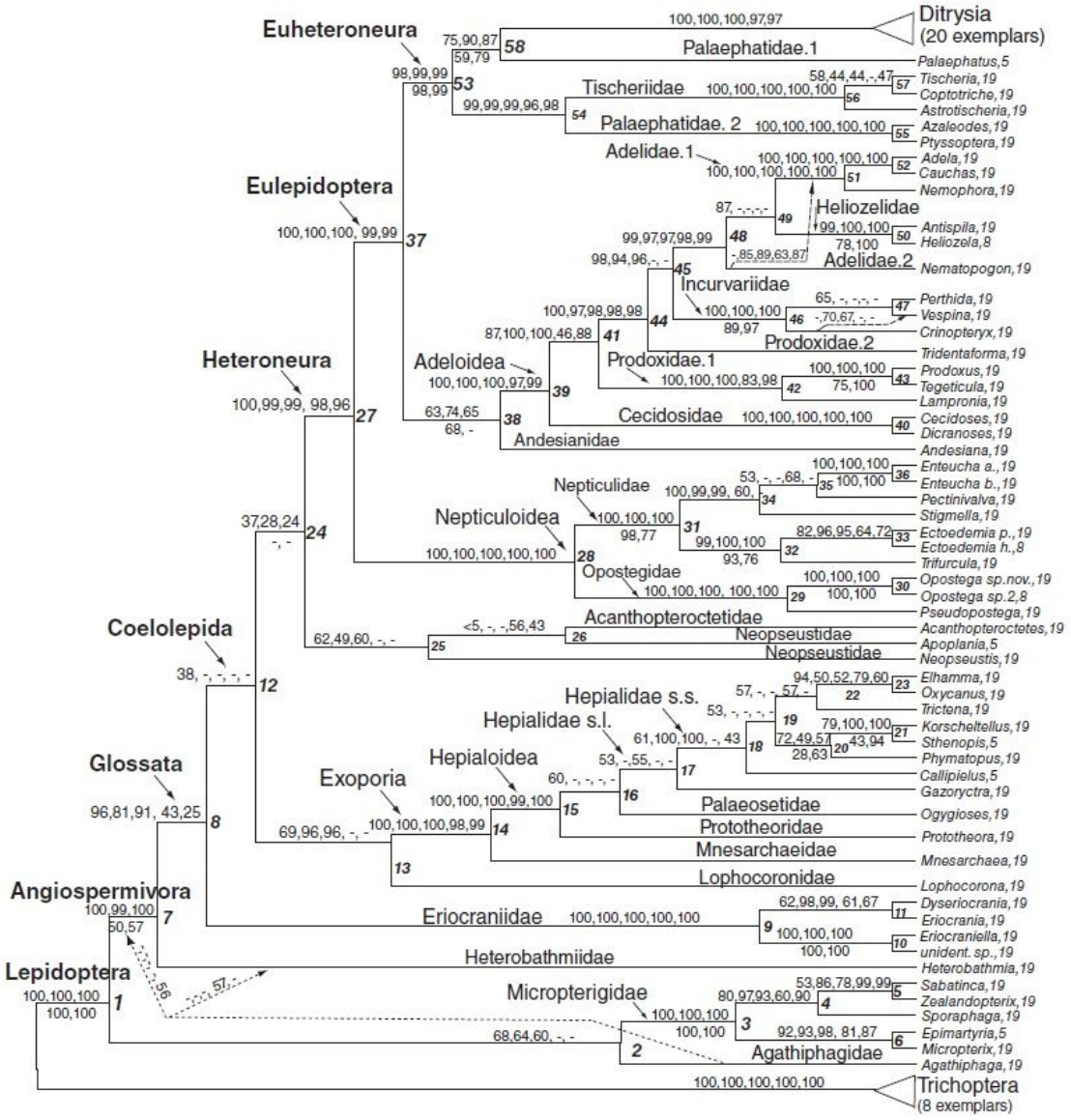

Figura 1. Filogenia de Lepidoptera mostrando as relações entre as linhagens não-Ditrisia. Fonte: Regier et al. (2015).

Atualmente, seis gêneros e 18 espécies são reconhecidos (Pellmyr e Leebens-Mack 1999, Hoare e Dugdale 2003, Mey 2011, Moreira et al. 2012). Na América do sul, quatro gêneros representam a família: *Cecidoses* Curtis, 1835, *Eucecidoses* Brèthes, 1916, *Oliera* Brèthes, 1916 e *Dicranoses* Kieffer & Jörgensen, 1910; os três primeiros sendo monotípicos e o último incluindo apenas duas espécies (Davis 1998, Moreira 2012, San Blas e Davis 2013). Na Nova Zelândia, somente um gênero monotípico, *Xanadoses* Hoare & Dugdale, 2003 é registrado, distinguindo-se de outros cecidosídeos por apresentar hábito minador (Hoare e Dugdale 2003). Para o continente africano, atualmente 12 espécies representam o único gênero, *Scyrotis* Meyrick, 1909; sete destas

espécies foram reconhecidas apenas nesta última década (Mey 2007), e nenhuma delas possui os estágios imaturos descritos.

Cecidosidae foi proposto como família pela primeira vez por Brèthes (1916), onde dois gêneros descritos pelo autor na ocasião, *Eucecidoses* e *Oliera*, foram adicionados juntamente com os gêneros *Cecidoses* e *Clistoses* Kieffer & Jörgensen, 1910. Por muito tempo essa posição ficou indefinida, até Becker (1977) sinonimizar *Eucecidoses* com *Cecidoses*, e esclarecer a relação dessas espécies com a superfamília Adeloidea (Nieukerken et al. 2011, Regier et al. 2015), propondo Cecidosidae como pertencente a uma subfamília de Incurvariidae (Incurvariinae). *Oliera* também foi proposto por Parra (1998) como sinônimo de *Cecidoses*. No entanto, todas essas sinonímias não foram universalmente aceitas, principalmente por falta de informações filogenéticas, dentre outras (Moreira et al. 2012).

Semelhanças encontradas com *Scyrotis* levou Davis (1987) a reestabelecer Cecidosidae como família, este mesmo autor em 1998, incluiu sete espécies ao grupo, *Cecidoses, Eucecidoses, Oliera, Ptisanora* Meyrick, 1913, *Scyrotis*, e *Dicranoses* com duas espécies, todas exclusivamente galhadoras no hemisfério sul. Outro gênero, *Xanadoses,* proveniente da Nova Zelândia foi proposto por Hoare e Dugdale 2003, para Cecidosidae por apresentar algumas sinapomorfias compartilhadas com a família, entretanto, a espécie é a única que não apresenta hábito galhador, minando ramos de diferentes plantas hospedeiras. Além da descrição de *Xanadoses nielsini* Hoare & Dugdale 2003, estes autores fornecem um *checklist* para a família, e adicionam mais duas espécies descritas para *Scyrotis*.

Posteriormente, *Oliera* foi revalidada por Moreira et al. (2012) utilizando dados morfológicos e moleculares. Neste mesmo estudo, uma filogenia molecular de Cecidosidae com os supostos integrantes da região Neotropical foi também fornecida, onde além de *Oliera*, *Eucecidoses* é também suportado como monofilético sendo ambos revalidados. Até o momento, o estudo de Moreira et al. (2012) é o único que aborda a filogenia de Cecidosidae através de dados morfológicos e análises moleculares, mostrando a importância que este tipo de metodologia representa hoje para o grupo e para a sistemática como um todo.

Os cecidosídeos neotropicais apresentam uma forte associação com plantas hospedeiras de Anacardiaceae (Davis 1998). As galhas são induzidas em distintas espécies de *Schinus* L., pequenas árvores e arbustos conhecidos popularmente como aroeiras (Wille 1926, Fleig 1989, Davis 1998). A taxonomia do gênero é confusa e de

difícil identificação, abrangendo 15 espécies na América da Sul, e está atualmente em revisão (C.L.S. Luz, USP, pers. com.) (Steibel e Troiani 2008). Para *Schinus polygamu*s (Cavanilles) Cabrera, por exemplo, hospedeira de *E. minutanus* Brèthes, 1916 e *C. eremita* Curtis, 1835, alguns autores atribuem a variação espacial encontrada à plasticidade fenotípica, tratando o grupo como espécie única (sensu Cabrera 1938, Fleig 1987 e 1989); para outros, apenas as populações localizadas à oeste dos Andes deveriam ser consideradas como *S. polygamus*, e o restante dividido em espécies proximamente relacionadas (sensu Barkley 1957).

As galhas formadas podem assumir formas esféricas ou fusiformes, de acordo com o gênero indutor e a planta hospedeira, e são abandonadas após a emergência do adulto. O desenvolvimento larval ocorre inteiramente no interior da galha, mais precisamente, dentro de uma câmara inclusa em uma expansão do tecido vegetal (Wille 1926, Moreira et al. 2012, San Blas e Davis 2013). Esse hábito galhador peculiar remete a especializações morfológicas para tal modo de vida. Dentre as principais modificações estão às peças bucais e ovipositor. As peças bucais são fortemente reduzidas ou até mesmo ausentes, potencialmente sugerindo que não se alimentam durante o curto período de vida como adultos (1 a 12 dias) (Wille 1926, Davis 1998). A presença de um ovipositor especializado para perfurar o tecido vegetal e depositar os ovos é típica para o grupo (Noort et al. 2006, San Blas e Davis 2013), assim como a redução das pernas torácicas e ausência de pseudopódios nas larvas. As estruturas da cabeça prognata também são reduzidas, como antenas e estemas (Davis 1998, Moreira et al. 2012).

A especialização às distintas espécies de *Schinus* poderia levar à especiação e surgimento de distintas linhagens de cecidosídeos. Apesar do conhecimento a respeito dos processos envolvidos na formação de novas espécies ainda ser escasso, esse tipo de especiação (também chamado de co-especiação) é conhecido para diferentes grupos taxonômicos. Um exemplo clássico é a polinização mutualista das mariposas da família Prodoxidae e hospedeiras do gênero *Yucca* (Agavaceae), que por consequência de especiação simpátrica e diversificação sugere que a especialização em novos hospedeiros gera adaptações de cunho morfológico, e mostra que as diferenças interespecíficas em nível macroevolutivo podem ser acumuladas rapidamente como resultado da colonização de um novo hospedeiro (Groman e Pellmyr 2000). Outro reconhecido sistema é o de um grupo de tripes (*Kladothrips* Froggatt, 1906) indutoras de galhas em acácia na Austrália, as quais divergiram utilizando diferentes hospedeiras

mostrando um padrão de co-especiação através de radiação planta-hospedeira (McLeish et al. 2007).

Filogenia molecular

Estudos de filogenia nos últimos 30 anos, i.e., antes da era de tecnologias de sequenciamento de DNA, foram quase que exclusivamente voltados para inferência das relações entre espécies (i.e., sistemática); entretanto, nos últimos 15 anos se tornou uma ferramenta indispensável nas diferentes áreas da biologia (Goldstein e DeSalle 2011, Yang e Ranalla 2012). Além da abordagem taxonômica, sequências de DNA também são utilizadas para inferir processos evolutivos, de especiação e diversificação associados à biogeografia (Groman e Pellmyr 2000, McLeish et al. 2010, Althoff et al. 2014, Gonçalves et al. 2015).

Estudos taxonômicos e ecológico-evolutivos utilizando dados moleculares com microlepidópteros são provenientes, de forma predominante, de marcadores moleculares simples ou multilocus, em especial genes mitocondriais (especialmente o Citocromo oxidase subunidade I*; CoI* e 16S) e nucleares (e.g., wingless, histona3, Ef1a) (Schmitz et al. 2007, Caterino e Sperling 2009, Moreira et al. 2012 e 2017, Reiger et al. 2015, Mutanen et al. 2015, Kawahara et al. 2016). Hebert et al.(2003) propuseram o uso de uma região de 658 pares de base (pb) do gene *CoI*, intitulada 'DNA barcode', e desde então, a maior parte dos estudos com filogenia molecular complementar à descrições/revisões de taxa utilizam tal fragmento, especialmente por apresentar i) alto grau de variabilidade em distintos níveis sistemáticos, isto é, tanto dentro de espécies (nivel populacional), como entre espécies e gêneros (Toussaint et al. 2015, Postaire et al. 2016), ii) baixo custo de sequenciamento, e iii) facilidade de amplificação por PCR em laboratório. Dados genômicos (sequenciamento de última geração; NGS), transcriptomas e genomas mitocondriais completos começaram a ser gerados em maior escala desde 2010, e tem auxiliado na resolução das relações acima do nível de gênero, com maior suporte estatístico (e.g., Breinholt et al. 2018).

Para investigar padrões de diversificação, as relações entre os taxa devem ser estudadas desde o tempo presente (isto é, espécies/linhagens devem ser delimitadas) até a origem do grupo (Grimaldi e Engel 2005, Misof et al. 2014), há centenas de milhões de anos no caso dos microlepidópteros. Assim, para reconstruir árvores filogenéticas mais robustas é importante incorporar dados adicionais a um único segmento gênico,

como *CoI*, por exemplo, outras regiões do genoma mitocondrial (e.g., Citocromo oxidase subunidade II [CoII] e rRNA 16s) que possuem adequada taxa mutacional. De forma geral, é mais eficiente contar com mais dados na matriz; a limitação é da ordem de tempo de processamento (isto é, capacidade computacional) e recursos financeiros (Regier et al. 2010, Kawahara et al. 2011).

A seleção de taxóns dentro de um determinado grupo depende da questão alvo de pesquisa bem como o número de marcadores genéticos usados, os quais devem ser equilibrados para incluir o maior número possível de representantes e, ao mesmo tempo, resolver as relações filogenéticas com alto suporte estatístico. Para estudos de diversificação, uma amostragem aleatória é importante, abrangendo o maior número possível de linhagens.

A automação em termos de geração de dados (i.e., mais rapidamente e com menor custo) nos últimos 10 anos levou a produção de um grande volume de sequências. Consequentemente, um dos aspectos mais desafiadores nos estudos de filogenia atualmente está na bioinformática e nos recursos computacionais para calcular as árvores a partir de grandes conjuntos de dados. Idealmente, todas as configurações de árvore possíveis são comparadas e pesadas (i.e., abordagem heurística), mas o número de combinações possíveis aumenta exponencialmente com o número crescente de dados e táxons, assim como o tempo computacional. Dois algoritmos usados com maior frequência em estudos evolutivos são a Máxima Verossimilhança e a Inferência Bayesiana, cada um deles sendo aplicado a diferentes metodologias para amostrar as possíveis combinações e calcular a árvore filogenética mais provável, como aqui proposto para a família Cecidosidae.

Justificativa

Além da suposta diversidade de espécies ainda não descritas formalmente, Cecidosidae é um grupo altamente relevante sob o ponto de vista ecológico e biogeográfico, uma vez que possuem hábitos alimentares altamente especializados. Sendo indutores de galhas, estão intimamente associados com plantas hospedeiras (*Schinus* L.; Anacardiaceae) na América do Sul, e as galhas induzidas formam um complexo sistema multi-trófico, podendo estar associadas com outras guildas de insetos. Além disso, apresentam distribuição gondwânica, e a maioria dessas espécies habita diferentes biomas dentro de uma dada região biogeográfica. Essas características os

tornam candidatos ideais para testar hipóteses relacionadas ao papel da biogeografia histórica na diversificação de linhagens, assim como demonstrar como a diversidade e ecologia desses insetos galhadores podem contribuir para a conservação da biodiversidade Neotropical.

OBJETIVOS

Objetivo Geral

Explorar a história evolutiva de uma espécie de Cecidosidae da região Neotropical, *Eucecidoses minutanus* Brèthes, 1916, através de uma abordagem filogeográfica, e descrever novas espécies da família.

Objetivos específicos

- Explorar a história evolutiva de *Eucecidoses* na região Neotropical, através de uma abordagem filogeográfica a partir da distribuição geográfica, associação com a planta hospedeira, e estrutura genética de populações deste táxon;

- Descrever uma nova espécie pertencente a um novo gênero de Cecidosidae do sul do Brasil;

- Descrever duas novas espécies de gêneros diferentes descobertas para a região central do Chile;

-Fornecer dados relacionados à história de vida, plantas hospedeiras e distribuição para as novas espécies;

- Estabelecer as relações filogenéticas das novas espécies com as já descritas na literatura.

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PLOS ONE

RESEARCH ARTICLE

Phylogeography of the gall-inducing micromoth *Eucecidoses minutanus* Brèthes (Cecidosidae) reveals lineage diversification associated with the Neotropical Peripampasic Orogenic Arc

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OPEN ACCESS

Citation: Silva GT, San Blas G, Peçanha WT, Moreira GRP, Gonçalves GL (2018) Phylogeography of the gall-inducing micromoth *Eucecidoses minutanus* Brèthes (Cecidosidae) reveals lineage diversification associated with the Neotropical Peripampasic Orogenic Arc. PLoS ONE 13(8): e0201251. https://doi.org/10.1371/journal.pone.0201251

Editor: Tzen-Yuh Chiang, National Cheng Kung University, TAIWAN

Received: January 6, 2018

Accepted: July 11, 2018

Published: August 8, 2018

Data Availability Statement: All sequence files are available from the Genbank database, accessions numbers MH667739 - MH667811 (CoI, tRNA-Leu, CoI) and MH667669 - MH667738 (rRNA16S).

Funding: This research was supported in part by a PRONEX project (process 16/2551-0000485-4) from Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Conselho Nacional de Desenvolvimento Científico e

Abstract

We investigated the molecular phylogenetic divergence and historical biogeography of the gall-inducing micromoth *Eucecidoses minutanus* Brèthes (Cecidosidae) in the Neotropical region, which inhabits a wide range and has a particular life history associated with *Schinus* L. (Anacardiaceae). We characterize patterns of genetic variation based on 2.7 kb of mitochondrial DNA sequences in populations from the Parana Forest, Araucaria Forest, Pampean, Chacoan and Monte provinces. We found that the distribution pattern coincides with the Peripampasic orogenic arc, with most populations occurring in the mountainous areas located east of the Andes and on the Atlantic coast. The phylogeny revealed a marked geographically structured differentiation, which highlights a first split into two major clades: western (Monte and Chacoan) and eastern (Pampean and coastal forests). Together with AMOVA and network analysis, phylogeny revealed the existence of six well-defined lineages, which are isolated by distance. The TMRCA for *Eucecidoses* was estimated at ca. 65 Mya, and the divergence among major clades occurred by the Plio-Pleistocene ca. 20–25 Mya, with the extant six lineages emerging about 0.9 to 5.7 Mya (later than the rise of *Schinus*). These results are associated with a diversification pattern of either a late burst of speciation or early extinction. Population range expansion for some lineages concurring with major climatic changes that occurred during the wet–dry events of the Pleistocene in the region was recovered in both neutrality tests and past dynamics through time analysis. A possible biogeographic scenario reconstructed suggests that *Eucecidoses* likely emerged from a central meta-population in the south and later dispersed (ca. 38 Mya) using western and eastern as two major routes. Thus, a combination of dispersal and vicariance events that occurred in the ancestral populations might have shaped the current distribution of

Tecnológico (CNPq). G. T. Silva was supported by a CAPES doctoral scholarship. G.L. Gonçalves received a fellowship from FAPERGS (process 16/2551-0000485-4). G.R.P. Moreira was supported by a research grant from CNPq.

Competing interests: The authors have declared that no competing interests exist.

extant lineages. Speciation driven by host plant shift is potentially involved in the evolutionary history of *Eucecidoses*.

Introduction

The Neotropical region is well known for its biodiversity, biome heterogeneity, geological history and the complex pattern of species distribution [1, 2]. Biogeographic hypotheses regarding the distribution of species in this highly diverse region are mostly associated with climatic and geological disturbances (*e.g.* tectonic events, variation in sea level and temperature) in association with periods of geographic isolation and fusion [3, 4, 5]. However, there are some conflicts regarding the historical and ecological processes responsible for the observed diversity [6]. Phylogeographic patterns responsible for shaping species diversity in South America are highly complex, forming a mosaic, and are largely unknown. Furthermore, studies have been conducted for specific areas only, as for example the Amazon, Atlantic forest, Pampas and Patagonia; little attention has been given to comparing them regarding the existence of a common faunal history (for a review, see [5]).

Although recognized as an area of 'biotic hybridization' between Neotropical and Andean regions, the South American transition zone (*sensu* Morrone, [7]) has been little explored in this regard, particularly in relation to the Argentinian mountainous systems located on the east side of the Andes (in the Prepuna and Monte provinces). These areas and the mountainous ones located in the Atlantic coast of Brazil (Parana dominion) apparently share extant lineages for a number of endemic species, a pattern supposedly determined by Tertiary tectonics [8–10]. Furthermore, it has been proposed recently that these mountainous areas were historically connected in the southernmost portion of the Pampean province from a faunistic perspective, thus forming a U-shaped distribution pattern for species involved in association with the existence of the Peripampasic orogenic arc (for a review, see [11]). However, in testing for the influence of this arc on diversity and species distributions no study has included divergence dates of the lineages involved (e.g. [11, 12, 13]). Furthermore, the influence of orogenic events on fauna diversity in those regions has not been tested for insects yet, and remains unexplored from a phylogeographic perspective at the specific level for any taxon. Here all these aspects are considered jointly to explore the existence of the orogenic arc and corresponding influence, if any, on the diversity of a gall-inducing micromoth *Eucecidoses minutanus* Brèthes (Cecidosidae), associated with *Schinus polygamus* (Cavanilles) Cabrera (Anacardiaceae).

The Cecidosidae is an ancient group of monotrysian Heteroneura micromoths [14, 15]. It is currently composed of seven genera (*Cecidoses* Curtis, *Scyrotis* Meyrick, *Dicranoses* Kieffer & Jörgensen, *Eucecidoses* Brèthes, *Oliera* Brèthes; *Xanadoses* Hoare & Dugdale, and *Cecidonius* Moreira & Gonçalves) and 19 species [14, 16, 17]. Despite hiding a putative species diversity that is yet to be discovered, cecidosids are very interesting from a geographical and ecological perspectives: 1) they have Gondwanic distribution ranges, thus limited to Southern Hemisphere (*Scyrotys* in Southern Africa, *Xanadoses* in New Zealand and the remaining genera in southern South America); 2) two highly specialized feeding habits are found (bark-mining in *Xanadoses* and gall-inducing in the other genera); and 3) particularly the gall inducers are intimately associated with their Anacardiaceae host plants (*Schinus* Linnaeus and *Searsia* F.A. Barkley, in South America and Africa, respectively). In addition, most of the South American species 4) inhabit several biogeographic provinces, 5) have localized populations determined

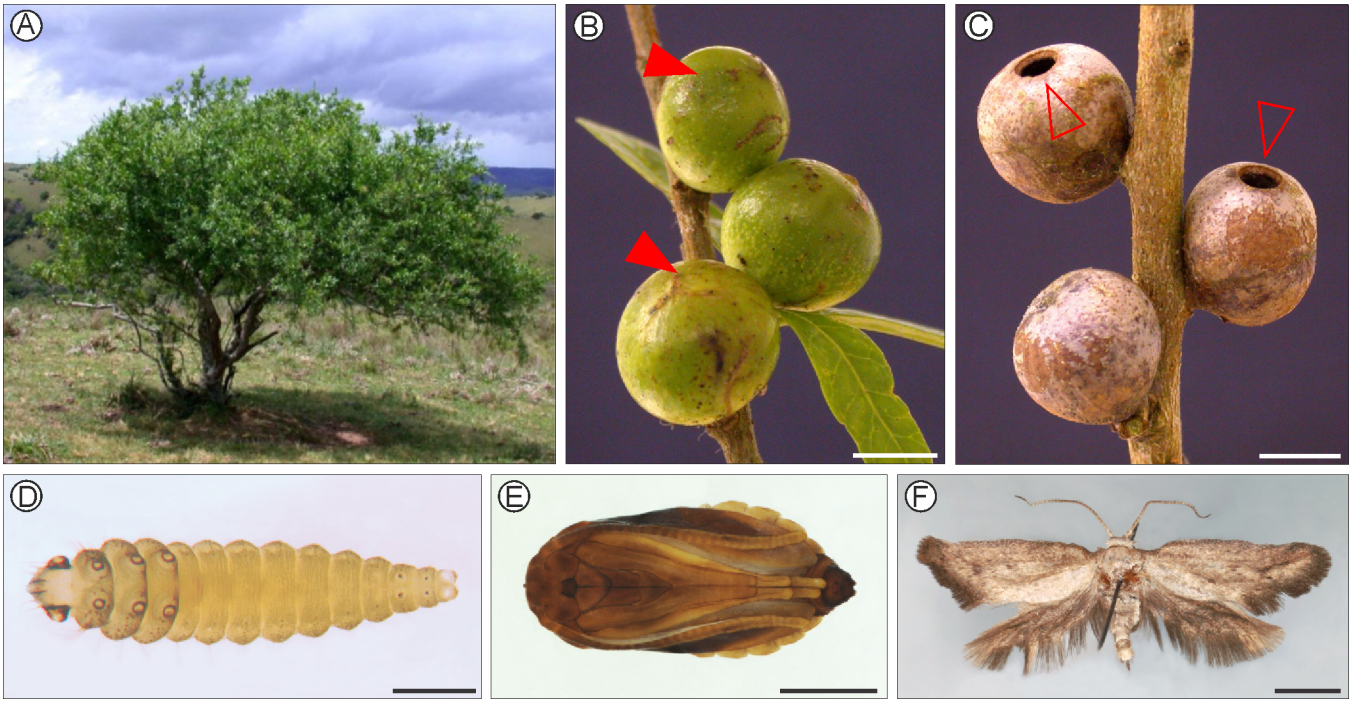

Fig 1. Natural history of *Eucecidoses minutanus* on *Schinus polygamus* plants from populations of the Chacoan domain, Pampean province, Brazil (Ch1_3; See Table 1). A, Isolated host plant on hilltop; B, mature galls on host plant branch (arrows point to developing opercula); C, empty, senescent galls (arrows indicate open opercula); D, last instar larva; E, pupa; F, pinned, dried adult (female, LMCI 175–34). Scale bars = 5, 5, 1, 2, 2 mm, respectively.

https://doi.org/10.1371/journal.pone.0201251.g001

by a patchy distribution of their hostplants, and 6) adults are short lived and supposedly do not disperse much. These characteristics make them ideal candidates for testing hypotheses related to the role of historical biogeography in lineage diversification.

Eucecidoses in particular is ideal to be explored within such a phylogeographic scenario since it includes only one species (*E. minutanus*; Fig 1B–1F), uses only one highly polymorphic species as hostplant (*S. polygamus*; Fig 1A) and has a wide geographic distribution. It ranges from east to west through most of southern South America with scattered populations located in the South American Transition Zone, Chaco and Parana dominions that include five provinces (*sensu* Morrone [18]), thus intraspecific divergence is expected in this case. In addition to the major tectonic events mentioned above, putative vicariant processes driven by past climate oscillations during glacial and interglacial periods might have influenced the genetic diversity in *E. minutanus*, as predicted by the climate refuge hypothesis for Neotropical species (e.g. [19–21]). Additionally, the particular life history of these gall-inducing micromoths in association with localized host plant distributions might limit dispersal among populations and lead to differences even in the absence of physical barriers.

In this case study, we take a phylogeographic approach to understand better the evolutionary history of *Eucecidoses* in the Neotropical region. We began by mapping the historical geographic distribution of this cecidosid moth in association with that of its host plant, using material preserved in herbaria. Then we sampled specimens of extant populations of *E. minutanus* from most of its distribution range, which were used for DNA sequencing of mitochondrial loci. We address two main questions specifically: (1) how strong is the genetic structure in this species across distinct biogeographic provinces and (2) what is the temporal depth of the mtDNA genealogy. In addition to these questions, phylogenetic and phylogeographic data together with estimates of divergence times were used to develop hypotheses for the historical

processes that have shaped lineage diversity in *E. minutanus*. Our results may be reconciled with two scenarios to explain current patterns found in *Eucecidoses*: (i) individuals from a centrally distributed population dispersed to colonize new areas, or (ii) a highly connected population lost connectivity with its peripheral populations and thus strengthened its genetic structure. We also estimated the areas of ancestral distribution and the events of diversification among the lineages of *E. minutanus* using the Bayesian Binary Method (BBM) and Statistical Dispersal–Vicariance analysis (S-DIVA). Findings are discussed in a broader context, thus being informative about Neotropical diversification in general.

Material and methods

Study area and sampling

Prior to the beginning of this study, *E. minutanus* was known to exist in four populations (Fig 2A) located within the Buenos Aires (type locality) and Mendoza provinces, Argentina [22–24], and in Paraná state, Brazil [25]. These records came from either reared adults or collected galls associated with *Schinus polygamous* (Cavanilles) Cabrera (Anacardiaceae) *sensu lato*, as discussed below. These biogeographic dominions were visited from 2011 to 2014; populations of *S. polygamus* were progressively located and searched for the presence of *E. minutanus* galls. Mature galls (lignified and operculate; Fig 1B) were collected from 21 sites across three biogeographic dominions of South America (*sensu* Morrone [18]); the Parana (Pr), Chacoan (Ch) and South American Transition Zone (Sa).

These sites include eight populations covering most of the range of *Eucecidoses*, corresponding to biogeographic provinces of Parana forest (Pr_{1_2}), Araucaria Forest (Pr_{3_11}),

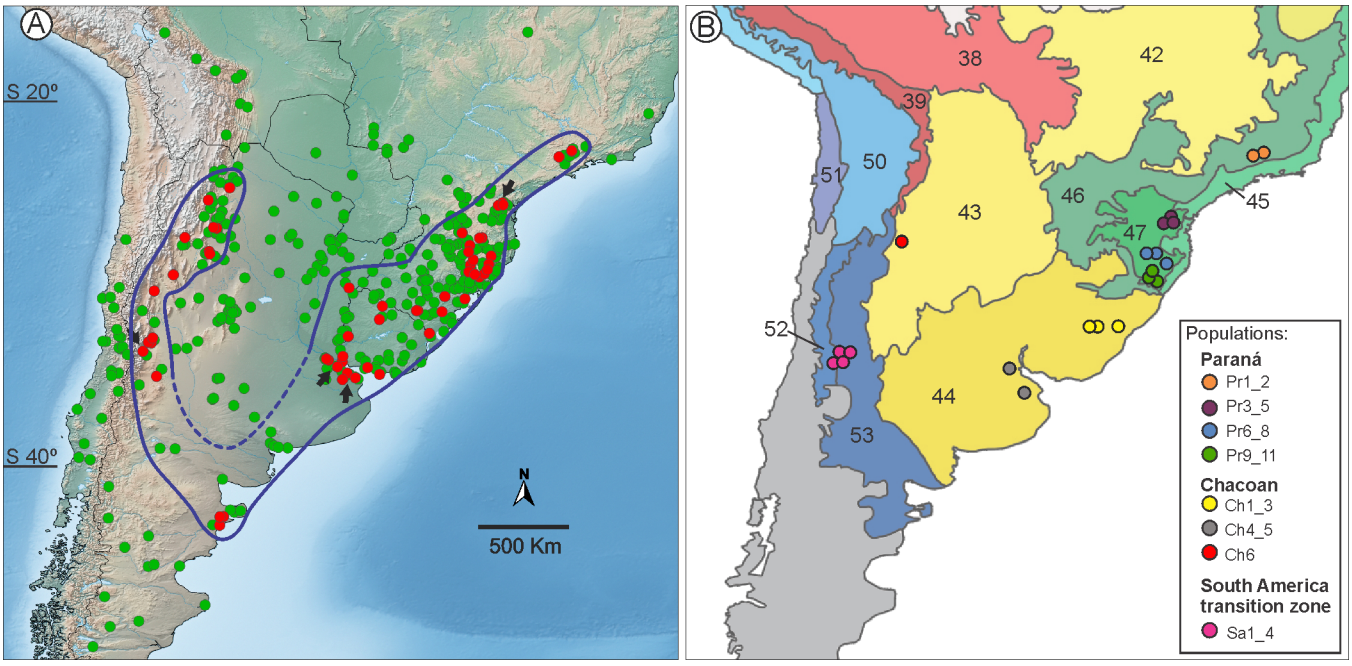

Fig 2. Geographic distribution of *Schinus polygamus* (green dots) and *Eucecidoses minutanus* (red) in South America (A), and populations sampled in this study (B), according to dominions proposed by Morrone [18]. The distribution of *E. minutanus* is circumscribed by solid and dashed blue lines on the left map; the latter type of line represents a hypothetical section. The only four records known for *E. minutanus* prior to this study are indicated by black arrows. See Table 1 and S1 Appendix for complete description of localities. Areas in shades of red, green, yellow and blue on the right map represent South Brazilian (Sb), Parana (Pr), Chacoan (Ch) and South American transition zone (Sa) dominions, respectively. Numbers represent corresponding provinces: 38, Rondonia; 39, Yungas; 42, Cerrado; 43, Chacoan; 44, Pampean; 45, Atlantic; 46, Parana Forest; 47, Araucaria forest; 50, Puna; 51, Atacaman; 52, Prepuna; 53, Monte.

https://doi.org/10.1371/journal.pone.0201251.g002

Pampean (Ch_{1_5}), Chacoan (Ch_6), and Monte (Sa_{1_4}) (Table 1, Fig 2B). Distinct localities (up to 4, at least 50 km apart) were chosen to replicate site sampling (Table 1). Immature stages (either last instar larva or pupa) dissected from galls (eight/locality) were kept frozen at -20 ^{o}C for later DNA extraction.

Field collections in Brazil were made under IBAMA/ICMBio license number 2024629, granted to G. R. P. Moreira. Specific authorization for each locality was not required since we collected galls on plants located either on road borders or private farms, with the permission of owners. None of these plants was located within or nearby protected areas, and samples did not involve endangered or protected species. Permission for field study in Argentina was approved by the Dirección de recursos naturales renovables, Mendoza (Res. 196/13 and 1109/17) and Dirección de flora y fauna silvestre y suelos, Tucumán (Res. 189/14). In Buenos Aires we collected in private lands (owners gave permission to conduct the study on sites). Field studies in Argentina did not involve any endangered or protected species. Adults were reared under room temperature in the laboratory from additional field-collected mature galls that were maintained in small plastic vials. Rearing followed the recommendations of the Animal Care and Use Committee (CEUA) of the Federal University of Rio Grande do Sul (UFRGS). After emergence they were pinned and dried, and assigned to *Eucecidoses* based on comparative analysis using wing venation and genitalia [22, 25]. Adult cecidosids are rarely represented in insect collections, which would preclude using only them in any robust biogeographic analysis. However, our preliminary observations indicated this is not the case regarding their galls existing in plant herbaria (S1 Fig). Only two cecidosid species (*Cecidoses eremita* Curtis and *E. minutanus*) induce external, spherical galls on *Schinus* branches [14, 22, 23]. Although similar in shape, these galls can be distinguished by their general size, wall thickness and operculum shape. Those of *C. eremita* are larger and have a thicker wall; they have double the diameter and thickness of those of *E. minutanus* ([22]; for precise dimensions, see Loetti et al., [24]). The operculum in *C. eremita* looks like a stopper that was described by Curtis [26] as *". . .having the diameter of the inside less than that of the external surface, which forms a broader rim*". This characteristic allows prompt separation of these galls independently of their general size, since by contrast the operculum of *E. minutanus* looks like a thin, flat cover (S1 Fig).

These aspects allowed us to search for additional geographic records (and extant populations) of *E. minutanus* by examining the dried material of *S. polygamus* preserved in the main herbaria existing in the region (complete list is presented in S1 Appendix).

The presence of one or more *E. minutanus* galls in a given *S. polygamous* exsiccate was used as evidence of its presence in that locality. Conversely, the absence of such galls in exsiccates would not necessarily demonstrate its historical absence in corresponding populations, since its presence there depends on other uncontrolled factors such as the collector and curator (i.e. their own decision about whether or not to collect and include this kind of plant material (galls) in collections). We assumed that this effect would be randomly distributed in this case, and that it should be diluted by increasing the number of herbaria visited. Thus, the method we adopted here may have underestimated the frequency and boundaries of the geographic distribution of *E. minutanus*, but it should still be valid to infer its pattern compared to that of the host plant. In South American herbaria, *C. eremita* and *E. minutanus* galls are found on dried-preserved material belonging to *Schinus* Linnaeus, subgenus *Duvaua* (Kunth) F. A. Barkley, section *Euduvaua* F. A. Barkey, which includes ca.15 species having single leaves and spine branches [27]. The taxonomy of species of *Schinus* is confused, however; species identification is difficult—the genus is under review (C.L.S. Luz, USP, pers. com.). For some authors (*e.g.* [28–30]), spatial variation in corresponding populations results from phenotypic plasticity, and thus they should all be treated in this section as a single species, *S. polygamus*. However, for others (*e.g.* Barkley, [31]), only those populations located west of the Andes should be

Table 1. Characterization of *Eucecidoses* specimens used in this study. *Province*, biogeographic province; *Dominion*, biogeographic dominion (both assigned according to Morrone [18]).

Dominion	Province	Pop.	Site	Location	Lat (S); Long (W)	Vouchers	Clade
Parana							
	Parana Forest	Pr_{1_2}	1	BR: São Paulo, Campos do Jordão	22° 44′ 34″; 45° 35′ 47″	LMCI 270-3A, B, C, D	Lineage 3
			2	BR: São Paulo, São Bento do Sapucaí	22° 24′ 41″; 45° 26′ 07″	LMCI 271-8A, B, C, D	Lineage 3
	Araucaria Forest	Pr_{3_5}	1	BR: Paraná, Almirante Tamandaré	25° 19′ 09″; 49° 18′ 15″	LMCI 213-2A, B, C, D	Lineage 2
			2	BR: Paraná, Curitiba	25° 25′ 43″; 49° 16′ 01″	LMCI 14-51A, B, C, D	Lineage 2
			3	BR: Paraná, Campo Largo	25° 27′ 34″; 49° 31′ 38″	LMCI 214-2A, B, C, D	Lineage 2
		Pr_{6_8}	1	BR: Santa Catarina, São Cristóvão do Sul	27° 16′ 01″; 50° 26′ 21″	LMCI 201-19A, B, C, D	Lineage 4
			2	BR: Santa Catarina, Curitibanos	27° 16′ 58″; 50° 34′ 51″	LMCI 205-37A, B, C, D	Lineage 4
			3	BR: Santa Catarina, São Joaquim	28° 17′ 33″; 49° 56′ 16″	LMCI 204-12A, B, C, D	Lineage 4
		Pr_{9_11}	1	BR: Rio Grande do Sul, Vacaria	28° 30′ 50″; 50° 56′ 02″	LMCI 206-8A, B, C, D	Lineage 4
			2	BR: Rio Grande do Sul, Campestre da Serra	28° 47′ 34″; 51° 05′ 40″	LMCI 207-4 to 207-6	Lineage 4
			3	BR: Rio Grande do Sul, S. F. Paula	29° 26′ 45″; 50° 34′ 50″	LMCI 165-2 to 165-5	Lineage 4
Chacoan							
	Pampean	Ch_{1_3}	1	BR: Rio Grande do Sul, Canguçu	31° 23′ 47″; 52° 40′ 43″	LMCI 175-3A, B, C, D	Lineage 1
			2	BR: Rio Grande do Sul, Bagé A	31° 19′ 41″; 54° 02′ 01″	LMCI 268-6	Lineage 1
			3	BR: Rio Grande do Sul, Bagé B	31° 19′ 47″; 54° 06′ 00″	LMCI 269-5	Lineage 1
		Ch_{4_5}	1	AR: Buenos Aires, Zarate	34° 50′ 42″; 59° 01′ 26″	LMCI 240-36 to 240-39	Lineage 1
			2	AR: Buenos Aires, Brandsen	35° 10′ 30″; 58° 14′ 11″	LMCI 240-22,240-24,240-25	Lineage 1
	Chacoan	Ch_6	1	AR: Tucuman	26° 43′ 57″; 65° 16′ 01″	LMCI 298-6A, B, C, D	Lineage 5
South America transition zone							
	Monte	Sa_{1_4}	1	AR: Mendoza, Manzano Historico	33° 35′ 52″; 69° 22′ 56″	LMCI 240-1,240-3,240-5,240-6	Lineage 6
			2	AR: Mendoza, Cuesta Cerillos	33° 70′ 20″; 68° 55′ 01″	LMCI 197-3,197-4,197-7,197-8	Lineage 6
			3	AR: Mendoza, Lujan de Cuyo	33° 20′ 02″; 68° 52′ 56″	LMCI 163-21A, B, C, D	Lineage 6
			4	AR: Mendoza, Las Heras	32° 51′ 02″; 68° 50′ 25″	LMCI 240-14,240-17 to 240-19	Lineage 6

Pop, general code used to identify a given population site within a biogeographic dominion.

Site, replicate (= identification number within a given population).

Location, Country, State, Municipality/political Province where samples were collected (BR = Brazil, AR = Argentina)

Coordinates, geographic coordinates (degrees, minutes, seconds—DMSo)

Vouchers, individuals collected and deposited in the LMCI = Laboratório de Morfologia e Comportamento de Insetos (UFRGS), Porto Alegre, RS, Brazil. Clade, Lineage groups defined in the phylogenetic analysis.

https://doi.org/10.1371/journal.pone.0201251.t001

considered as belonging to *S. polygamus*. The remainder would be split into closely related species that are differentiated from each other by details of leaf anatomy and reproductive structures. The latter include *S. fasciculatus* (Griseb.) I.M. Johnst., *S. longifolius* (Lindl.) Speg. and *S. johnstonii*in Argentina [27, 31], and *S. engleri* Barkley in the mountainous areas of southern Brazil [32], all presumably used as host plant by *Eucecidoses*. This aspect precluded us from approaching the role of the host plant in speciation, if any, at a small geographical scale. To overcome this taxonomic limitation and make analysis from a broad geographic perspective feasible, we treated all as *S. polygamus* (*sensu lato*), taking into account as synonyms species listed by Cabrera [28] and Fleig [29, 30] (see S1 Appendix).

Laboratory procedures

Total genomic DNA was extracted from tissue samples (larvae, pupae and/or adults) using the PureLink Genomic DNA Kit (Life Technologies). About 2.7 kb of mitochondrial loci were amplified via polymerase chain reaction (PCR), including the cytochrome oxidase subunit I (*CoI*), tRNALeu, cytochrome oxidase subunit II (*CoII*) and rRNA16S [33, 34]. PCR was performed using 10 ng genomic DNA, 10 pmol each primer, 1 U Taq polymerase (Life Technologies, California, USA) and 1.5 mM $MgCl_2$ in a volume of 20 uL using a ABI thermal cycler (Applied Biosystems, Foster City, California, USA). The cycling parameters of the PCR, primer sequence and amplified length of each locus are described in S1 Table. PCR products were purified using the enzymatic method of Exonuclease and Alkaline Phosphatase IT (ThermoFisher Scientific, USA). The sequencing products were separated in an ABI 3730xl DNA analyzer (Applied Biosystems, California, USA). Sequences and raw sequence chromatograms were visualized, edited and aligned using CodonCodeAligner v5.1.5 (Centerville, MA, USA). All sequences generated in this study are available in the GenBank database, accession numbers: MH667739—MH667811 (*CoI*, tRNA-Leu, *CoI*) and MH667669—MH667738 (rRNA16S) (S2 Table).

Phylogenetic analysis and divergence times

Maximum likelihood (ML) and Bayesian approaches were used for phylogenetic inference on the mtDNA-concatenated dataset. The appropriate substitution model and optimal partitioning were determined using PartitionFinder v1.1 [35]. The GTR + Gamma model proved most suitable according to the Bayesian information criterion for all markers individually as well as the combined dataset. We first reconstructed ML trees using the PhyML plugin in Geneious v.11.0.4 [36] for each genetic marker individually and assessed them for contamination issues or conflicting signals, and then we repeated that approach for the combined markers. There was no incongruence between the phylogenetic signals of different datasets. In all subsequent analyses, the dataset was analyzed following the partitioning from PartitionFinder: four partitions mostly following the division of gene fragments, as well as the second and third codon positions of *CoI*. Searches were based on 100 bootstrap replicates, followed by a thorough ML search. Clades with bootstrap values $> 70\%$ were considered as strongly supported, following Hillis and Bull [37]. Bayesian inference was performed in the BEAST v2.4.3 software [38]. Three independent Markov Chain Monte Carlo (MCMC) runs were performed, each with four streams per 50 million steps of the MCMC, sampled every 5,000 generations and discarding 5 million burn-in (about 10% of trees discarded), starting the initial trees randomly, without restriction. We assessed chain convergence by comparing the results of independent runs, and considered MCMC sampling sufficient when ESS reached > 200 for all parameters. Convergence and the effective sample size of all MCMC runs were checked in Tracer 1.6 (http://tree.bio.ed.ac.uk/software/tracer/). The software DensiTree [39] was used to draw the 8,000

tree set transparently. This allows one to evaluate properties of the tree such as well-supported clades and topological uncertainty. The tree set was processed in TreeAnnotator v1.8 (supplied with the BEAST package); a consensus tree was obtained, displayed and edited with FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/). The time of the most recent common ancestor (TMRCA) for relevant nodes and major mitochondrial clades was reported as the mean value of node height with 95% highest posterior density interval (HPD). Support of nodes was provided by clade posterior probabilities (BPP) directly estimated from the consensus topology. Those nodes with BPP > 0.95 were considered strong according to Erixon *et al.*, [40]. The cecidosid species *Oliera argentinana* and *Cecidoses eremita* were incorporated in the analysis based on the sister relationship proposed by Moreira *et al.* [23]. The tree was rooted with representative species of Adeloidea: *Prodoxus quinquepunctellus* (Prodoxidae) and *Incurvaria masculella* (Incurvariidae) [15, 41, 42]. Divergence times were estimated in BEAST 2. For a speciation tree prior we ran the Birth-Death Process, Yule Pure Birth, Coalescent Constant Size and Coalescent Exponential Population under a comparative framework; all retrieved the same tree topology and highly similar parameters (Table 2). Plots of parameters for stationarity and for effective sample (ESS) appeared stable and with high values (>200) in all cases. We repeated the analyses to ensure that topologies were consistent between independent MCMC chains. Since the birth-death tree has successfully been used in modeling speciation and extinction—it is used as a prior distribution when inferring phylogenies using Bayesian methods [38], and this was the lowest likelihood modelled with our dataset, we chose it as the prior. We tested whether our data followed a strict or a relaxed molecular clock; then we examined the coefficient of variation of the branch rates using the lognormal relaxed molecular clock model. The coefficient of variation was 1.78 (HPD 1.47–2.02), suggesting departure from a strict molecular clock (a condition in which the coefficient of variation equals zero). Therefore, divergence times were estimated allowing branch lengths to vary under a lognormal relaxed clock [43], following a normal distribution centered on the fossil age, reflecting the bi directionality of uncertainty inherent in such calibrations [44]. We applied a calibration to set the prior on 120 ±10 Myr [45] for the crown clade of the Adeloidea (Cecidosidae (Prodoxidae, Incurvariidae)), using a fossil from the Early Cretaceous [46] assigned to Incurvariidae. Evolutionary relationships among families within Adeloidea are not fully resolved [42].

Genetic and geographic structure

Standard diversity indices (number of different haplotypes, haplotype and nucleotide diversity) were estimated in the program Arlequin v3.5 [47]. This program was also used to assess the level of genetic structure (i.e., gene flow) among subpopulations using φST, which is analogous to Wright's *F*-statistics but takes into account the genetic distance among haplotypes [47]. The influence of biogeographic scenarios (i.e. major geographic distances [western *vs.* eastern groups], dominions and provinces) in the allocation of intraspecific variation was investigated through the analysis of hierarchical levels of molecular variance (AMOVA;

Table 2. Speciation tree priors run in Beast 2. *Eucecidoses* dataset comprised by 2,720bp (CoI = 1560 bp, CoII = 688 bp, and rRNA = 470 bp) of mitochondrial DNA sequences.

Model	LnL	TreeHeight	Kappa	Rate	
				Mean	Variance
Birth-Death	-7215.164	118.936	3.090	$1.092E^{-3}$	$1.447E^{-7}$
Calibrated Yule	-7228.528	119.907	3.092	$1.539E^{-3}$	$1.975E^{-7}$
Coalescence Constant population	-7230.097	119.961	3.091	$1.128E^{-3}$	$1.642E^{-7}$
Coalescence Exponential population	-7230.223	119.896	3.095	$1.111E^{-3}$	$1.898E^{-7}$

https://doi.org/10.1371/journal.pone.0201251.t002

Excoffier *et al*. [48]) also in the program Arlequinv3.5. Pairwise genetic distance among lineages was estimated based on Kimura 2-parameter (K2P) model [49], with 1000 bootstrap of replication, and are presented in percentage.

We constructed an intraspecific genealogy using the concatenated dataset to represent evolutionary relatedness among individuals. A median-joining method [50] was implemented using the software Network 6 (http://www.fluxus-engineering.com/) to test the influence of isolation by distance (IBD) on the genetic structure of *E. minutanus*. The relationship between genetic and linear geographic distances of populations was evaluated using Mantel tests [51]. The significance of IBD values was assessed by the Mantel procedure (1,000 randomizations) using Arlequinv3.5.

Since historical demography might also have had an essential role in generating the variability found in *E. minutanus*, we performed a population expansion for each lineage. Deviations from the null hypothesis of constant population size were tested using Tajima's D and Fu's Fs neutrality tests [52] in the program Arlequin v3.5. Model fit to the data was tested using the sum of squared deviations (SSD) and the raggedness index (*Hri*) [53] with 1000 bootstrap replicates. We also performed a Bayesian Skyline plot (BSP) analysis, which does not assume an *a priori* growth model and infers effective population size through time based on coalescent theory [43]. The BSP was estimated in the program BEAST 1.8.4, run for 50 million iterations and sampled every 5000 steps, assuming the relaxed clock model and a normal distribution for the substitution rate, with a mean of 1.12% Myr^{-1} (Table 2) and a standard deviation of 0.13% Myr^{-1} to allow for some uncertainty in the evolutionary rate. The first 10% of the iterations were discarded to allow for burn-in. To assess the robustness of parameter estimates, two independent chains were run with identical settings. Log-files were analyzed in Tracer 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) [52], and effective sample sizes were used to evaluate MCMC convergence within chains.

Ancestral reconstruction and diversification

We reconstruct the ancestral areas and estimate diversification patterns within the genus *Eucecidoses*. Historical biogeographic processes likely shaped the current distribution of lineages and we used this approach to infer current patterns and to test whether extant populations derived from an ancestral species that colonizes novel areas (dispersal), or diverged from ancient geographical barriers (vicariance). We thus used two approaches for ancestral character reconstructions: Statistical Dispersal-Vicariance Analysis (S-DIVA) v 2.0 [54, 55] and the Bayesian Binary MCMC method (BBM) [56] as implemented in RASP v.3.0 [57]. The RASP (Reconstruct Ancestral State in Phylogenies) is a tool to reconstruct evolutionary histories using Bayesian phylogenetic inference, which reveals the probability of each possible range of an ancestral area to each node based on previously indicated biogeographic areas. This method consists of using all generated trees from the MCMC output and calculates the average frequency of an ancestral range at a node in ancestral reconstructions. The BBM method was performed using 500,000 generations with 10 chains, sampling every 100 generations; the first 500 trees were eliminated (burn-in). Fixed JC (Jukes-Cantor) were used. We defined five areas to assess the historical biogeography of the *Eucecidoses*: (A) Parana Forest, (B) Araucaria Forest, (C) Pampean, (D) Chacoan and (E) Monte, based on the classification of provinces proposed by Morrone [18].

The chronogram obtained by BEAST analysis was used to construct semi-logarithmic lineages through time (LTT) plots in TRACER version 1.5. To examine confidence in the estimated dating, confidence intervals (95%) were estimated using 2000 trees from the pool of converged Bayesian trees in the BEAST analysis. To test for significant departures from the

constant speciation rate model we used the γ-statistic [58]. The gamma statistic of Pybus and Harvey [58] relates to the distributions of internode distances through time, and under the pure birth model follows a standard normal distribution with a mean of zero.

Results

Phylogenetic analysis

The total dataset comprised 2,720bp (CoI = 1560 bp, CoII = 688 bp, and rRNA = 470 bp). The alignment was straightforward for protein-coding genes, as no internal stop codons were detected. Bayesian and ML analyses revealed the same topology with six strongly supported clades (hereafter defined as lineages 1 to 6) with marked phylogeographic structure. Since we also estimated divergence time, we selected the Bayesian tree as the basis for discussions (Figs 3 and 4). Lineage 1 is composed of populations Ch_{1_3} + Ch_{1_5} from the Pampean province, which covers the type locality and therefore the original description of *E. minutanus*. Novel monophyletic groups found within *Eucecidoses* are represented by lineages 2 to 6 (Figs 3 and 4). The South American Transition Zone presented only one (lineage 6), while the Chacoan and Paraná dominions showed two (lineages 1 and 5) and three lineages (lineages 2, 3 and 4), respectively. We found highly divergent lineages in the Pampean (1 [Ch_{1_5}]) and Chacoan provinces (5 [Ch_6]), both within the Chacoan dominion (Table 3). Similarly, within the Paraná dominion (Araucaria and Paraná Forest) we found lineages 2 [Pr_{3_5}], 3 [Pr_{6_8} + Pr_{9_11}] and 4 [Pr_{1_2}], with different levels of divergence. The K2P genetic distance between these six lineages ranged from 2% to 11% (Table 2). Within each lineage variability varied from 0.1% to 1%.

Estimation of divergence times, biogeography and diversification analyses

The mtDNA substitution rate observed in *Eucecidoses* was slow, ca. 1% per Myr (Table 2). Divergence time estimate indicated that the TMRCA of *Eucecidoses* split back in the Paleocene, ca. 65 Mya (95% HPD: 37.94–89.32) (Fig 3). The initial diversification event occurred ca. 38 Mya (95% HPD: 23.70–53.73) giving rise to two major clades. The first one diversified ca. 25 Mya (95% HPD: 10.95–39.47) and is formed by lineages 5 and 6 in the Chacoan (Ch_6) and Monte (Sa_{1_4}) provinces, respectively; the second major clade split around 20.5 Mya (95% HPD: 12.69–29.26), and originated lineages 1 to 4 in the Parana and Araucaria forests and Pampean provinces (Fig 3). The clade that includes groups from the forest provinces (lineages 2, 3 and 4) emerged around 17 Mya (95% HPD: 10.10–24.47). Although most of these clades emerged in the Paleogene Period, the date estimated for each of six particular lineages is quite recent as the Late Pliocene, including the Quaternary period (<3 Mya) (Table 4; Fig 4).

The oldest lineage is 6 from the Monte region near the Andes Mountains, and the youngest ones are found in the forest provinces (lineages 2, 3 and 4), except lineage 5 from the Chacoan. A marked geographic structure was evident within the Paraná domain. Lineage 2 was divergent from the others within the same province in ca. 4%). Results from the BBM analysis suggest a complex biogeographical history in which both dispersal and vicariance have been important in shaping the current distribution pattern in *Eucecidoses* (Fig 5).

Lineages 5 and 6 indicated by the phylogeny are derived from the DE area (Chacoan + Monte). Thus a vicariance event is evident at this node, resulting in the present Chacoan (D) and Monte (E) populations. For lineages 1 to 4 the BC area is suggested (Araucaria Forest + Pampean) as a likely origin of these groups. From this node, events of vicariance and dispersal are suggested. Accordingly, lineage 1 (Pampean province [C]) might have diverged from the others through a vicariance event. Other lineages (2 to 4) shared area B (Araucaria forest) as ancestral, thus from a dispersal event lineages probably dispersed and occupied new areas through provinces (B) [Araucaria forest] and AB [Parana and Araucaria forest]. Later,

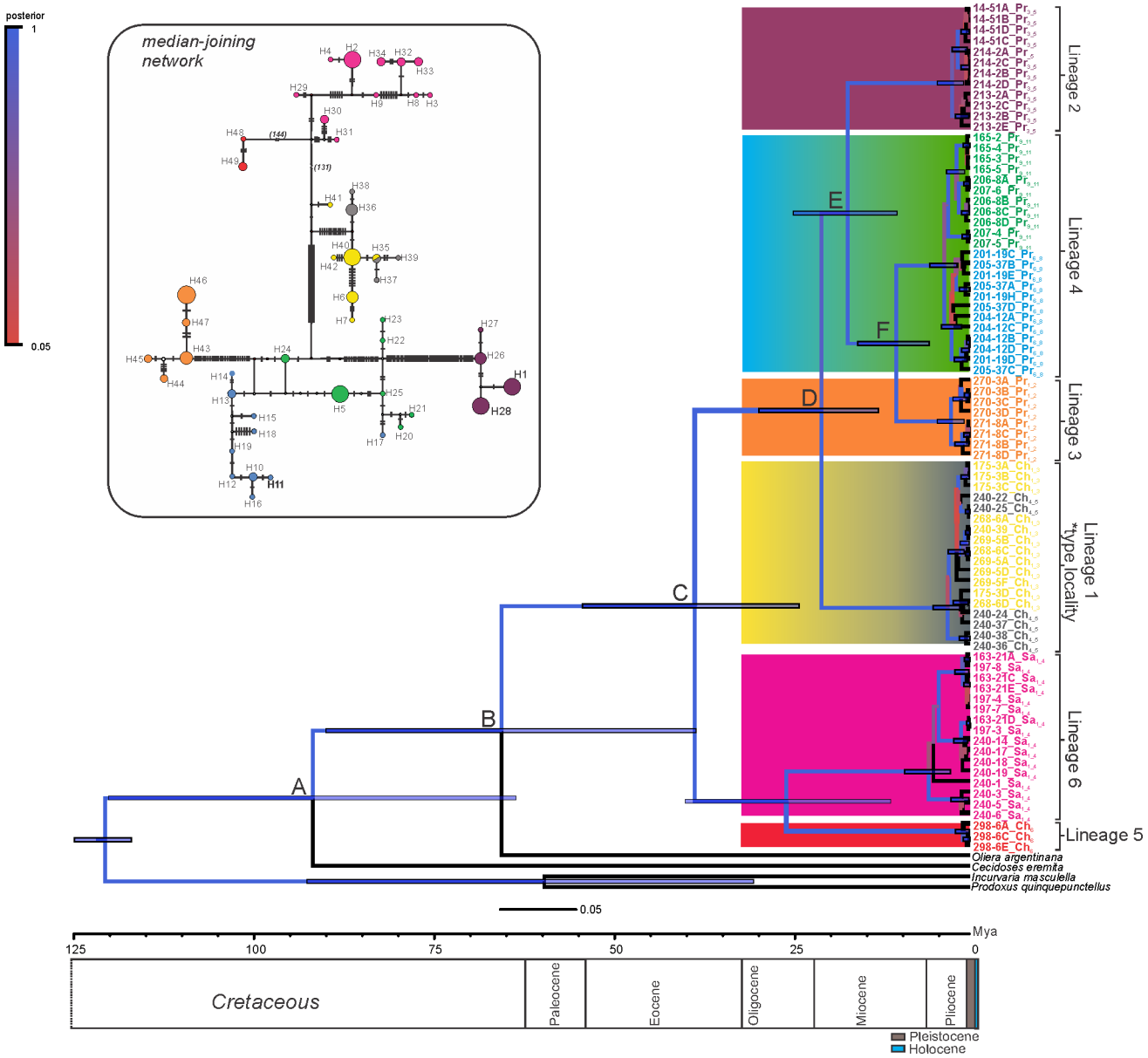

Fig 3. Evolutionary relationships within *Eucecidoses minutanus*. Phylogenetic tree under the relaxed uncorrelated lognormal clock Bayesian analysis reconstructed based on 2.7 Kb of mitochondrial sequences using birth-death tree prior. Capital letters at nodes (A-F) indicate major clades referred to in Table 4. Populations are indicated by the colored terminals (See Table 1 for further description). Colored squares represent the six lineages inferred; those that encompass more than one population are indicated by a gradient of colors. Posterior probabilities are indicated by the colored branches and the legend inside the figure. Mean time to the most recent common ancestor is indicated in the middle of the branches, and 95% credibility intervals (95% HPD) are represented by solid blue bars, in millions of years (Mya). The ruler at the bottom represents the rate of substitution per site. A median-joining network of haplotypes found in all populations in presented on left top. Corresponding bars along branches represent mutational steps. Circle size is proportional to haplotype frequency. Small red circles represent median vectors.

https://doi.org/10.1371/journal.pone.0201251.g003

there was likely a vicariance of lineages 3 and 4, between A (Parana forest) and B (Araucaria forest) areas.

Node 161 in Fig 5 represents all *Eucecidoses* lineages, suggesting that the ancestor originated in area C (Pampean), with a marginal probability of 100%. Similarly, as in the S-DIVA analysis, dispersal events associated with vicariance are evident in the common ancestor node of

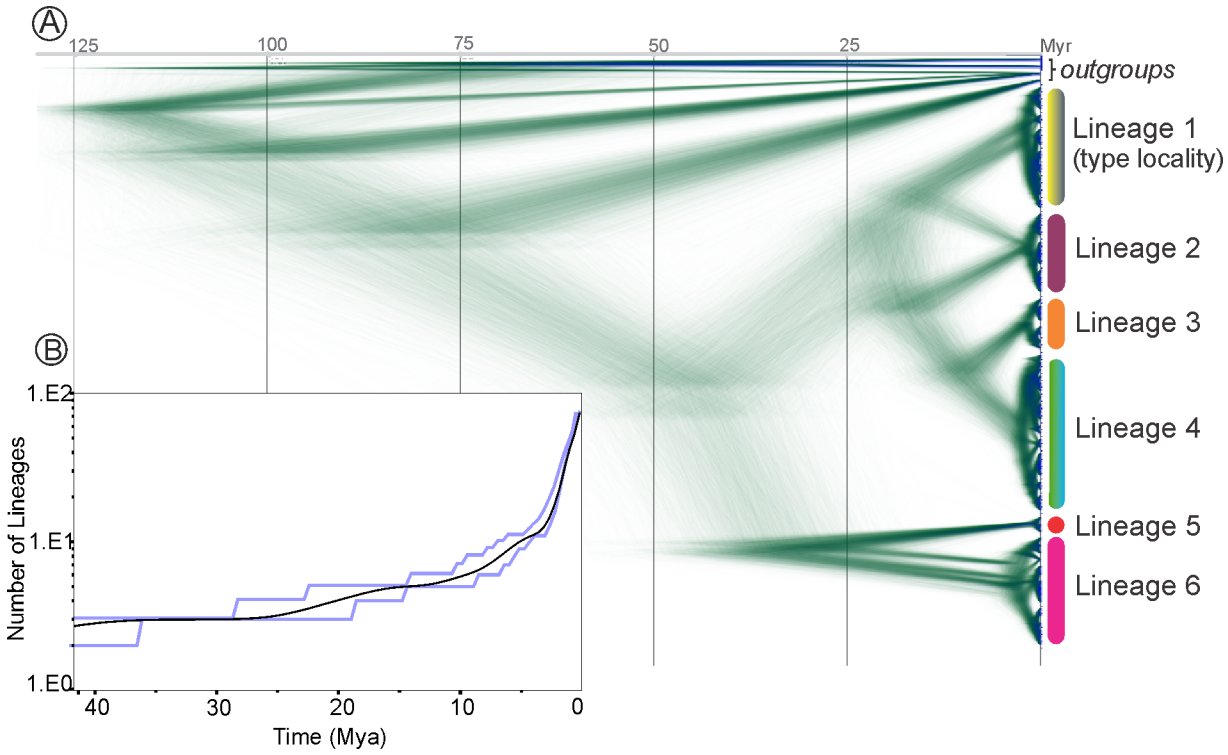

Fig 4. Evolutionary diversification in *Eucecidoses minutanus* resulting from Bayesian analysis based on 2.7 Kb of mitochondrial sequences. A, Densitree, a consensus posterior density of trees representing the entire posterior distribution of populations (after 8,000 trees of burnin) from the BEAST analysis. Areas where species trees agree on topology and/or branch lengths are densely colored. Variation in timing of divergences (in Myr) is shown as fuzziness along the *x*-axis. All nodes have support values > 0.99. The six clades found in the phylogenetic reconstruction (see Fig 3) are indicated by the color bar on the right, defined as lineage 1 to 6 (see Fig 3 for details). B, Lineage through time (LTT) plot (black line) highlighting a late speciation (or early extinction) pattern of diversification. Confidence intervals (95%), estimated using 2000 trees from the pool of converged Bayesian trees in BEAST analysis, are also indicated (blue lines).

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Eucecidoses. However, node 160 is ambiguous, and ancestral reconstruction for lineages 5 and 6 could not be determined. There are three possibilities: D (marginal probability of 42.22%), E (25.56%) and C (21.29%). Dispersal events associated with vicariance are also suggested by this node. The ancestral reconstruction at node 142 that represents members of lineages 1 to 4 postulates that the ancestors probably originated in the C (Pampean) area, with 79.38% marginal probability. Dispersion events associated with vicariance are evident in this node. In addition, as indicated in the S-DIVA analysis, linage 1 split from the other three lineages. Node 124 concentrates the other lineages present in the provinces of Parana forest (A) and Araucaria forest

Table 3. Genetic divergence among six lineages of *Eucecidoses* based on 2.7 Kb of mitochondrial sequences. Above diagonal: pairwise phi (ST) values based on haplotype frequency only, which indicates low rates of gene flow among all lineages. All comparisons were statistically significant; $P \leq 0.05$). Below diagonal, pairwise K2P distance (numbers in brackets indicate diversity within populations). See Fig 3 for detailed description of lineages.

Lineage	1.	2.	3.	4.	5.	6.
1.	[1.2%]	0.8539	0.7989	0.7766	0.9153	0.8955
2.	4.3%	[0.2%]	0.9577	0.8839	0.9850	0.9358
3.	3.4%	4.0%	[0.2%]	0.7581	0.9816	0.9259
4.	3.5%	4.0%	1.9%	[0.6%]	0.9417	0.9157
5.	8.5%	8.7%	8.2%	8.4%	[0.08%]	0.9230
6.	10.7%	10.8%	10.0%	10.1%	9.4%	[0.1%]

https://doi.org/10.1371/journal.pone.0201251.t003

Table 4. Estimates of the time to the most recent common ancestor (TMRCA) for the main nodes recovered in the phylogenetic reconstruction of *Eucecidoses* using 2.7 kb of concatenated mitochondrial sequences. Clade letters are indicated in the Fig 3. populations included in each lineage are presented in brackets (for details see Table 1).

Clade	TMRCA (Mya)	95% HPD
A	91.09	62.92-119-47
B	64.98	37.94–89.32
C	38.15	23.7–53.73
D	20.56	12.69–29.32
E	16.97	10.1–24.47
F	10.24	5.67–15.54
Lineage 1 [Ch_{1_3} + Ch_{4_5}]	3.1	1.37–5.11
Lineage 2 [Pr_{3_5}]	2.48	0.87–4.5
Lineage 3 [Pr_{1_2}]	2.63	0.8–4.54
Lineage 4 [Pr_{6_8}+ Pr_{9_11}]	3.64	1.74–5.61
Lineage 5 [Ch_6]	0.91	0.1–1.98
Lineage 6 [Sa_1 + Sa_{2_4}]	5.72	2.69–9.08

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(B). Lineage 2, as well as the ancestral node 124 (with high marginal probability, 94.65%) remained in the province Araucaria forest zone. Finally, node 123 presents dispersion associated with vicariance. However, despite a marginal probability of 87.97% for Araucaria forest as the ancestral area of lineages 3 and 4, the dispersal from B (Araucaria forest) to (A) Parana forest is evident, suggesting that once the species reached a new area (through dispersion), the lineages split. The different sets of the analyses did not change the historical distribution patterns of ancestors, except that BBM analysis and S-DIVA suggest different ancestral ranges at basal nodes (S2 Fig). The maximal S-DIVA value determining support for ancestral range was 1752.48.

The lineage accumulation plot shows smooth increases in the number of lineages from the Late Pliocene (Fig 4B). The γ-statistic showed a positive value (γ = 1.941), rejecting the model of constant diversification rate (p = 0.02), and suggests accelerated diversification rate in the recent history of *Eucecidoses*.

Genetic differentiation and demographic changes

High levels of genetic variability were observed in the six lineages found (Table 5).

The number of haplotypes varied from 2 to 17 per lineage, with a total of 47 (Fig 3). Nucleotide diversity was highest in lineages 1 (Pampean) and 5 (Monte). Haplotypes are clearly geographically structured, except for lineages 4 and 1 that shared Pr_{6_8} /Pr_{9_11} and Ch_{1_3} /Ch_{4_5} haplotypes among distant populations (Fig 3). AMOVA results showed that almost half of the variation is allocated among groups (48.07%) when eastern and western groups are defined, indicating that highly divergent populations were clustered together (Table 6). Differentiation was comparatively slightly lower among groups (29.46%) when provinces and dominions were defined *a priori* but higher among populations within groups (61.4%), suggesting differentiation due to the heterogeneity of physiognomies (Table 6). The lowest portion of variance was found within populations of each biogeographic subdivision, and a non-significant difference was observed in both biogeographic groups. The genetic differentiation based on the fixation index revealed high estimates (Fst = 0.61, p<0.05) for most comparisons, indicating some level of gene flow among the six lineages (Table 3).The IBD analysis indicated significant correlation between genetic and linear geographic distance (r = 0.50, p<0.001) of the collection sites (Fig 6).

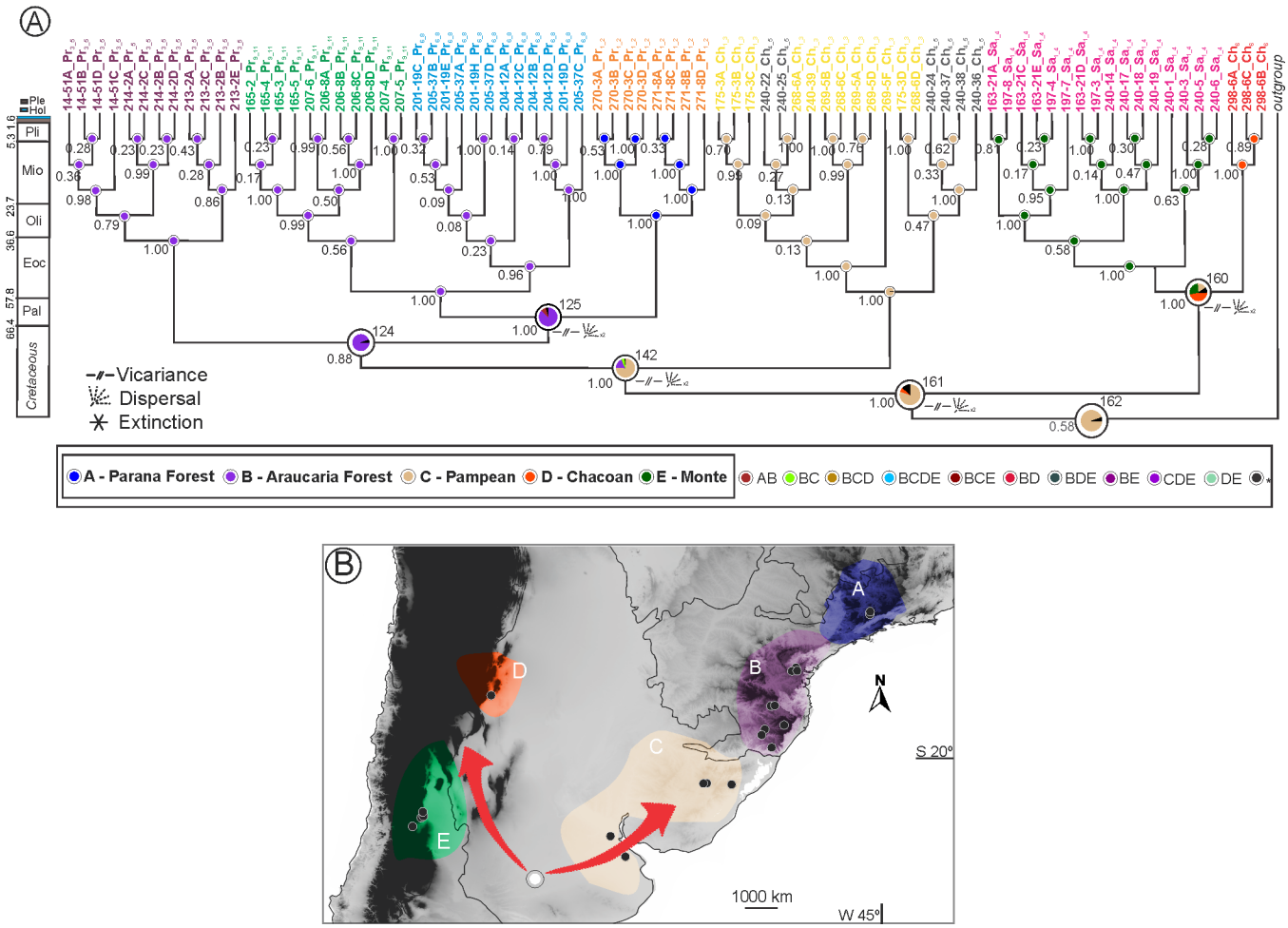

Fig 5. Ancestral area reconstruction using BBM analysis for *Eucecidoses* lineages considering five biogeographic groups. (A) Graphic output of ancestral distribution at each node of the Bayesian phylogeny of *Eucecidoses minutanus*. The tips indicate populations sampled (see Table 1 for details), while colored nodes represent geographic area estimated (indicated by the legend at the bottom of the figure). Circle sizes at nodes refer to frequency of ancestral range occurrence, with values indicated below the branch. Events of dispersal and vicariance, and extinction are indicated by graphic symbols B, Depiction of the historical biogeography scenario estimated; large circle and associated arrows refer to likely center of origin and colonization pathways.

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Tajima's D-test and Fu's Fs-test yielded non-significant results in most of the lineages. Neutrality tests results were significantly negative in four populations (Table 5), which suggests an excess of low frequency variants. Since positive selection can be neglected, we infer that the significantly negative values of the neutrality test could arise from demographic expansion. This is in agreement with recent range expansion evidence during the Pleistocene as shown by BSP plots (Fig 7).

Coalescent-based inference of demographic history through BSP indicated congruent responses among lineages. A history of stability of ancestral population sizes across the evolutionary past was observed, with relatively narrow confidence intervals (Fig 7). Recent growth around 0.5 Myr for lineage 1 and 6 is supported. BSP indicated variation in effective population size among lineages, and differences in their timing of expansion. This implies some level of differences in population trajectories, particularly with respect to topography and microclimate. However, the broader confidence intervals around most recent evolutionary nodes suggest that these inferences should be made with caution.

Table 5. Genetic variability of six *Eucecidoses* lineages based on 2.7 Kb of mitochondrial sequences. See Table 1 for detailed description of populations.

						Neutrality test			
Lineage [Population(s)]	**N**	**S**	**H**	**Hd**	**Pi**	**Tajima's *D***	**Fu's *Fs***	**SSD**	**Raggedness index**
1 [Ch_{1_3} + Ch_{4_5}]	13	91	12	0.987±0.03	0.0113±0.0020	-0.018	0.088	0.0447	0.0309
2 [Pr_{3_5}]	11	20	10	0.982±0.04	0.0025±0.0003	-0.115	-3.473*	0.0214	0.0492
3 [Pr_{1_2}]	8	12	7	0.964±0.07	0.0071±0.0016	0.598	-1.826	0.0510	0.0982
4 [Pr_{6_8}+ Pr_{9_11}]	22	66	16	0.970±0.02	0.0082±0.0012	-1.017	-1.501*	0.0211	0.0536 *
5 [Ch_6]	3	5	3	1.00±0.272	0.0035±0.0035	-	-0.077	0.2312	0.6666
6 [Sa_{1_4}]	17	251	16	0.993±0.02	0.0005±0.0073	-1.935*	-1.120*	0.0220	0.0325

N, number of specimens

S, variable sites

H, number of haplotypes

Hd, haplotype diversity (±standard error)

Pi, nucleotide diversity (±standard error)

Neutrality tests using Tajima's D and Fu's Fs. SSD and the raggedness index were used for statistical support

*P indicates significance < 0.05

https://doi.org/10.1371/journal.pone.0201251.t005

Discussion

Geographic distribution

The 54 additional geographical records provided in this study demonstrated that the distribution of *E. minutanus* is concentrated in the mountainous systems of central and northern Argentina, east of the Andes (Monte province of the South American transition zone and Chacoan dominion/province), and southern Brazil (Araucaria and Parana forest provinces of the Parana dominion). Thus, based on records presented herein its distribution range was extended north in these mountainous regions to the Tucumán and Campos do Jordão areas, respectively in Argentina (west) and Brazil (east). Additional populations were also found in the southernmost Pampean province, within the Chacoan dominion, in Rio Grande do Sul State (Brazil), Uruguay and the Buenos Aires area (Argentina), thus forming a U-shaped

Table 6. Analysis of Molecular Variance (AMOVA) using ϕ-statistics based on concatenated mitochondrial sequences (2.7 Kb) for lineages of *Eucecidoses* (defined on Bayesian phylogeny, see Fig 3) considering distinct geographical scenarios.

				Variance*		
#	**Level**	**# Groups**	**Definition**	**Va**	**Vb**	**Vc**
i)	Major geographic distance	2	Western [L5+L6] *vs*. Eastern [L1+L2+L3+L4]	48.07% ϕ_{ST} = 0.48 (p = 0.072)	44.51% ϕ_{CS} = 0.85 (p = 0.000)	7.43% ϕ_{CT} = 0.92 (p = 0.000)
ii)	Biogeographic dominions	3	Chacoan [L1+L5], Parana [L2+L3+L4], South America Transition Zone [L6]	29.46% ϕ_{ST} = 0.29 (p = 0.116)	61.42% ϕ_{CS} = 0.87 (p = 0.000)	9.12% ϕ_{CT} = 0.90 (p = 0.000)
iii)	Biogeographic provinces	5	Pampean (L1); Araucaria Forest (L2+L4); Parana Forest (L3); Chacoan (L5); Monte (L6 [Sa_{1_4}])	34.03% ϕ_{ST} = 0.34 (p = 0.235)	56.32% ϕ_{CS} = 0.85 (p = 0.000)	9.64% ϕ_{CT} = 0.90 (p = 0.000)

* Source of variation

ϕ_{ST}, Among groups (major lineages or regions)

ϕ_{SC}, Among populations within groups

ϕ_{CT}, Within populations (both regional and individual-population levels).

https://doi.org/10.1371/journal.pone.0201251.t006

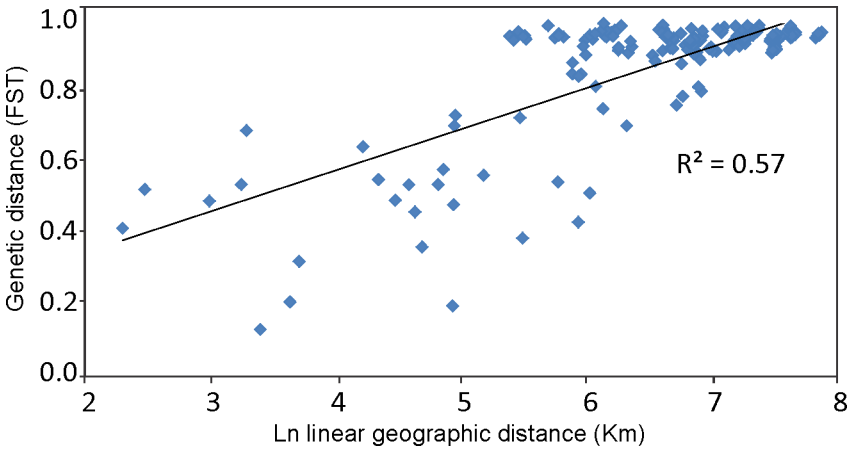

Fig 6. Isolation by distance plots of pairwise values for log geographic distance and genetic distance across collected sites of *Eucecidoses*. Genetic distance is given by φ-statistics (φST). Geographic distance (linear) is given in km. Statistical significance was assessed using the Mantel test ($r = 0.57$, $p<0.001$).

https://doi.org/10.1371/journal.pone.0201251.g006

distribution pattern, inferred provisionally in connection with those located in the Mendoza area. Efforts to localize extant populations of *E. minutanus* south of these regions in Argentina were unsuccessful. We predict that herbarium records from Chubut (Argentina) represent relict populations of *E. minutanus*, which should be further explored. Despite the existence of several populations of *S. polygamus* in the central areas located further north, we found no records for *E. minutanus* within the remaining Pampean and Chacoan provinces. Interestingly, species from other cecidosid genera such as *Cecidoses* Curtis and *Dicranoses* Kieffer & Jörgensen are known to occur within this area, for example in the Espinhal [59]. *Cecidoses* is the closest related genus to *Eucecidoses* [17], and uses the same host plant in this area. The absence of *E. minutanus* was also noted from the Patagonia area, where at least one species of *Cecidoses* is found [60]. Furthermore, *Eucecidoses* seems to have never occurred west of the Andes. *Schinus polygamus* is relatively abundant in the central regions of Chile, where in recent years we have intensively searched for cecidosids. Gall-inducing micromoths are represented there by undescribed species belonging to different genera, all associated with *S. polygamus*. However, they are not closely related to *Eucecidoses*, and will be treated elsewhere (for further discussion, see Moreira *et al.*, [17]). Thus, we provided strong evidence that extant distribution pattern of *E. minutanus* cannot be explained by variation in distribution of the host plant, but it is instead related to historical geographic events.

In association with the phylogenetic results, our study gives also strong support for the existence of a common diversification history for the two main extant *E. minutanus* lineages that are isolated in distribution in the western and eastern mountain systems. This pattern has already been found for other arthropods, including scorpions [21], freshwater crabs [13] and spiders [11]. However, as already mentioned, these studies were not conducted within species, but in higher taxonomic categories (genus and/or family), and were not based on estimates of divergence times between lineages. Here, using a phylogeographic approach jointly with a Bayesian divergence time estimation we estimated that two *Eucecidoses* moth lineages started to diverge in these mountainous systems, ca. 38 Mya. Thus, divergence might have occurred concurrently in time with fragmentation of the corresponding peripampasic orogenic arc, due supposedly to Tertiary tectonics associated with the uplift of the Andes. This event not only might have led to the divergence of these two clades, separating them geographically, but also maintained the western one (central and northern Argentina) restricted to east of the Andes.

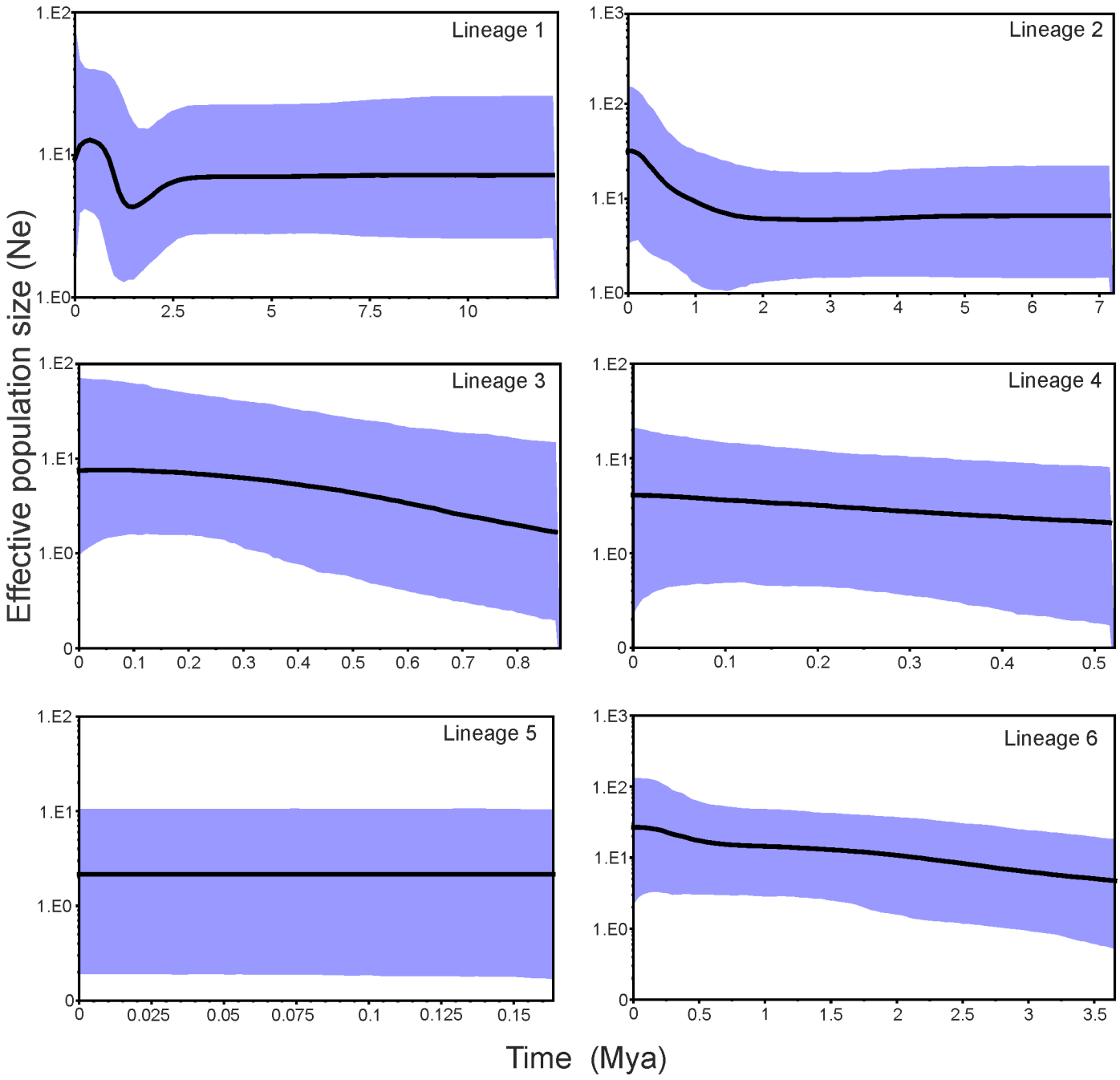

Fig 7. Bayesian Skyline Plots illustrating the demographic history pattern of six lineages of *Eucecidoses* along evolutionary time. The x-axes represent time in millions of years and the y-axes correspond to the product of effective population size and generation time in millions of years. The upper and lower purple shadows represent the 95% HPD and black line indicates mean population size.

https://doi.org/10.1371/journal.pone.0201251.g007

Cryptic diversity and taxonomic implication

The molecular phylogeny of *Eucecidoses* presented here revealed a strong genetic structure, which showed a deep evolutionary history for mtDNA haplotypes. We found six well-supported lineages, that is, five new phylogenetic units in addition to the single one *E. minutanus* currently recognized. The criteria used here to distinguish lineages (i.e., phylogenetic units), in

addition to reciprocal monophyly, was the threshold of genetic distance >2% that has been used to separate species in other micromoths, for example 1.39–2.37% in gracillariids [61]. We also considered as a cut off the inter-lineage distance (2 to 10%) being two times higher than intraspecific divergence (0.08%-1.2%) observed in our data. Whether all the six phylogenetic units found in this study should be described as distinct species remains as an open question that requires availability of morphological data to be addressed. Preliminary observation showed that adults of the two major clades (east *vs*. west groups) present stable differences in wing venation, pupal and larval integumentary morphologies. Additionally, within these clades males of each lineage may differ in their genitalia. This is the case for specimens of lineage 2 (Curitiba, Brazil) compared to those reared from the others (lineage 3 and 4) located in the east (Parana dominion), which might represent geographic structure of the same taxon. A separate publication with at least two of such lineages described as a different species, in conjunction with a taxonomic revision of *E. minutanus* will be further proposed.

The genetic differentiation found might be attributed either to isolation by distance or to marked heterogeneity of physiognomies and ecological characteristics of each region. These pathways are not necessarily mutually exclusive, and should be explored further. Despite variation in flight ability, for most lepidopteran species this is the main form of dispersal [62]. However, *Eucecidoses* is expected to present a very low dispersion capacity; like other cecidosids they may restrict their movement solely around their host plants, searching for mates and oviposition sites [63, 15]. Adults of all cecidosids have atrophied mouth appendages, and thus supposedly do not feed and live for a short time. Those of *Dicranoses capsulifex* Kieffer and Jörgensen reproduce and die in the same day of emergence [64]. These low dispersion capabilities in addition to the genetic distance observed among across provinces reinforce that populations of *Eucecidoses* can be considered different lineages, as restricted gene flow was observed, and thus fixed differences at the genotypic level.

Time of diversification and historical biogeography

Slow nucleotide substitution rates have been found in other basal Lepidopterans [45]. The divergence rate found in *Eucecidoses* was ca. 1% per My, lower than the 'standard' 2.3% estimated for mtDNA in insects [65]. This likely results from old calibration point used here and by not assuming a linear relationship of sequence divergence over this period, considering the higher rate of diversification in recently split lineages. The rate of substitution (non-synonymous) is generally considered a decreasing function of effective population size [66]. Accordingly, values of genetic diversity and demographic changes in *Eucecidoses* suggest important differences in population dynamics between biogeographic regions. Populations from the central part of the distribution are more variable and have had larger population size changes, although the credible intervals are broad since only mtDNA sequences were used in this analysis [67]. The population expansion that affected groups from the central part of the distribution is roughly coincident with the origin of several subclades within the forest lineage. Given that there is no haplotype sharing and that all subclades are restricted to a single geographic population, it may be inferred that several suitable areas were colonized (or re-colonized) during this expansion, with genetic drift and population isolation later restricting each population.

Divergences leading to extant Cecidosidae genera occurred primarily in the Cretaceous; however, diversification within each genus has mainly occurred in the Cenozoic [17]. Accordingly, molecular analyses suggest that *Eucecidoses* originated during the early Paleogene, more than 60 Mya, and since its origin has experienced a long period (± 25 Mya) without speciation. This pattern might result from widespread extinctions caused by the Cretaceous–Paleogene

event; therefore, an equal number of lineages could have become extinct. All extant lineages of *Eucecidoses* appear to have begun diversifying around 5 Mya.

Although the credibility intervals associated with diversification plot are broad, date estimated still fits within the general trend of major geological changes and climatic shifts.

Because there were no evident topographic barriers in the southern portion of the continent, the advance of an Atlantic transgression, the Salamanca Sea, covered a great part of the continent during this period; this event might have had played a role on the early timing of diversification of the group [68].

Eucecidoses first diverged into two main lineages ca. 38 Mya, during the middle Eocene. One of these lineages includes the current populations from the South America Transition Zone (Lineage 6) and the Chacoan dominion (Lineage 5), which are highly divergent. Their split coincides with the 'Inca phase' of the Andean uplift and is correlated with an intense marine regression [68]. The other major lineage that subsequently diverged into other populations from the Parana forest and Chacoan provinces began this process during the Miocene, ca.20 Mya, coinciding with three more marine transgressions known as the Paranaean Sea, which separated a few land areas and gave rise to flooded regions that later came to the surface as plains. These newly formed ecosystems, combined with the tectonic "Quechua phase" of the Andes, created a rain shadow effect that led to the first stages of differentiation of the current biogeography of South America. Furthermore, during this same period of time and climatic conditions xeric environments became established in the south, and many plant families became more abundant, including the Anacardiaceae [68]. Although not the focus of the present study, altitude may play an important role in the distribution of *E. minutanus*, since *S. polygamus* populations are located at higher elevations. Plants located on hilltops are mainly used as hosts in the Pampean province (Southernmost Brazil, Uruguay and Buenos Aires region).

During the Quaternary, climate changes in the Neotropics contributed significantly to the diversification of fauna and flora [69]. Climatic oscillations and the glaciations may have caused the fragmentation of many habitats, creating temporary and isolated areas that resulted in refuges for many groups of species [70–72]. By detecting a possible origin of the *Eucecidoses* stock lineage in the south range in the Argentinian Monte we also inferred the existence of further recent northern diversification within both derived clades. This diversification is possibly associated with vicariant and dispersion events related either to forest refuge [73] or the emerged continental shelf during the Last Glacial Maximum (around 21 kyr), which allowed forests and forest-adapted species to expand [74]. We considered the scenario of recurrent dispersal from the same source population in the case of lineages 2, 3 and 4, which likely originated from the Pampean province and lately occupied eastern forests. This hypothesis is reinforced by the result of reciprocally monophyletic populations. The current distribution of lineage 2, which follows the same biogeographic province as some other populations of the Parana dominion, is clearly genetically isolated.

In vicariance, interacting lineages usually present the same distribution range, and consequently experience similar geological events that could lead to allopatric speciation, hence showing a concordant biogeographic pattern. The Cenozoic Andean uplift had a major impact on the evolution of South American landscapes, and was a fundamental event in generating the high biodiversity of the continent, providing ecological and vicariant events of speciation [75, 76]. Quaternary climatic fluctuations may also be indicative of vicariance that took place during the *Eucecidoses* speciation, as demonstrated for several other species [77–81].

Host plant as driver of diversification

Geographically structured populations combined with genetic drift may be also going through a process of co-evolution (e.g., related to its host-plant), ultimately leading to isolation and

speciation [77]. *Eucecidoses* date to the Early Cretaceous and *Schinus* the middle Miocene [82], which reveal a discrepancy in timing for co-diversification. Thus, we suggest that *Eucecidoses* may have originated first in distinct Anacardiaceae host and secondarily used *Schinus*. The significant increase in diversification rate of *Eucecidoses* around 5 Mya, is concordant with the rise of *Schinus*. Thus, the late diversification of *Eucecidoses* did not imply that they colonized already diversified *Schinus* host. Current knowledge of the *Eucecidoses*-*Schinus* interaction, based on insect-host ranges and their intimate relationship, suggest that ecological speciation may also have been driving species shifts in this case.

As already mentioned, specimens of *Schinus* used as hosts by *E. minutanus* have been identified in many herbaria as *Schinus polygamus* (Cav.) Cabrera (*sensu* Cabrera [28]; Fleig [29, 30]). The identity of these plants is however controversial, and the genus need to be reviewed (for a discussion, see [29–31, 83]). We have strong evidence from an ongoing study (Luz C.L.S., USP, unpublished data) that this taxon may be divided into several species in the near future. Consequently, *Schinus* specimens from Mendoza, Buenos Aires, Tucumán and Curitiba used in this study may in fact correspond respectively to *S. fasciculatus* (Griseb.) I.M. Johnst., *S. longifolius* (Lindl.) Speg., *S. gracilipes* I.M. Johnst. and *S. engleri* Barkley. In a follow-up study we will be looking for correspondence between times of divergence between hosts (*Schinus*) and gall inducers (*Eucecidoses*), as well as regarding their cladogenesis in a co-speciation scenario, taking advantage of a more accurate age recently proposed for Anacardiaceae, particularly *Schinus* [82]. Finally, to make robust inferences on host specialization more than co-phylogeny analysis might be required. An experiment involving phenotypic plasticity and relative fitness of *Eucecidoses* populations on local vs. non-local *Schinus* populations using transplants would be also important to clarify further the role of host plants in relation to evolution of the micromoths.

In summary, we clearly demonstrated in this study that variation in geographic distribution of this cecidosid moth in South America cannot be explained only by that of its host plant, which is much broader. The distribution pattern of *E. minutanus* coincides with the Peripampasic orogenic arc, with most populations occurring in the mountainous areas located east of the Andes (Argentinean Monte biogeographic province and Chacoan dominian/province) and on the Atlantic coast (Brazilian Parana and Araucaria forest provinces). Phylogeny and dating of clades based on molecular data performed with populations covering these areas corroborated this scenario from a historical perspective. Two *E. minutanus* clades began to split early (ca. 38 Mya) in association with these mountainous areas, and a few lineages differentiated further within each of these clades later in time (ca. 20 Mya). Thus, we associated the initial cladogenesis (first major clade) of *E. minutanus* with the Tertiary tectonics occurring in the area, starting with the uplift of the Andes. The second major clade, which includes lineage 1 (type locality) and lineages 2, 3 and 4, likely originated from recurrent dispersal of a metapopulation from the central area north to the Pampean and further to coastal forests. The demographic dynamics of the six lineages across biogeographic regions was markedly different and the isolation by distance found among lineages reveals instability during glacial and interglacial periods. In this case study we clarified the evolutionary pathway from a phylogenetic perspective that this gall-inducing moth may have gone through in association with orogenic events that molded the mountainous regions located in the west and east South American coasts. Thus, our findings can be used to explain not only the evolutionary history of *E. minutanus*, but generally for regional Neotropical fauna.

Supporting information

S1 Fig. A, Dried-preserved *Schinus polygamus* branches from the Herbário do Instituto de Ciências Naturais, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do

Sul, Brasil (ICN 043172), bearing open galls of *Cecidoses eremita* (indicated by open arrow) and *Eucecidoses minutanus* (closed arrows), shown in detail with their detached opercula in B and C, respectively. Scale bars = 3 and 2 mm, respectively.
(TIF)

S2 Fig. Comparative assessment of BBM and S-DIVA analyses for ancestral area reconstruction in *Eucecidoses minutanus*.
(TIF)

S1 Appendix. Geographic data of *Schinus polygamus* (sensu lato) and *Eucecidoses minutanus* compiled for the biogeographic analysis.
(PDF)

S1 Table. Primers and PCR conditions to amplify the three mitochondrial loci (CoI, CoII and 16s) surveyed this study.
(DOCX)

S2 Table. Genbank accession numbers for individuals analysed in this study.
(XLSX)

Acknowledgments

We are grateful to Thales R. O. Freitas (UFRGS) for allowing the use of molecular laboratory facilities. Clara Vaz (EMBRAPA), Osmar S. Ribas (MBM), Gabriel Melo, Lucas Beltrami and Eduardo Carneiro (UFPR), Fernando Luz (UFRGS) and Silvio Nihei (USP) kindly assisted with field collections. We thank Ademir Reis (HBR), Alexandre Salino (BHCB), André Luís Gasper (FURB), Diego Gutiéreez (BA), Élen Nunes Garcia (PEL), Laura Iharlegui (LP), Mara R. Ritter (ICN), Maria Salete Marchioretto (PACA), Mauricio Bonifacino (MVFA), Mayara Krasinski Caddah (FLOR), Norma Deginani (SI), Osmar S. Ribas (H), Paulo Henrique Labiak Evangelista (UPCB), Rosana M. Senna (HAS), and their respective staff, for allowing access and providing assistance for work in the herbaria under their care.

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S1 Fig. **A**, Dried-preserved *Schinus polygamus* branches from the Herbário do Instituto de Ciências Naturais, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brasil (ICN 043172), bearing open galls of *Cecidoses eremita* (indicated open arrow) and *Eucecidoses minutanus* (closed arrows), that are showed in detail with their detached opercula in **B** and **C**, respectively. Scale bars = 3 and 2 mm, respectively.

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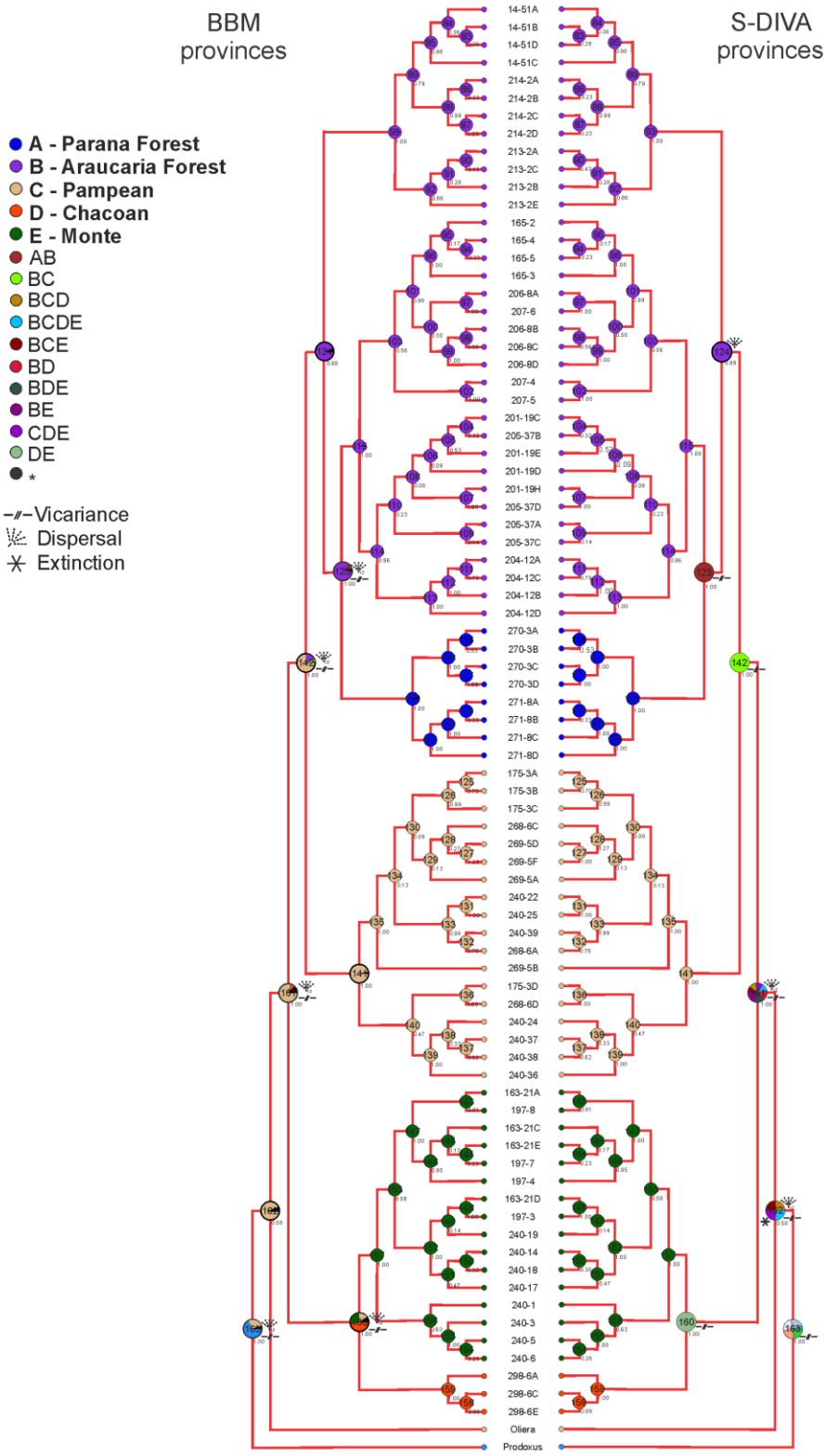
S2 Fig. Comparative assessment of BBM and S-DIVA analysis for ancestral area reconstruction in *Eucecidoses minutanus*.



Silva et al. - Figure S2

S1 Appendix. Geographical data of *Schinus polygamus* (lato sensu) and *Eucecidoses minutanus* compiled for the biogeographical analysis. Data from literature are followed by the corresponding references within brackets, as follows: 1 – SpeciesLink (splink.cria.org.br); 2 - Fleig (1989); 3- Barkley, F. A. (1944); 4 – German San Blas, UNLP, inedit data; 5 – Loetti et al. (2016); 6 – Plants Jstor (plants.jstor.org); 7 – Museo de La Plata; 8 – Intituto de Botánica Darwinion; 9 – Museo Argentino de Ciencias Naturales Bernardino Rivadavia; 10 – Facultad de Agronomia de Montevideo; 11 – Gilson R.P. Moreira, UFRGS, inedit data (dried material preserved at Laboratório de Morfologia e Comportamento de Insetos (LMCI); to be donated to ICN). a – as *S. longifolius*; b – as *S. spinosus*; c – as *S. dependens*; d – as *S. johnstonii*; e – as *S. polygama*; f – as *S. sinuatus*; g – as *S. piliferus*; h – as *S. fasciculatus*; i – as *S. cabrerae*; j – as *S. engleri*; k – as *S. apparicianus*; l – as *S. ferox*; m – as *S. praecox*; n – as *S. bumelioides*; * indicates records of *E. minutanus* galls. Abbreviations follow Thiers, B. [continuously updated], except for BCTw (Xiloteca Calvino Mainieri, Instituto de Pesquisas Tecnológicas, São Paulo, Brazil), DVPR (Herbário da Universidade Tecnológica do Paraná, Dois Vizinhos, Paraná, Brasil), FPS (Fototeca Paulo Schwirkowski, São Bento do Sul, Santa Catarina, Brasil). Latitude (S) and longitude (W) are given at the end in parentheses.

Schinus polygamus: ARGENTINA: **Buenos Aires:** *Atalaya* – [9], Izunieta, BA68381, 20/03/1970, (-35.0333, -57.5333); *Atucha* – [1], Krapovickas A 3283, SP 54306, 26/01/1947, (-33.9736, -59.2025); *Bahia Blanca* – [3,d], Eyerdam, Beetle & Grondona 23449, GH, 16/12/1938, (-38.7166, -62.2666); *Berisso* – [3,a], Cabrera 507, GH, 11/1928, (-34.8666, -57.8666); *Brandsen* – [4*], GSB, R. & E. San Blas, C. Amaya, 06/09/2012 (-35.1750, -58.2365); *Bernal* – [7,a], Elián L. Guerrero, LP 114, 28/08/2011,(-34.7, -58.2833); *Coronel Rosales* – [6,d], Boelcke, O.11936, 08 /12/1964, Múlgura de Romero, M. E., (-38.8666, -62.0666); *Estancia José Santos Arevalo* – [7], L. R. Miccio, LP 795, 14/11,(-35.1667, -59.1667); *Estanción San Isidro* – [7,a], Torres Robles, LP 342, 06/12/2000, (-35.1458, -57.3738); *Isla Martin Garcia* – [7,a*], Torres Robles, P. Simon, N. González, LP 2022, 30/10/2004, (-34.1798, -58.25); *Laguna Chasilauquen* – [7], A. L. Cabrera, H. A. Fabris, LP 14809, 17/11/1962, (-37.2727, -63.1536); *La Plata* – [6,a], SI045512, 17/10/1929, Muñoz, J. D. det., (-34.9213, -57.9544); *Las Palmas* – [6,a*], Boelcke, O. 4951, SI045514, 18/10/1951, Muñoz, J. D. det., (-34.0194, -59.1341); *Lima* – [8,h], Múlgura de Romero et al., SI 324, 08/12/1982, (-34.033333, -59.2); *Lujan* – [2], F. A. Barkley 8227, LIL, 26/09/1942, (-34.5666, -59.1); *Magdalena* – [5,a*], 04/2013, (-35.0833, -57.5166); *Monte Hermoso* – [7,d], H. A. Fabris, H. Schwabe, LP 4788, 24/11/1962, (-38.9833, -61.3); *Punta Lara* – [3,a*], 1815, GH, 25/10/1931, (-34.8166, -57.9666); *Quilmes* – [7,a], Nieves Baldaccini, LP 32, 16/11/2014, (-34.7333, -58.2666); *San Isidro* – [6,a*], Hicken, C. M., SI045513, 12/12/1901, Muñoz, J. D.det., (-34.4666, -58.5166); *Sierra de las Tunas* – [7,h], Laura A. Pertusi, LP 52, 25/11/1979, (-38.9494, -61.8155); *Tigre* – [6,a], Lanfranchi, A. E. 1685, SI045506, 15/09/1967, (-34.4258, -58.5966); *Tornquist*– [7,h], Proyeto Ventania, LP 233, 26/11/1978, Laura Pertusi, det., (-38.1, -62.2333); *Zarate* – [4*], GSB, R. y E. San Blas, C. Amaya, 08/09/2012 (-34.0952, -59.0241); **Catamarca:** *Aldalgalá* – [2], Vervoort 3428, LIL, (-27.6, -66.3166); *Belén* – [7*], A.L. Cabrera, E. M. Zardini, N. Deginani, LP 24654, 27/01/1974, (-27.65, - 67.0333); *Capayan* – [2], Risso 870, LIL, (-28.7636, -66.0683); *El Alto* – [6,a], Venturi, S. 7063, SI033348, 10/01/1928, Múlgura de Romero, M. E. det., (-28.3, -65.3666); *El Durazno* – [8,h*], A. R. Cuezzo 9494C., SI, 14/11/1972, (-27.2538, -67.0380); *La Merced* – [4*], GSB, 09/07/2014, 27/08/2014, 23/11/2015 (28.1644, -65.6516); *La Puerta* – [7], A. L. Cabrera, LP 9523, 17/08/1969, (-28.1666, -65.7833); *San Antonio* – [8*], Sara T., SI, 25/01/1957, (-28.0333, -65.6833); *Shincal* – [7,h], Capparelli Aylen, LP 31, 23/05/1991, Laura Iarleghi det., (-27.6861, -67.1805); **Chaco:** *Barranqueras* – [3,f], Curran 15, US, 12/11/1913, (-27.4833, -58.9333); *Chacabuco* – [1,h], Scarpa, G 797, SPF 214453, 04/03/2009, Scarpa, G det., (-27.2166, -61.2); *Colonia Benitez* – [3,f], Schulz 848, 03/1935, (-27.3333, -58.9333); *Las Breñas* – [8,h], Aliscioni, S.S. et al., SI 663, 21/03/2006, (-27.0666, -61.0666); *La Fidelidad* – [3,f], Jorgensen 1950, FAB/NY, 09/04/1918, (-24.9677, -60.9666); *Resistencia* – [2], Margarita Belen, LIL 201713, 13/03/1947, (-27.4513, -58.9866); *Villa Angela* – [3,f], Bogga 8, NY, 03/01/1930, (-27.5833, -60.7166); **Chubut:** *Colhue Hualpi* – [8,d], Martin T., SI 11, 26/12/1972, (-45.4938, -68.7572); *Futaleufú* – [6,d], Illin, N. 82, SI033252, (-42.9, -71.3166); *Gaiman* – [1,d], Seijo, G 1509, MBM 242133,

20/01/1999, Schinini, A det., (-43.2833, -65.4833); *Istmo Carlos Ameghino*– [7,d], Juan Daciuk, LP 126, 3/10/1969, Juan Daciuk det., (-42.4526, -64.5); *Nueva Lubecka* – [8,h], Zuloaga, F. O., SI 13926, 19/11/2012, Rau det., (-44.5333, -70.4)*; Paso de Indios* – [6,d], Biganzoli, F.; Larsen, C. 1930, SI033248, 01/11/2008, Múlgura de Romero, M. E. det., (-43.9, -69.0666); *Península Valdés* – [7,d], Juan Daciuk, LP 47, 15/08/1969, Juan Daciuk det., (-42.5, -63.9333); *Pico Salamanca* – [7], Castellanos, LP 6086, 25/01/1932,(-45.5733, -67.3372); *Puerto Madryn* – [8,d*], L. Hauman, SI 14, 07/01/1914, F. A. Barkley det., (-42.8295, -65.0823); *Puerto Pirámides* – [3,d], Eyerdam, Beetle & Grondona 23570, GH, 21/12/1938, (-42.5666, -64.2833); *Punta Gales* – [6,d], Daciuk, J. 21, SI033249, 11/09/1969, Múlgura de Romero, M. E. det., (-42.4236, -5358); *Punta Loma* – [9,d*], J. Daciuk, BA 9824, 11/01/1968, M. J. Dimitri det., (-42.8166, -64.8833); *Rawson* – [7*], M. M. Job, LP 3011, 20,11/1951, (-43.3, -65.1); *Sarmiento* – [6,d], Kreibohm, E. 273, SI033259, 31/10/1965, Terrazas, T. det., (-45.6, -69.0833); **Cordoba:** *Capilla del Monte* – [7,h], Maria M. Job, LP, 18/08/1952, (-30.85, -64.5166); *Córdoba* – [7,h], J. H. Hunziker, LP 2654, 01/1950, (-31.4166, -64.1833)*; El Brete* – [3,f], Lorentz 1477, F, 02/02/1878, (-30.6905, -64.8747); *La Cumbre* – [6,h], Cuezzo, A.R & Balegno, B. 2128, E00089920, 10/02/1950, (-31.1, -64.483); *La Falda* – [3,h], Job 545, NY, 01/1936, (-31.0833, -64.5); *Malagueño* – [8], A. M. Fuchs, SI 17099, 12/08/1949, (-31.4638, -64.3575); *Mina Clavero* – [8,h], Cordo, H., SI 77-A-56, 03/12/1976, (-31.7238, -65.005); *Nono* – [1,h], Silva-Luz, CL; Luz, LF 278, SPF 212447, 22/03/2014, Silva-Luz, CL det., (-31.8104, -64.9928); *Punilla* – [1,m], Hunziker, AT 7451, MBM 144399, 17/10/1947, F. A. Barkley, det., (-31.25, -64.45); *Rio Primero* – [3,a], Hyeronymus, F, 30/08/1877, (-31.1333, -63.3666); *San Javier* – [1,m], Castellanos, A 3, SPF 214454, 15/12/1927, Silva-Luz, CL det., (-31.9333, -65.2); *San Roque* – [3,d], Rose & Russel 21059, US, 09/09/1915, (-31.3730, -64.4416); *Sierra Achala* – [3,d], Hyeronymus, NY, 11/11/1878, (-31.6826, -64.8371); *Sierra de los Condores* – [8], Kiesling, R., Terrazas, T., SI 10115, 04/11/2004, M. E. Múlgura, det., (-32.3211, -64.28); *Tanti* – [1,m], Silva-Luz, CL; Luz, LF 280, SPF 212432, 23/03/2014, Silva-Luz, CL det., (-31.3333, -64.6); *Unquillo* – [3,f], Bruch 2935, NY, 1926, (-31.2333, -64.3166); *Villa La Merced* – [3,h], Hieronymus, NY, 11/1891, (-31.8238, -64.5194); **Corrientes:** *Arroyo Timboy* – [4*], GSB, 21/01/2012, (-30.0638, -57.8722); *Concepción* – [7,j], Troels Myndel Pedersen, LP 4632, 24/09/1974, (-28.3666, -57.8666); *Curuzú Cuatiá* – [8,h], A. Schinini, O Ahumada, SI 13917, 08/01/1977, (-29.7833, -58.0833); *Empedrado* – [7,h], Troels Myndel Pedersen, LP 1755, 18/06/1952, (-27.9333, -58.7833); *Garruchos* – [7,n], Krapovickas, Cristóbal, Tressens, Schinini, Quarin, CTES 25062, 12/04/1974, (-28.1736, -55.6511); *Gioya* – [3,f], Curran 335, US, 04/09/1913, (-29.1333, -59.25); *Itatí* – [7,h], A. Schinini, L. Mroginski, LP 4486, 16/04/1972, (-27.2666, -58.25); *Mburucuyá* – [7,n], Troels Myndel Pedersen, LP 4499, 16/03/1958, (-28.05, -58.2333); *Mercedes* – [1], Marchiori, J. N. C. s.n., HDCF 503, 26/08/1981, Longhi, S. det., (-29.1838, -58.0733); *San Cosme* – [1,e], Paula-Souza, J.; Ferrucci, M.S.; Meza Torres, E.I. 8060, ESA098907, 31/01/2007,(-27.5166, -58.5666); *San Luis del Palmar* – [1], Krapovickas, A; Quarín, C; Fernández, A 21836, MBM 23887, 08/03/1972, (-27.5108, -58.5569); **Entre Ríos:** *Concepcion del Uruguay* – [3,a], Lorentz, GH, 11/11/1877, (-32.4844, -58.2369); *El Espinillo* – [7,a], Muñoz, J.D., SI 611, 30/01/1980, Malúlgura det., (-33.525, -58.3555); *Gualeguaychú* – [2], Meyer 10276, LIL, (-33.0077, -58.5111); *Parana* – [1,f], Silva-Luz, CL 330, SPF 216474, 21/12/2014, Silva-Luz, CL det., (-31.7372, -60.3284); *Santa Elena* – [7,h], A. Burkart et al., SI 28736, 23/10/1971, (-30.9413, -59.7833); *Yuqueri* – [7,] Maria M. Job, 03/11/1949, (-31.3833, -58.1192); **Formosa:** *Espinillo* – [6,h], Morel, I. 7776, E00089918, 27/05/1949, F. A. Barkley det, (-24.9666, -58.5666)*; Formosa* – [3,f], Jorgensen 1971, FAB/NY, (-26.1833, -58.1833**)**; *Guaycolec* – [8,h], H. Maturo, D. Prado, SI 53, 20/09/2004, H. Maturo det., (-25.9845, -58.1614); *Patiño* – [8,h], P. Arenas, SI 1961,, 18/01/1982, P. Arenas det., (-25.3333, -59.6833); **Jujuy:** *Cachipunco* – [8,g*], Rotman A., SI 898, 10/12/1983, (-24.4766, -64.535); *Coiruru* – [1,g], Silva-Luz, CL; Luz, LF 290, SPF 212454, 27/03/2014, Silva-Luz, CL det., (-23.8765, -65.4589); *Rio San Francisco* – [3,g], Venturi 9735, CAS, 19/10/1929, (-23.3041, -64.0791); *Santa Barbara* – [3,g], Fries 293, US, 09/07/1901, (-24.3086, -64.6603); *Sierra de Zapla* – [8,n], A. L. Cabrera, S. Botta, A. M. Cialdella, A. Rotman, SI 32003, 14/11/1980, Cabrera A. L. det., (-24.1833, -65.0666); *Tilcara* – [1], Kiesling, R; et al. 1157, SP 250141, (-

23.5772, -65.3936); Tumbaya – [7,g], A. Burkart et al. 30524, SI, 7/11/1974, (-23.8527, -65.4661); *Valle Grande* – [2], Cabrera 27874, SI, 14/09/1976, (-23.4755, -64.9469); **La Pampa:** *General Pico* – [8,f], A. Buckart, SI 9847, 10/11/1939, (-35.6666, -63.7333); *Santa Rosa* – [7,h], H. Schwabe, H. Fabris, LP 2123, 28/11/1959, (-36.6205, -64.3075); *Telén* – [3,f], Cabrera 4367, GH, 16/03/1938, (-36.2667, -65.5); *Victorica* – [9], Pérez Moreau – Perrone, BA 30107, 13/01/1951,(-36.2167, -65.434); **La Rioja:** *General Belgrano* – [7,h], G. Covas 1208, LP 45082, 25/02/1941, F. A. Barkley det., (-30.6316, -66.2663); *Miranda* – [8,n*], A. Buckart, SI 12450, 26/02/1941, F. A. Barkley det., (-29.3333, -67.6833); **Mendoza:** *Cacheuta* – [4*], GSB, 19/07/2014, (-33.0858, -69.0786); *Cuesta Cerrillos* – [4*], GSB, 30/10/2012 (-33.1224, -68.9171)*; La Paz* – [7], E. L. Gautier, A. L. C. det., (-33.4666, -67.55)*; Las Heras* – [4*], GSB, 26/11/2011 (-32.8507, -68.8404); *Lujan del Cuyo* – [4*], GSB, 13/02/2012 (-33.0339, -68.8822); *Maipu* – [7,d], Carette, Ruiz, LP 3749, 25/01/1936, F. A. Barkley det., (-32.9666, -68.75); *Malargüe* – [8,d], A. Prina, G. Alfonso, E. Morici, W. Muiño, SI 2349, 12/12/2003, (-35.4744, -69.5852); *Manzano Historico* – [4*], GSB, 30/10/2012 (-33.5979, -69.3823); *San Carlos* – [1,d], Silva-Luz, CL; Luz, LF 266, SPF 212449, 17/03/2014, Silva-Luz, CL det., (-33.8156, -69.1815); *San Rafael* – [2*], Lourteig 763, LIL, (-34.6, -68.3333); *Tunuyan* – [7,h], LP 2111, 29/01/1934, F. A. Barkley det., (-33.5666, -69.0166); **Missiones:** *Candelaria* – [6,a], Rodríguez, F. M. 51, SI045504, 02/02/1920, (-27.45, -55.7333); *General Manuel Belgrano* – [1,l], Keller, H.A. 3640, HUEFS 133311, 27/08/2006, (-26.25, -53.65); *Loreto* – [2], Rodriguez 244, SI, 27/03/1910, (-27.3319, -55.5231); *San Ignacio* – [2], Schwarz 2019, LIL, 04/02/1946, (-27.2583, -55.5392); *Santa Ana* – [3,a], Rodriguez 51, F, 03/02/1930, (-27.3694, -55.5822); **Neuquén:** *Senillosa* – [8,d], Sede, S. M., SI 288, 04/01/2012, (-39.01, -68.43)**; Rio Negro:** *General Roca* – [3,d], Fischer 11, F, 09/1914, (-39.0333, -67.5833); *San Antonio Oeste* – [8,d], Eskuche, Klein, SI 166, 17/01/1968, (-40.73, -64.9388); *San Carlos de Bariloche* – [7], Stuessy, Crawford, Crisci, Cigliano, Gentilli, LP 6746, 02/12/1984, (-41.1436, -71.2908); **Salta:** *Alemanía* – [3,g], 9830, GH, 27/11/1929, (-25.6238, -65.6133); *Anta* – [1,n], Ragonese 362, SPF 214447, 06/1934, F. A. Barkley, det., (-24.1136, -64.1247); *Cafayate* – [2], R. Winkler, LIL 277100, (-26.0833, -65.9666); *Campo Duran* – [3,g], Parodi 9171, GH, 23/01/1930, (-22.2333, -63.7); *Chicoana* – [1,g], Paula-Souza, J.; Ferrucci, M.S.; Rando, J.G.; Meza Torres, E.I. 7780, ESA056590, 25/01/2007, (-25.1, -65.55); *Cuesta del Ovispo* – [4*], GSB, F. Navarro, H. Beccacece, 19/04/2010 (-25.1663, -65.7338); *La Candelaria* – [6,n], Venturi, S. 5494, SI033347, 24 /07/1927, Múlgura de Romero, M. E, det., (-26.1, -65.1); *Las Juntas* – [3,i], Bruch 2874, NY, 15/12/1926, (-28.1333, -65.9); *Lumbreras* – [1,n], Ragonese 357, SPF 214448, 08/1934, F. A. Barkley, det., (-25.2, -64.9166); *Rosario de Lerma* – [7,f], M. M. Job, LP 1559, 01/1937, Barkley det., (-24.9833, -65.5833); *Rosario de la Frontera* – [2], Villa Carenzo 2696, LIL, (-25.7983, -64.9741); *San Ramón de la Nueva Orán* – [1,g], Mello-Silva, R; Forzza, RC; Marcato, AC 1908, SPF 159369, 13/12/2001, Novara, LJ det., (-23.9, -64.3383); *Tanti* – [3,g], Venturi 9573, GH, 20/09/1929, (-31.3333, -64.6); **San Juan:** *Calingasta* – [2], Castellanos, LIL 61827, 27/01/1950, (-31.336, -69.42); Jáchal – [8*], Tombesi, T. S. et al., SI 72, 16/02/2000, (-30.2333, -68.75); *Valle Fertil* – [2*], Cuezzo 2941, LIL, (-30.9080, -67.285); **San Luis:** *Alto Pencoso* – [3,h], Bruch-Carette, NY?, 02/1914, (-33.43, -66.93); *Lujan* – [1,n], Silva-Luz, CL; Luz, LF 277, SPF 212460, 22/03/2014, Silva-Luz, CL det., (-32.3708, -65.9269); *Nueva Galia* – [3,h], Rodriguez 1468, F, 24/11/1915, (-35.1166, -65.25); *Pedernal* – [7,f], A. P. Rodrigo. LP 2006, 11/1941, (-31.9833, -68.7333); *San Luis* – [1,h], Silva-Luz, CL; Luz, LF 276, SPF 212459, 22/03/2014, Silva-Luz, CL det., (-33.0388, -66.3233); limite *San Luis – La Pampa* – [8,d], Cordo, Ferrer, SI 77-d-48, 24/11/1977, (-35.0947, -65.1066); **Santa Cruz:** *Cerro Alto* – [2], José Steinbach, SI, 21/12/1921, (-48.65, -69.6166); *Lago Ghio* – [8,d], Zuloaga, F. O., SI 14771, 21/11/2013, C. Zanotti det., (-47.2836, -71.525); *Puerto Deseado* – [2], Odonell 3579, LIL, (-47.75, -65.9166); **Santa Fe:** entre *Vera* y *Margarita* – [7,f], Ragonese, LP 2913, 19/11/1938, F. A. Barkley, det., (-29.5847, -60.2308); *Lago Argentino* – [6,a], Molina Massey, E. 48, SI033226, 11/1909, Múlgura de Romero, M. E. det., (-50.0702, -72.1794); *Lago Cardiel* – [6,a], Scott de Birabén, María Isabel, Birabén, Max 150, LP005406, 27/02/1936, Angel Lulio Cabrera det., (-48.95, -71.2166); *Lanteri* – [3,f], Job 1256, NY, 01/01/1936, (-28.8333, -59.65); *Reconquista* – [6,j], Kermes LP011017, 1900, F. A. Barkley det., (-29.2333, -59.9333); *Villa Guillermina* – [7,f],

Ragonese, LP 2920, 14/11/1938, F. A. Barkley, det., (-28.2403, -59.4667); *Tostado* – [1], Krapovickas, A; Vanni, R 43685, UB, 30/03/1990, (-29.2263, -61.7719); *Villa Ocampo* – [3,f], Venturi 153, FAB/NY, (-28.4833, -59.35); **Santiago del Estero:** Choya – [7], R. Maldonado Bruzzone, LP 1525, 27/09/1944, (-28.495, -64.8569); *Jiménez* – [3,g], 10113, GH, 16/02/1930, (-27.1633, -64.4908); *Moreno* – [2], Castellanos, LIL 268977, (-27.3702, -62.2655); *Ojo de Agua* – [7], Maldonado Bruzzone, LP 1504, 27/07/1944, (-29.5019, -63.6925); *Parque Nacional Copo* – [8,h], J. P. 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S1 Table. Primers used in this study and PCR conditions.

Locus	Primer	Sequence (5´–3´)	Reference	Size (pb)	Annealing temp.
CO-I	k698	F: TACAATTTATCGCCTAAACTTCAGCC	Caterino & Sperling 1999	732	55
	Nancy	R: CCCGGTAAAATTAAAATATAAACT	Caterino & Sperling 1999		55
	Jerry	F: CAACATTTATTTTGATTTTTTGG	Caterino & Sperling 1999	831	52
	PatII	R: TCCATTACATATAATCTGCCATATTAG	Caterino & Sperling 1999		52
CO-II	George	F: ATACCTCGACGTTATTCAGA	Caterino & Sperling 1999	750	47
	Eva	R: GAGACCATTACTTGCTTTCAGTCATCT	Caterino & Sperling 1999		47
16S	16Sar	F: CCCGCCTGTTTATCAAAAACAT	Palumbi (1996)	990	55
	Ins16Sa	R: CCCTCCGGTTTGAACTCAGATC	Palumbi (1996)		55

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S2 Table. Mitochondrial sequences generated in this study and deposited in the Genbank database.

Clade	Province	Pop.	Vouchers	Genbank No	
				CoI, tRNA-Leu, CoII	16S
Lineage 3	Parana Forest	Pr1_2	LMCI 270-3A, B, C, D	MH667801-04	MH667728-31
Lineage 3	Parana Forest	Pr1_2	LMCI 271-8A, B, C, D	MH667805-08	MH667732-35
Lineage 2	Araucaria Forest	Pr3_5	LMCI 213-2A, B, C, D	MH667776-79	MH667703-06
Lineage 2	Araucaria Forest	Pr3_5	LMCI 14-51A, B, C, D	MH667739-42	MH667669-72
Lineage 2	Araucaria Forest	Pr3_5	LMCI 214-2A, B, C, D	MH667780-82	MH667707-09
Lineage 4	Araucaria Forest	Pr6_8	LMCI 201-19A, B, C, D	MH667757-60	MH667684-87
Lineage 4	Araucaria Forest	Pr6_8	LMCI 205-37A, B, C, D	MH667765-68	MH667692-95
Lineage 4	Araucaria Forest	Pr6_8	LMCI 204-12A, B, C, D	MH667761-64	MH667688-91
Lineage 4	Araucaria Forest	Pr9_11	LMCI 206-8A, B, C, D	MH667769-72	MH667696-99
Lineage 4	Araucaria Forest	Pr9_11	LMCI 207-4 to 207-6	MH667773-75	MH667700-02
Lineage 4	Araucaria Forest	Pr9_11	LMCI 165-2 to 165-5	MH667747-49	XXX
Lineage 1	Pampean	Ch1_3	LMCI 175-3A, B, C, D	MH667750-52	MH667677-79
Lineage 1	Pampean	Ch1_3	LMCI 269-5	MH667797-00	MH667724-27
Lineage 1	Pampean	Ch4_5	LMCI 240-36 to 240-39	MH667794-96	MH667721-23
Lineage 1	Pampean	Ch4_5	LMCI 240-22,240-24,240-25	MH667791-93	MH667718-20
Lineage 5	Chacoan	Ch6	LMCI 298-6A, B, C, D	MH667809-11	MH667736-38
Lineage 6	Monte	Sa1_4	LMCI 240-1,240-3,240-5,240-6	MH667783-86	MH667710-13
Lineage 6	Monte	Sa1_4	LMCI 197-3,197-4,197-7,197-8	MH667753-56	MH667680-83
Lineage 6	Monte	Sa1_4	LMCI 163-21A, B, C, D	MH667743-46	MH667673-76
Lineage 6	Monte	Sa1_4	LMCI 240-14,240-17 to 240- 19	MH667787-90	MH667714-17

CAPÍTULO II

Cecidonius pampeanus, gen. et sp. n.: an overlooked and rare, new gall-inducing micromoth associated with *Schinus* in southern Brazil (Lepidoptera, Cecidosidae)*

Gilson R.P. Moreira, Rodrigo P. Eltz, Ramoim B. Pase, Gabriela T. Silva, Sérgio A.L. Bordignon, Wolfram Mey, Gislene L. Gonçalves

*Artigo publicado na revista *Zookeys* em 04 de setembro de 2017

ZooKeys 695: 37–74 (2017)
doi: 10.3897/zookeys.695.13320
http://zookeys.pensoft.net

RESEARCH ARTICLE

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Cecidonius pampeanus, gen. et sp. n.: an overlooked and rare, new gall-inducing micromoth associated with *Schinus* in southern Brazil (Lepidoptera, Cecidosidae)

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Academic editor: *E. van Nieukerken* | Received 20 April 2017 | Accepted 7 August 2017 | Published 4 September 2017

http://zoobank.org/F9D9BD39-1346-4F18-8EA1-7572C480F300

Citation: Moreira GRP, Eltz RP, Pase RB, Silva GT, Bordignon SAL, Mey W, Gonçalves GL (2017) *Cecidonius pampeanus*, gen. et sp. n.: an overlooked and rare, new gall-inducing micromoth associated with *Schinus* in southern Brazil (Lepidoptera: Cecidosidae). ZooKeys 695: 37–74. https://doi.org/10.3897/zookeys.695.13320

Abstract

Galls induced by the larval stage of cecidosids (Lepidoptera: Cecidosidae) are complex, multi-trophic systems, still poorly studied. They may be associated with other insect feeding guilds, including inquilines, kleptoparasites, cecidophages, parasitoids, and predators. By causing death of the gall inducer early in life and altering the gall phenotype, inquilines may lead to misidentification of the true gall inducers. Here, we describe through light and scanning electron microscopy *Cecidonius pampeanus*, a new genus and species of cecidosid moth, from the Pampa biome, south Brazil. It induces unnoticed, small galls under swollen stems of *Schinus weinmannifolius* Mart. ex Engl. (Anacardiaceae). Such galls are severely attacked early in ontogeny either by unidentified parasitoids belonging to *Lyrcus* Walker (Pteromalidae) that feed upon the inducer, or by inquiline wasps of the genus *Allorhogas* Gahan (Braconidae). The inquilines modify the galls into large ones that last longer and promptly call attention. Free-living galls are rare and dehiscent, pupation of *C. pampeanus* occurring on the ground. Due to these reasons the true inducer has been

overlooked in this case for more than a century. Additionally we inferred a phylogeny for Cecidosidae using sequences from mitochondrial and nuclear loci, and characterized genetic variation and gene flow across ten populations. Despite its natural history similarities with the African genus *Scyrotis*, *Cecidonius* is a much younger lineage, more closely related to the Neotropical cecidosids. *C. pampeanus* populations, which are now confined to a few mountain areas within its distribution range due to habitat destruction, are also genetically isolated, requiring conservation measures.

Keywords
Anacardiaceae, cecidosid moths, conservation, insect galls, Neotropical region, taxonomy

Introduction

Insect-induced galls may consist of very complex, multitrophic-level systems including not only the gall inducers themselves, but also predators, cecidophages, parasitoids, kleptoparasites and inquilines, among other insects such as successors that use them for shelter. Kleptoparasites in particular invade galls, usurping the cecidogenous species and become stationary, feeding upon gall tissues until they complete their larval development, and may prey upon the inducer and other insects that eventually enter the usurped gall (e.g., Morris et al. 2000, Luz et al. 2015). They do not induce differentiation and growth of new tissues, only feeding on those that were induced to develop by their precursors. Inquilines, however, induce the development of new tissues, either similar to or different from original ones when they take over a given gall, generally killing the inducer by inanition (e.g., Brooks and Shorthouse 1988, van Noort et al. 2007). Thus, they may change substantially the size and shape of the gall they invade. Little attention has been paid to the important taxonomic consequences of this phenomenon, a potential difficulty factor in identification of hidden diversity in gall communities. Misidentification of the true gall inducers in such cases is obviously likely, since the inducer is eliminated from the system early in the gall ontogeny and no conspicuous trace of it may be left inside the gall. In addition, contrary to galls attacked either by kleptoparasites or inquilines that may stay attached to host plants, those free of them still containing the growing inducer may be dehiscent, with later development of immature stages occurring on the ground (e.g., van Noort et al. 2007, Luz et al. 2015). In this case, by altering the place of gall development in the field and thus enhancing the encounter of attacked galls by kleptoparasites and inquilines that stay attached to the host plant compared to free, detached ones, the possibility of missing the presence of the true inducers is substantially increased. Furthermore, depending on the rate of attack by other parasitoids and predators in association, natural densities of the true gall inducer would be reduced further, even becoming rare, and thus may be unnoticed. As a case study, here we describe one example of such a peculiar system, where the induction of a non-conspicuous, dehiscent gall by a cecidosid moth has been overlooked for more than a century, erroneously believed to be induced by their hymenopteran inquilines who do not originally induce galls but in fact modify them early in development into large and colorful, visually appealing galls.

Cecidosidae are poorly known monotrysian Heteroneura moths (*sensu* Davis 1998), comprising six genera and 18 species, all with ranges restricted to the southern hemisphere. They are among a few lepidopteran lineages with a Gondwanic distribution: one occurs in New Zealand, the monotypic genus *Xanadoses* Hoare & Dugdale; twelve in southern Africa, all belonging to *Scyrotis* Meyrick, and five in South America, two in *Dicranoses* Kieffer & Jörgensen, and three in the monotypic genera *Cecidoses* Curtis, *Eucecidoses* Brèthes, and *Oliera* Brèthes. *Xanadoses nielseni* Hoare & Dugdale is a bark-miner of several New Zealand bark trees, particularly within *Weinmannia* Linnaeus (Cunoniaceae). Larvae of African *Scyrotis* form galls on species of *Searsia* F.A. Barkley (Anacardiaceae) (van Noort et al. 2007). In this case, they may also be located in the leaves; these galls are known as "jumping-beans". They exfoliate from the hostplant and drop to the ground, where they are propelled for short distances by the active pupa inside, a supposed adaptation to avoid excessive heat from the sun (Meyrick 1917, Davis 1998). Unfortunately, none of the immature stages of *Scyrotis* species have been described in detail yet. South American cecidosids induce galls either on the stem or on axillary buds of *Schinus* Linnaeus (Anacardiaceae), particularly *S. polygamus* (Cav.) Cabrera (*sensu* Cabrera 1938, Fleig 1987, 1989). Gall morphology and life history of *C. eremita* Curtis have been treated in detail by Wille (1926). The taxonomy was reviewed and immature stages and galls of *O. argentinana* Brèthès, and *D. capsulifex* Kieffer & Jörgensen were described respectively by Moreira et al. (2012) and San Blas and Davis (2013). Information gathered recently by the first author suggested that diversity of cecidosids is much greater in the Neotropics, and not only additional species of *Schinus* are used as host but also other Anacardiaceae, such as species of *Lithraea* Miers ex Hook. & Arn.

This study concerns the galls of *Schinus weinmannifolius* Mart. ex Engl., which are induced by an undescribed genus and species of Cecidosidae in southern Brazil. Although not fully explored yet, the existence of these galls has been known for a long time; their induction was wrongly associated with cynipid wasps (Tavares 1909, Wille 1926, Houard 1933, Sáiz and Núnez 1997). Here the gall, the immature stages, and adults of the true inducer are described under both light and scanning electron microscopy and provided information on its natural history, in conjunction with a parasitoid and an inquiline wasp frequently found in association with these galls. By conducting an analysis of concatenated mitochondrial (COI and 16S) and nuclear (Wingless) DNA sequences including putative members of all known Neotropical cecidosid lineages, we provide further support for the proposition of the new taxon. Considering the possibility that the new species could be closely related to the African lineages, two *Scyrotis* species are also included for the first time for comparison in the phylogenetic analysis of Cecidosidae. Given that extant populations of the new taxa are in low numbers and restricted to a reduced distribution range, a genetic structure analysis was carried out using *ca*. 1.5 kb of COI gene sequences. Statistical analysis was performed to describe the genetic diversity of this rare species. Data are discussed in the context of importance regarding use of integrative taxonomy, including molecular analyses, in the discovery of hidden insect diversity in gall communities and the corresponding conservation scenario.

Materials and methods

Morphology

Adult specimens used in this study were reared from galls in small plastic vials, which were maintained under controlled conditions (14 h light/10 h dark; 25 ± 2 °C) in the Laboratório de Morfologia e Comportamento de Insetos (**LMCI**), Departamento de Zoologia, Universidade Federal do Rio Grande do Sul (**UFRGS**), Porto Alegre city, RS. Dehiscent galls (approx. 20 in total) were collected from the ground, in the surroundings of *S. weinmannifolius* plants of an old grass field, located in a farm belonging to Antonio Malta, Coxilha das Lombas, 30°01'46"S, 50°36'40"W, 86m, 29.V.2012, Santo Antônio da Patrulha Municipality, Rio Grande do Sul State (RS), Brazil. Pupae were obtained later (September) by dissecting some galls under a stereomicroscope in the laboratory. Larvae were obtained by dissecting *S. weinmannifolius* branches, either from galls located under swollen bark (early instars) or erupted from the stem (later instars). Adults were pin-mounted and dried. Immature stages were fixed in Dietrich´s fluid and preserved in 75% ethanol. Larvae used for DNA extraction came from several additional populations (listed below), and were preserved in 100% ethanol at -20 °C.

For descriptions of adult morphology the specimens were cleared in a 10% potassium hydroxide (KOH) solution, stained with Chlorazol black E and slide-mounted in either glycerine jelly or Canada balsam. Last instar larvae were prepared similarly for description of chaetotaxy. Observations were performed with the aid of a Leica® M125 stereomicroscope. Structures selected to be drawn were previously photographed with a Sony® Cyber-shot DSC-H10 digital camera attached to the stereomicroscope. Vectorised line drawings were then made with the software Corel Photo-Paint® X7, using the corresponding digitalized images as a guide. Additional specimens were used for scanning electron microscope analyses. They were dehydrated in a Bal-tec® CPD030 critical-point dryer, mounted with double-sided tape on metal stubs, coated with gold in a Bal-tec® SCD050 sputter coater and examined and photographed in a JEOL® JSM6060 scanning electron microscope at the Centro de Microscopia Eletrônica (CME) of UFRGS.

Molecular analysis

Mitochondrial and nuclear DNA sequences were used for two different levels of analysis of the undescribed genus and species: 1) to infer the phylogenetic status and relationships within Cecidosidae, and 2) to describe the genetic diversity and population structure of this rare taxon. For the first approach we used representative species of all members of Cecidosidae except *Xanadoses*, the corresponding samples coming from the tissue collection of LMCI: i.e., *C. eremita*, *Dicranoses congregatella* Kieffer & Jörgensen, *Eucecidoses minutanus* Brèthes, *O. argentinana*, an undescribed lineage from Chile (previously known to be closely related based on morphology) and *Scyrotis* (*Scy-*

rotis sp. and *S. granosa* Meyrick), a genus from Africa included for the first time in a molecular phylogeny. For the second approach we sampled 10 populations across the distribution range of *Cecidonius pampeanus* sp. n. (P1 to P10), including six individuals per site (Suppl. materials 3, 5). Previous analyses indicated there was no substantial addition of genetic variation by increasing corresponding sample size. Total genomic DNA was purified from fresh collected larval tissue of all Cecidosidae surveyed except *Scyrotis* (dried museum adult specimens were used), using the PureLink genomic DNA kit (Life, Invitrogen, USA) following the manufacturer's instructions.

For cecidosid phylogeny we used nucleotide sequences obtained from different molecular markers, selected because they evolve at different rates and provide phylogenetic resolution at different, overlapping taxonomic levels: two mitochondrial (1421 bp of the cytochrome oxidase subunit I [COI] and 474 bp of the 16S ribosomal RNA [16S] genes), and one nuclear (395 bp of the Wingless [Wg] gene) loci. For the genetic structure and variability approach, we amplified COI in 60 individuals, six from each population sampled. The selected molecular markers were amplified by polymerase chain reaction (PCR); primers and conditions used are described in the supplementary material (Suppl. material 1). PCR products were purified using the enzymatic method (exonuclease and alkaline phosphatase), sequenced with BigDye chemistry, and analysed in an ABI3730XL (Applied Biosystems Inc.). Chromatograms obtained from the automatic sequencer were read and sequences were assembled using the software CodonCode Aligner (CodonCode Corporation). Sequences generated in this study were deposited in the GenBank database (Suppl. material 3).

Sequence data were used for the reconstruction of a concatenated phylogenetic tree (COI+16S+Wg) with the Bayesian method in BEAST 2.02 (Bouckaert et al. 2014). The tree prior was set as a Yule calibrated process, using GTR + I for the COI partition and TN92+G for both 16S and Wg, selected with the Bayesian information criterion (BIC; Schwarz 1978) for each data set in jModelTest 2.1.2 (Darriba et al. 2012). The branch lengths were allowed to vary under a relaxed clock model with an uncorrelated log-normal distribution (Drummond et al. 2006). To adjust the molecular clock we used the fossil calibration point of Adeloidea (sensu van Nieukerken et al. 2011), about 120±10mya, with a log-normal distribution (Walhberg et al. 2013). The analysis was run for 10,000,000 Markov Chain Monte Carlo (MCMC) cycles and parameters were sampled every 1,000 cycles; this was repeated four times to test for MCMC convergence, and priors exceeded 200 to ensure effective sample sizes (ESS). Burn-in was determined in Tracer 1.5 (Drummond and Rambaut 2007) based on ESS and parameter trajectories, and the first 20% of trees were then removed with TreeAnnotator. Trees were observed and edited in FigTree v1.3.1 (Rambaut 2009). Clades with Bayesian Posterior Probability (BPP) ≥ 95% were considered strongly supported. Pairwise genetic distances (p-distances) among lineages were calculated in MEGA 7 (Tamura et al. 2013).

Nucleotide and haplotype (gene) diversity indices were estimated for individuals grouped into ten populations (P1 to P10) with DnaSP 5.1 (Librado and Rozas 2009). To investigate the evolutionary relationships among COI-haplotypes a median-joining haplotype network (Bandelt et al.1999) was constructed in NETWORK 5

(http://www.fluxus-engineering.com/sharenet.htm). Levels of genetic structure among populations were characterized using φST with Arlequin3.5 (Excoffier and Lischer 2010). Additionally, to investigate spatial patterns of genetic structure we assessed the correlation between genetic and geographic distances for all pairs of sampled individuals using a Mantel test (Mantel 1967). We also performed an Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) with Arlequin to assess more detailed quantitative differentiation among populations, performing two rounds of AMOVA employing different geographic clustering strategies: i) taking into account the vicariate effect of the Jacui River, and ii) using the genetic distance and haplotype relationship results. Finally, we investigated the demographic history of the new genus and species using neutrality tests (Tajima's D, Fu & Li' D* and F*, Fu's Fs) and mismatch distribution analysis (Rogers and Harpending 1992, Schneider and Excoffier 1999) with DnaSP.

Abbreviations of the institutions from which specimens were examined are:

DZUP Coll. Padre Jesus S. Moure, Departamento de Zoologia, Universidade Federal do Paraná, Curitiba, Paraná, Brazil.

LMCI Laboratório de Morfologia e Comportamento de Insetos, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

Results

Molecular phylogeny

The phylogenetic reconstruction corroborated our hypothesis of monophyly (well supported by posterior probability) for the new proposed genus (Fig. 1). Its sister taxon is the undescribed lineage from Chile (Cecidosidae sp.); it was close to *O. argentinana* among the described species of cecidosids. The dated phylogeny revealed the new genus as the youngest lineage among cecidosids, which emerged around 23.8 Mya (95% HDP 10.2–34.6 mya). Genetic distances of the new genus to other cecidosids ranged from 9 to 25%; less divergence was observed in relation to the sister species *Cecidonius* sp. and highest to *D. congregatella* (Table 1).

Taxonomy

***Cecidonius* Moreira & Gonçalves, gen. n.**
http://zoobank.org/5029391A-325F-4BB4-A726-8D5F9FB78476
Figs 2–9

Type species. *Cecidonius pampeanus* Moreira & Gonçalves, new species

Diagnosis. *Cecidonius* gen. n. bears several adult, pupal, larval, and gall features that in conjunction differentiate it from all cecidosid genera. Unlike other ce-

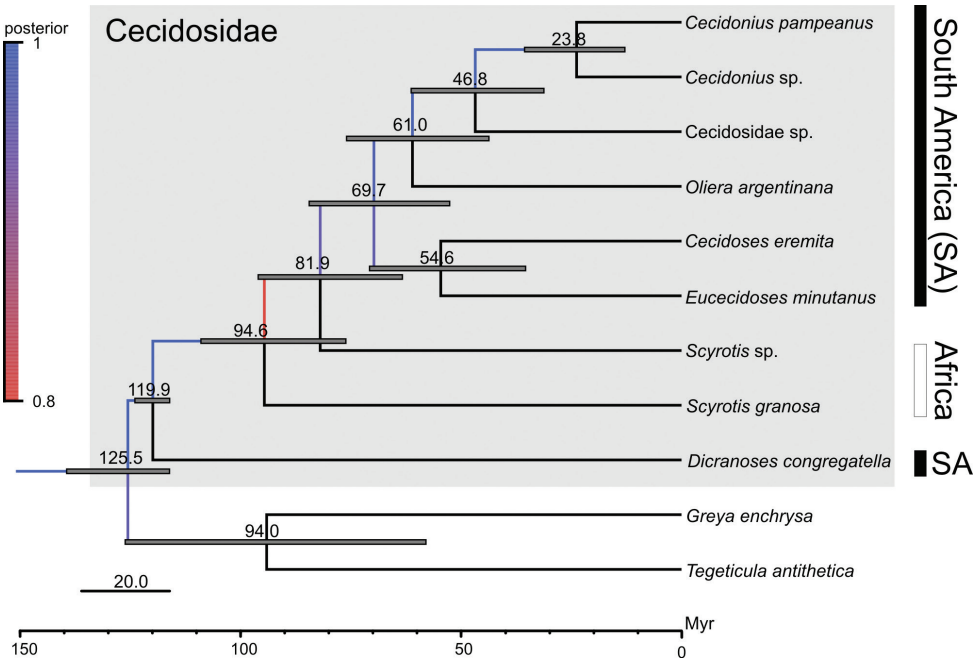

Figure 1. Molecular phylogeny of Cecidosidae. Bayesian time-calibrated consensus tree based on cytochrome oxidase subunit I (COI), r16S ribosomal (16S) and Wingless (Wg) genes. Prodoxidae (*Greya enchrysa* and *Tegeticula antithetica*) was used to root the tree. Colored branches indicate posterior probability support for the equivalent node following the legend. Dark gray bar indicates confidence interval for each node age estimate, presented in millions of years ago (Mya).

Table 1. Estimates of pairwise genetic distance (%) among nine Cecidosidae lineages based on DNA sequences (1420 base pairs of the cytochrome oxidase subunit I gene) using p-distance.

	Cecidonius pampeanus	*Cecidonius* sp.	*Cecidoses eremita*	*Dicranoses congregatella*	*Eucecidoses minutanus*	*Cecidosidae* sp.	*Oliera argentinana*	*Scyrotis* sp.	*Scyrotis granosa*
Cecidonius pampeanus	–								
Cecidonius sp.	9.0	–							
Cecidoses eremita	18.3	20.7	–						
Dicranoses congregatella	25.0	25.2	25.4	–					
Eucecidoses minutanus	13.8	18.6	18.3	23.2	–				
Cecidosidae sp.	16.1	18.3	18.5	23.7	17.8	–			
Oliera argentinana	13.7	16.4	17.5	21.8	16.3	15.1	–		
Scyrotis sp.	19.4	20.8	21.3	25.0	21.4	20.3	18.2	–	
Scyrotis granosa	23.7	27.4	26.9	28.8	27.8	25.8	24.5	27.3	–

cidosids, adults of *Cecidonius* have lateral cervical sclerites with anterior arms short and posterior ones with distal portion membranous. Females have a long ovipositor, bearing a large oviscapt cone with internal dorsal crest that extends cephalad within the seventh abdominal segment. In particular, they differ from those of the New Zealand *Xanadoses* that have a well-developed proboscis and five-segmented maxillary palpus (Hoare and Dugdale 2003) by having a vestigial proboscis and two-segmented maxillary palpus, among other characters. Unlike all species of the African *Scyrotis* that have forewings with four radial veins (Mey 2007), *Cecidonius* has five R-veins. With the exception of *Oliera*, which has small rudiments of galea (Moreira et al. 2012), other South American cecidosids show no vestiges of such structures. However, adults of *Cecidonius* have moderately well-developed galea. Contrary to those of *Oliera* where the maxillary and labial palpi are respectively three- and two-segmented, *Cecidonius* has the reverse; that is, two- and three-segmented maxillary and labial palpi, respectively. The pupa of *Cecidonius* is unique among all described cecidosids (those of *Scyrotis* are unknown), by having a stout and truncate cocoon cutter, flanked at the base by a pair of small, similarly shaped processes. In addition, in *Cecidonius* pupae the anterior margin of abdominal terga bear strong, posteriorly directed, transversally aligned spines that are much smaller in other genera. The larva of *Cecidonius* is also unique in having long thoracic setae, compared to short abdominal ones. They have two pair of stemmata; there is one in *Xanadoses*, and they are absent in other South American genera (larvae of *Scyrotis* are also unknown). Their woody, cylindrical galls are also unique, initially developing within swollen stems of *S. weinmannifolius* in southern Brazil. Later in ontogeny, they rupture the plant stem, thus growing externally. They are dehiscent, falling to the ground where pupation occurs. Contrary to those of *Scyrotis* (for detail, see von Noort et al. 2007), they do not exfoliate from the stem; they detach with their proximal base open, the corresponding orifice being clogged by larval feces.

Description of adults (Figs 2–4). Male and female similar in size and color; the body is covered with uniform, faded copper-coloured scales. Small moth, forewing length 4.16–4.58 mm (n = 4). *Head* (Fig. 3A): frons and vertex smooth, with sutures weakly developed; vestiture consisting of a pair of latero-dorsal scale tufts curved forward over the frons. Scales slender, lamellar, suberect and scattered over labrum, haustellum, maxillary, and labial palpi. Eyes relatively large, rounded; vertical diameter ~ 2.0x, minimum interocular distance across frons. Antennae median (~ 0.7x length of forewing); scape smooth except for medium dense pecten; flagellum filiform, with slender scales scattered only over dorsal half; ventral half with several elongate sensilla *ca*. 0.7× length of flagellomere. Labrum greatly reduced. Pilifers and mandibles absent. Haustellum moderately developed (~2/3 labial palpi length). Maxillary palpi short, 2-segmented; ratios of segments from base ~1.0:1.4. Labial palpi 3-segmented, bent anteriorly and upward (~2/3 eye width in length); ratio of segments from base ~1.0:1.8:1.6. *Thorax*: Anterior arms of laterocervical sclerites (Fig. 3B) short; posterior arms with distal portion weakly melanised. Metafurca (Fig. 3D, E) with slender, elongate postero-dorsal apophyses, free from secondary arms; antero-dorsal apodemes

Figure 2. Pinned-dried *C. pampeanus* adults, dorsal view: **A** male (holotype, LMCI 188-4) **B** female paratype (LMCI 188-6). Scale bars: 2 mm.

present. Wings (Fig. 3C) lanceolate; microtrichia reduced in number; accessory cell present; retinaculum absent. Wing coupling consisting of ~20 frenular scales arising in two to three irregular rows near base of costa. Veins 13 in number, all reaching the margin; L/W index ~2.9; Sc ending near midpoint of wing margin, radius with 5 free branches, M 3-branched, CuA 2-branched, CuA1 and M3 well separated from each other basally, CuP faint distally and not stalked with 1A+2A. Hindwing: ~0.8 forewing in length, L/W index ~2.9; Sc and R stalked and ending distally at midpoint of wing margin, Rs unbranched, M 3-branched, M1 and M2 well separated, CuA 2-branched, CuA1 and M3 well separated, CuP faded, not stalked with 1A+2A. Legs (Fig. 3F) with spurs 0-2-4; epiphysis present. Tibial length proportion (anterior / medium / posterior legs) ~ 0.6:0.7:1.0. *Abdomen*: Sternum 2 with broad, U-shaped caudal rim; tergosternal connection absent. Male with remaining pre-genital segments unmodified. Female with abdominal segment A7 ~ 4x the length of A6; caudal margin bearing a dense ring of stout, elongate setae.

Male genitalia (Fig. 4A, B). Uncus moderately bilobed. Socii consisting of a pair of setigerous, dorsally directed lobes. Valva long and slender, with an elongate pectinifer along ventral margin extending ~ distal half-length of valva. Vinculum Y-shaped. Phallus (Fig. 4B) simple, slender, and tubular, rosette-like shaped anteriorly; vesica without cornuti. Juxta (Fig. 4B) elongate (~ 2/3 phallus length), slender, slightly spatulate distally and encircling phallus caudally. Saccus stout and tubular, ~ 1.3× length of valve.

Female genitalia (Fig. 4C, D). Oviscapt cone (*sensu* Kristensen 2003, San Blas and Davis 2013) present, with internal dorsal crest long, reaching the anterior portion of tergum seven. Anterior apophyses long, extending beyond fifth abdominal segment. Posterior apophyses ~1.5× length of anterior apophyses, and with anteriorly attached apodemes of similar width. Posterior apophyses are caudally fused to form an acute ovipositor, whose apex is compressed and sagittate, the lateral ridge bearing minute serrations. A typical primitive monotrysian reproductive system, with cloaca and vestibulum each bearing a pair of slender apodemes that extend anteriorly within abdominal segment 7; vestibulum without sclerotized structures; ductus and corpus bursae membranous, the latter saculiform, without signum; spermatheca connected to small, saculiform utriculus by a slightly coiled, afferent canal.

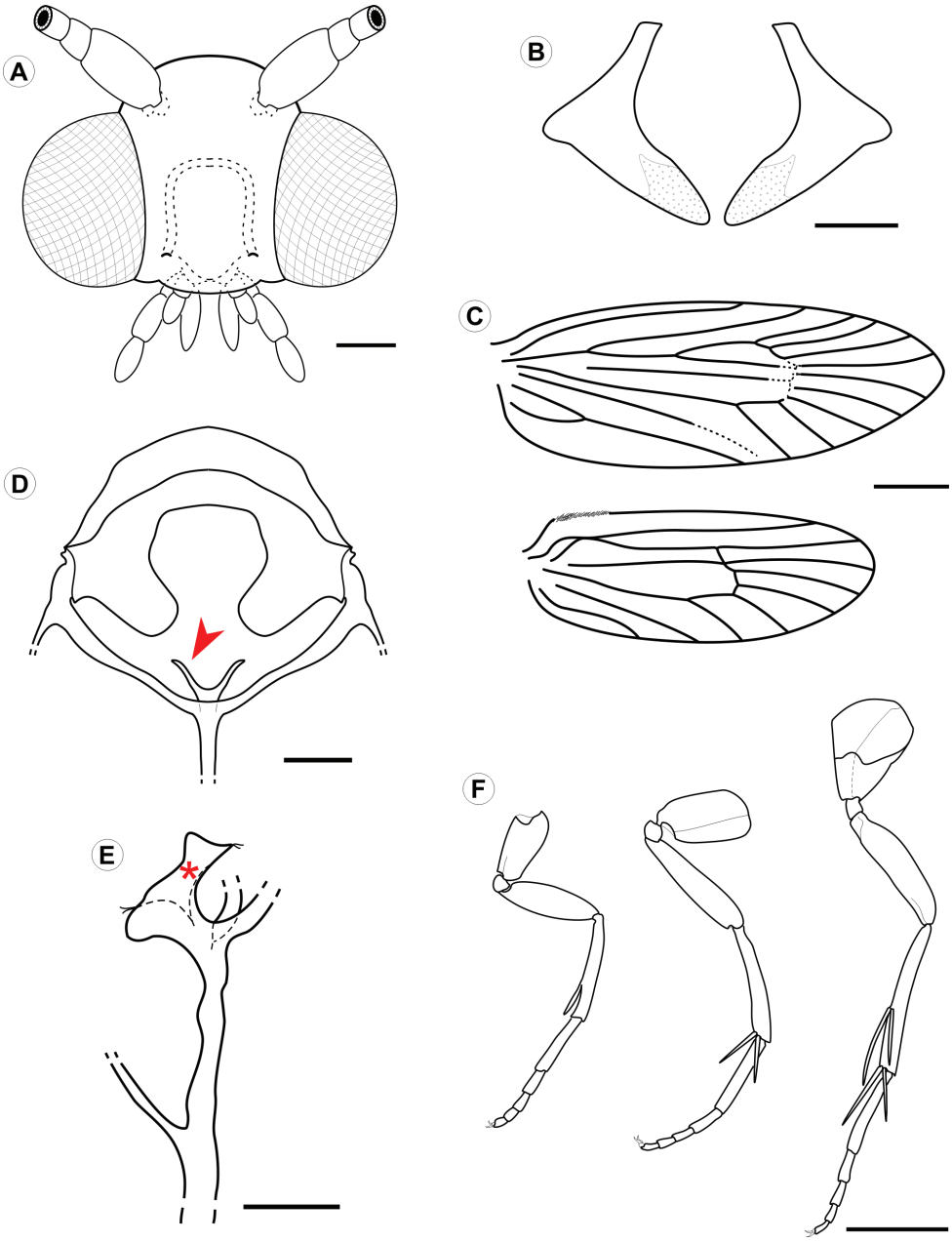

Figure 3. *Cecidonius pampeanus* adult morphology under light microscopy. **A** head, anterior view **B** lateral cervical sclerites, anterior; **C** fore- and hindwing venation, dorsal **D** metathoracic furcasternum, posterior (closed arrow points to left furcal apophysis) **E** metathoracic furcasternum in detail, lateral (asterisk indicates left furcal apophysis) **F** fore-, median- and hindlegs, from left to right, respectively. Scale bars: 0.25 (**A, D**); 0.1 mm (**B**); 1 mm (**C, F**); 0.2 mm (**E**).

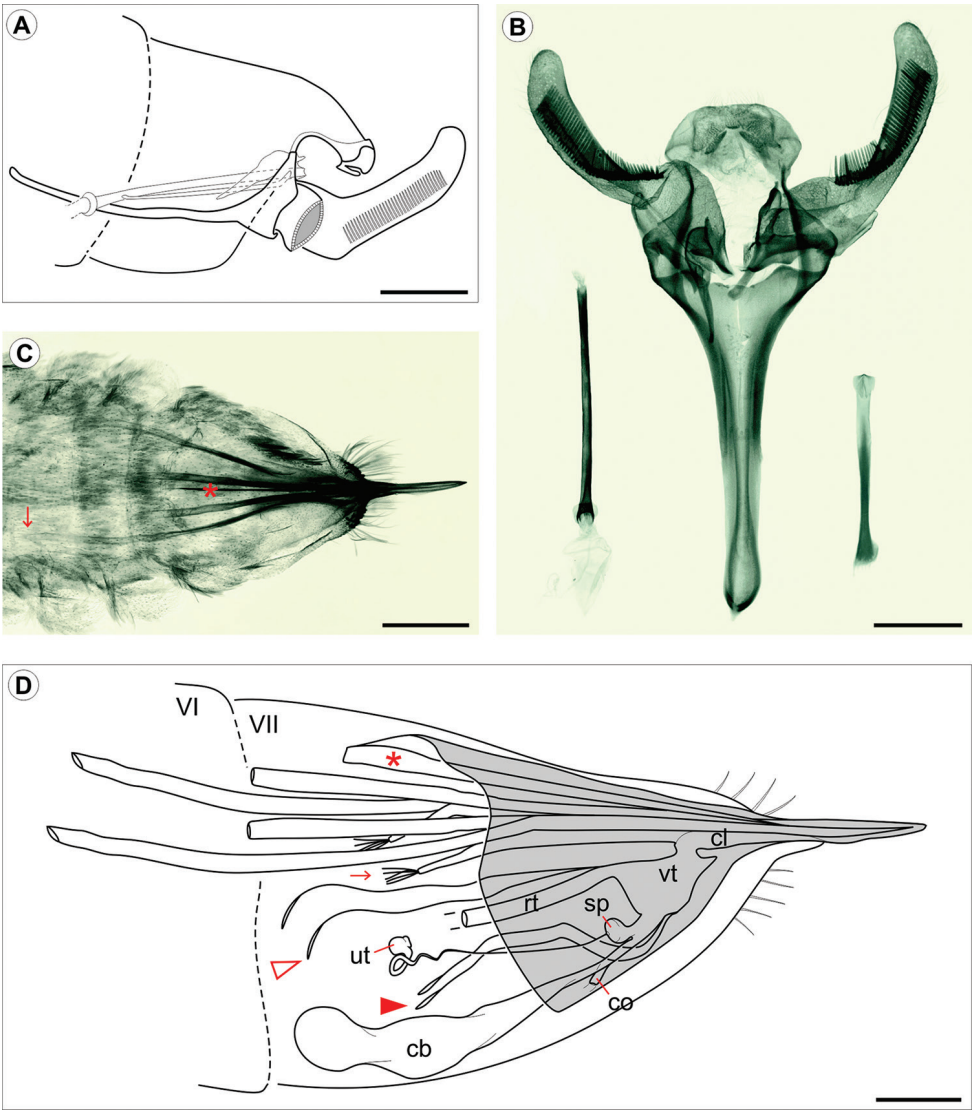

Figure 4. *Cecidonius pampeanus* genitalia morphology under light microscopy. **A** schematic representation of male genitalia, lateral view (left valve omitted) **B** dissected male genitalia, ventral, with detached phallus and juxta, on left and right side, respectively **C** female genitalia, dorsal **D** schematic representation of female genitalia, latero-dorsal. Roman numbers indicate abdominal segments. Oviscapt cone is represented in light gray in **D**. Arrows point to the end of left anterior apophysis in **C**, and to the apodeme of posterior apophysis in **D**. Asterisks indicate internal dorsal crest of oviscapt cone in **C** and **D**. Open and closed arrow heads point, respectively, to posterior apophyses and cloacal apodemes in **D**. Abbreviations: **cb** corpus bursae; **cl** cloaca; **co** common oviduct; **sp** spermatheca; **rt** rectum; **vt** vestibulum; **ut** utriculus of spermatheca. Scale bars: 0.25 mm.

Etymology. The genus name is derived from a composition between the Portuguese *Cecidia* (a gall; from the Greek, *kekídion*) with *Don* (an English nickname). Thus, the generic name means "Don`s gall", named after Donald Davis from the Smithsonian Institution, USA, in recognition of his great contribution to the development of world lepidopterology, and in particular for having kindly introduced the first author to the study of Neotropical cecidosids a few years ago. The name is to be treated as masculine.

***Cecidonius pampeanus* Moreira & Gonçalves, sp. n.**
http://zoobank.org/15DA6F09-4BF7-45CD-B25A-26DF60EDC383
Figs 2–9

Diagnosis. As discussed for the monotypic genus.

Description of adults. As described for the monotypic genus.

Type material. Brazil: Old grass field, private farm belonging to Antonio Malta, Coxilha das Lombas, 30°01'46"S, 50°36'40"W, 86m, Santo Antônio da Patrulha Municipality, Rio Grande do Sul State (RS), Brazil; G.R.P. Moreira, H. A.Vargas, R. Brito & S.A.L. Bordignon; 29.V.2012, pinned-dry preserved adults, reared by the first author from dehiscent galls collected on the ground around patches of *Schinus weinmannifolius* Mart. ex Engl. plants. Holotype ♂: LMCI 188-4, emerged on 9.XI.2012; donated to DZUP (33.342). Paratypes: 1♂ (LMCI 188-7), emerged on 21.XI.2012, donated to DZUP (33.352); 1♀ (LMCI 188-6), with genitalia on slide (GRPM 50-127), emerged on 19.XI.2012, donated to DZUP (33.362).

Additional specimens used for morphological descriptions, with the same collection data as the type material: 1♂ (LMCI 188-5), emerged on 18.XI.2012, mounted on three slides in Canada balsam, genitalia (GRPM 50-124), head and thorax (GRPM 50-125) and wings (GRPM 50-126); three pupae (LMCI 188-8), three last instar larvae (LMCI 188-11), and several galls, dissected from galls induced on *S. weinmannifolius* plants, fixed in Dietrich' fluid and preserved in 70% ethanol; two last instar larvae, mounted similarly on slides (GRPM 50-128 and 129).

Etymology. The epithet refers to Pampa, a biogeographic province within the Chacoan subregion (*sensu* Morrone 2006), predominantly composed of grasslands, and where *C. pampeanus* was first found.

Description of immature stages. *Larva* (Figs 5, 6, 9D, F): With five larval instars, which can be separated from each other by the head capsule width.

First instar (Fig. 6A, B). Head capsule width (average + standard error) = 0.066+0.009 mm; body length = 0.570+0.058 mm, n = 4. Head yellowish brown, with chewing mouthparts. Stemmata absent; antennae reduced, located close to mandibles; labrum subquadrate, with three pairs of minute setae; mandibles well developed, with four cusps along distal margin; maxilla with palpus and galea poorly developed; spinneret well developed, tubular; labial palpus one-segmented, bearing an apical sensillum. Thorax and abdomen creamy-white, cylindrical and U-shaped, with no developed primary setae; prothoracic shield, thoracic legs, prolegs, and abdominal calli absent.

Second instar (Fig. 9D). Similar in shape and color to fifth instar; head capsule width = 0.160+0.004 mm; body length = 1.060 + 0.134 mm, n = 3.

Third instar. Similar in shape and color to fifth instar; head capsule width = 0.217+0.005 mm; body length = 2.078 + 0.052 mm, n = 3.

Fourth instar. Similar in shape and color to fifth instar; head capsule width = 0.452+0.017 mm; body length = 3.990 + 0.700 mm, n = 4.

Fifth instar (Figs 5, 6C–L, 9F). Head capsule width = 0.898+0.031 mm; body length = 7.190 + 1.722 mm, n = 5. Head yellowish brown, with anterior margin orange-brown and lateral margin convex; frontoclypeus subtriangular, well-marked by pigmented adfrontal sutures, extending to apex of epicranial notch. Two well-developed, latero-located stemmata; antennae 2-segmented, with five sensilla, four short and one ~5x longer the others; labrum slightly bilobed, with three pairs of setae on distal margin; mandible well developed with four cusps along distal margin and one seta basally on external surface; maxilla with palpus and galea reduced; spinneret tubular to conical; labial palpus one-segmented, with well-developed apical seta. Chaetotaxy consisting of 14 pairs of setae: F group unisetose; C group unisetose; A group trisetose; AF group unisetose; P group bisetose, reduced in length; S group trisetose, one reduced in length; SS group trisetose.

Thorax (T) and abdomen (A) creamy-white, cylindrical, slightly curved, covered with microtrichia. Prothoracic shield light yellowish; thoracic legs and abdominal prolegs absent; abdominal segments A2 to A7 with well-developed calli, located on posterior margin of terga. A10 composed of three lobes, one dorsal and two latero-ventral. Circular spiracles without elevated peritreme, laterally on T1, A1–8. Thoracic segments surrounded by short setae interspersed with long ones (~5x longer). T1 with 12 pairs of setae: D group bisetose; XD unisetose; SD unisetose, outside prothoracic shield; L group trisetose, anterior to spiracle; SV group trisetose; MV unisetose; V unisetose. T2-3 with 10 pairs of setae: D group bisetose; SD bisetose; MSD unisetose; L group bisetose; SV group bisetose; V unisetose.

Abdominal segments (AB) with only short setae that are more or less aligned on the middle region of each segment, which are tentatively named. AB1-7 with 6 pairs of setae: D group bisetose; L group trisetose, posterior to spiracles; V unisetose. AB8 with 8 pairs of setae: D group bisetose; SD group unisetose; L group tetrasetose, posterior to spiracles; V unisetose. AB9 with 5 pairs of setae: D group unisetose; SD group unisetose; L group unisetose; SV unisetose; V unisetose. A10 with six pairs of setae: D group bisetose; SD group unisetose; SV trisetose.

Pupa (Figs 7, 8). Length = 6.44+0.52 mm; n = 3. Yellowish brown, with head, thorax, and abdominal spines becoming dark brown near adult emergence (Fig. 7C). Head with frontal process (gall-cutter) formed by three processes; one large, inverted U-shaped, located in the centre, which is flanked at the base by the other two that are ~5x shorter than the central one, directed laterally and slightly bent to the anterior side (Figs 7, 8A, B). Antennae narrow, long, slightly surpassing forewing apex; prothorax a narrow transverse band between head and mesothorax; hindwings concealed by forewings, reaching posterior margin of sternum A6; pro- and mesothoracic legs

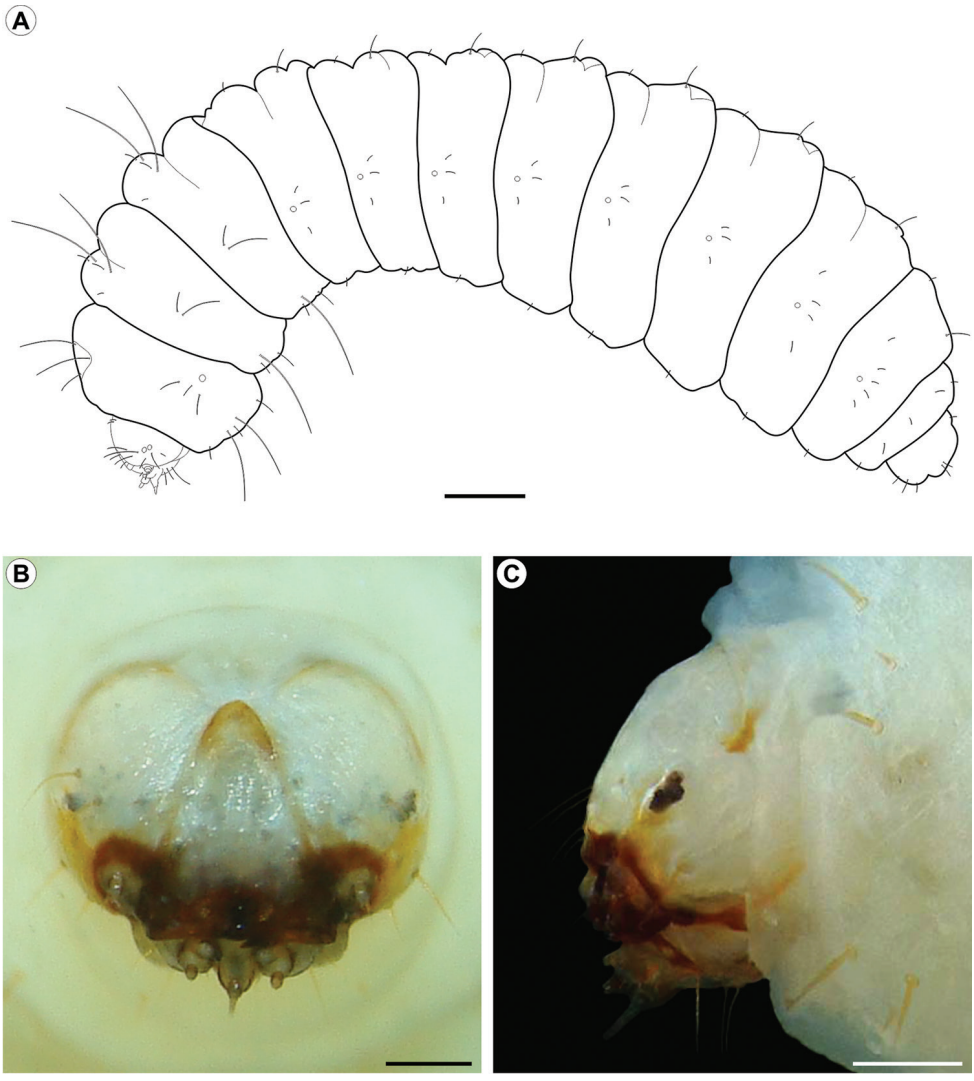

Figure 5. *Cecidonius pampeanus* last larval instar under light microscopy. **A** general schematic representation, lateral view **B, C** head, anterior, and lateral, respectively. Scale bars: 0.5 mm (**A**); 0.2 mm (**B, C**).

extended to A4 and A5, respectively; metathoracic legs reaching beyond forewing apex on segment A7 (Fig. 7). Frons and lateral portion of vertex with two pairs of setae each; tergum T2 with a pair of latero-dorsal setae; tergum T3 with a single seta on each side. Abdominal segments with central region covered by microtrichia; A2–9 with a transverse band of stout spines (Fig. 8E), near anterior margin of terga; tergum A10 with a pair of acute processes on posterior margin (Fig. 8F). Abdominal setae slightly shorter than thoracic, arranged in three rows (dorsal, supra- and subspiracular); one dorsal pair on segments A1–8; one supra-spiracular pair on segments A2–8; four subspiracular

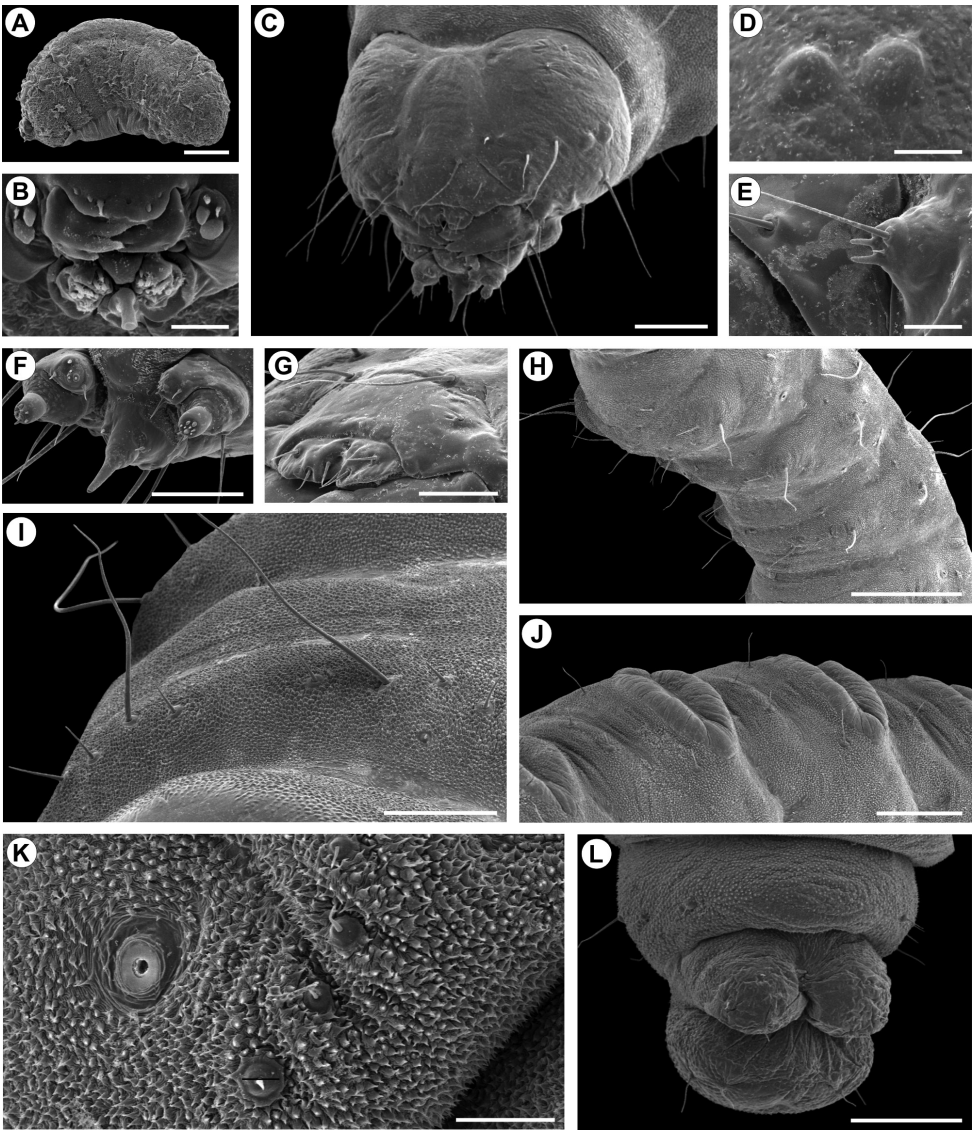

Figure 6. Morphology of *C. pampeanus* first (A, B) and last (C-L) larval instars under scanning electron microscopy. **A** general aspect, lateral view; B, buccal apparatus, anterior **C**, head, latero-dorsal **D** stemmata, lateral; **E**, left antenna, lateral **F** maxilla and labium, antero-lateral **G** labrum and clypeus, latero-dorsal **H** thorax, latero-ventral **I** meso- and metathorax in detail, with aligned setae of different lengths, antero-dorsal **J** second and third abdominal segments, showing tergal calli, latero-dorsal **K** eight abdominal segment in detail, showing aligned secondary setae (arrows) and spiracle, lateral **L** last abdominal segments, ventral. Scale bars: 100 μm (**A, F, G**); 10 μm (**B**); 200 μm (**C**); 50 μm (**D, E, K**); 250 μm (**J**); 1 mm (**H, L**); 0.5 mm (**I**).

pairs on segments A3–6 (Fig. 8D); seven subspiracular pairs on A7-8; six pairs latero-ventrally on A10; spiracles with slightly elevated peritreme, laterally on A2–8, spiracle on A8 partially closed.

Natural history. The unilocular, club-shaped, green galls of *C. pampeanus* develop initially enclosed within swollen stems of *S. weinmannifolius* branches (Fig. 9B, C). Later on in ontogeny, they erupt from the stem surface, either as isolated units or in small groups, and may reach a few tens per branch (Fig. 9F). The larval chamber is almost cylindrical in shape (maximum length = 7.99+0.58 mm; n = 6), and transverse to the stem axis. The external wall is shallow and thinner distally, formed as an expansion of the wood tissue under the bark (Fig. 9D–F). During the last larval instar, *C. pampeanus* galls have their wall somewhat annealed and ruptured at the base (Fig. 9G), when they fall freely to the ground containing the larva inside. The basal orifice left on these galls consequently is clogged by feces (Fig. 9I). These are continually deposited, then dry and solidify at the bottom of the gall chamber. After falling, the gall progressively dries up, turning a dark brown color (Fig. 9J). The external part may appear rotted in some old galls, when thin, longitudinally aligned groves are found on the gall surface. Like *O. argentinana* galls (Moreira et al. 2012), those of *C. pampeanus* also lack an operculum. With the action of the frontal process and body contortions, the pupa opens an irregular orifice on the distal, weaker wall (Fig. 9H). By continuing these movements and anchoring the body laterally with its abdominal spines, the pupa pushes itself partially out of the gall. During this process, the anterior portion of the exuviae is split, allowing adult emergence. In all cases of adult emergence followed under laboratory conditions, the anterior part of the pupal exuviae (head and thorax) was found protruding to the outside, while the posterior third remained in the chamber.

Field collections carried out during five consecutive years at the type locality indicated that *C. pampeanus* is a univoltine species, larvae growing during the summer when young galls are seen on *S. weinmannifolius* stems. Fully developed galls containing last instar larvae have been collected mainly during autumn. Based on several dissections of galls on the ground that were field collected during the winter, it can be inferred that the species overwinters in the larval stage, pupation occurring in spring, and adults emerging later on. This time of the year coincides with full vegetative activity of *S. weinmannifolius* host plants, including production of new sprouts. In the populations of *S. weinmannifolius* located in the study area, several plants can be attacked by *C. pampeanus*, and many branches within a patch of plants can bear galls induced by them. Under severe attack by *C. pampeanus*, *S. weinmannifolius* stems may wilt, die, and then fall, but the underground portion may stay alive. Under low gall densities, however, the aerial portion of plants stay green throughout the year, the signs of detached galls appearing as small, cylindrical craters on their stem surface.

In the populations studied here, *C. pampeanus* larvae are only common to find in yearly stages, within those galls still under the bark. Free-living larvae are rarely found in the external galls. These are severely attacked by unidentified parasitoids belonging either to *Lyrcus* Walker (Pteromalidae) or to *Allorhogas* Gahan (Braconidae), whose taxonomy and biology will be treated in detail elsewhere. Larvae of *Lyrcus* are ectopara-

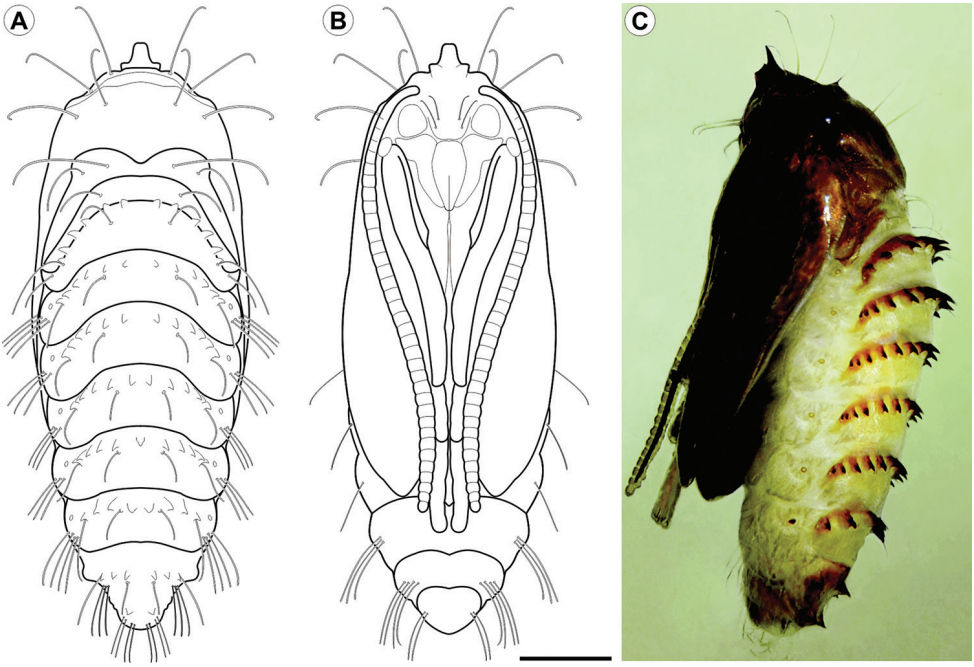

Figure 7. *Cecidonius pampeanus* pupa with light microscopy, under dorsal (**A**), ventral (**B**) and lateral (**C**) views. Scale bar: 1 mm.

sitoids found singly attached to *C. pampeanus* larvae inside the galls (Fig. 10A). They suck the internal contains of larvae, killing them and leaving only their exoskeletons intact. These parasitoids pupate inside *C. pampeanus* galls (Fig. 10B), which do not have their main shape changed, but turn a dark brown colour. In this case, galls stay attached to the stems for a longer time compared to ones free of parasitoids. After emergence, adults of *Lyrcus* open a characteristic, small orifice on the distal portion of the gall (Fig. 10C), through which they leave. By contrast, larvae of *Allorhogas* are gregarious and inquilines. They modify *C. pampeanus* galls, inducing production of additional tissue. When initially viewed externally in this case, *C. pampeanus* galls appear partially surrounded by this type of tissue (Fig. 10E). Later in ontogeny they are completely involved by such tissues, turning into globular, pinkish, large galls (up to 3.2 cm in diameter; n = 8) that last much longer in the field and promptly call attention (Fig. 10D, F). These galls are multilocular; larvae of inquilines are found individually in several chambers within (Fig 10G). Pupation also occurs inside galls, that then dry up and turn dark brown; adults of inquilines leave through small circular orifices that are found on the gall surface (Fig. 10H).

Host-plant and distribution. Galls of *C. pampeanus* have been found only on branches of *Schinus weinmannifolius* Mart. ex Engl. (Anacardiaceae). This is a small shrub (up to 50-cm tall), originally found scattered in open savannas (Fig. 9A), hill tops and forest borders of southern South America, including central and south Bra-

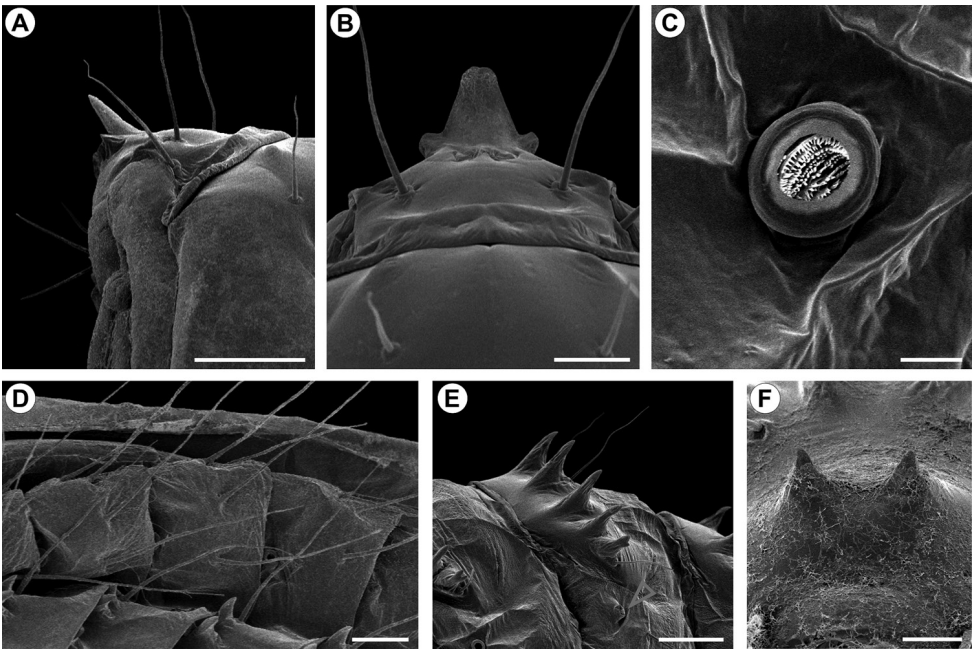

Figure 8. *Cecidonius pampeanus* pupal morphology with scanning electron microscopy. **A, B** head and prothorax, lateral and dorsal views, respectively **C** spiracle of sixth abdominal segment, dorsal **D** subspiracular setae from fourth to sixth abdominal segments, dorsal **E** tergal spines of eighth abdominal segment, lateral (arrow points to partially closed spiracle) **F** spines of tenth abdominal segment, posterior-dorsal. Scale bars: 0.5 mm(**A**); 0.25 mm (**B, C, E**); 0.1 mm (**D, F**).

zil, Paraguay, northeast Argentina and Uruguay (Barkley 1957, Luz 2011). However, populations of *S. weinmannifolius* bearing galls of *C. pampeanus* were found only in Rio Grande do Sul, the southernmost state of Brazil, particularly in the surroundings of Porto Alegre city (Fig. 11A) in the eastern limit of the Pampean province within the Chaco biogeographic region (*sensu* Morrone 2006). This region, also known as the Southeastern Highlands, since it reaches higher elevations than the remaining Pampean areas, includes several low-elevation hills (up to 300 m) that are more or less interwoven with fragments of semi-deciduous forests, herbaceous and shrub vegetation and single-layer grasslands, forming a mosaic. In this area a few, isolated, populations of *S. weinmannifolius* were found either as isolated plants or forming small patches (up to 3m in diameter), primarily located on hilltops and hill slopes, and a few scattered in the single-layer grasslands that prevail in the lower elevation areas.

Little is known about the biology or natural history of *S. weinmannifolius*. Although also found as isolated individuals, it usually forms small patches of plants, particularly in sandy soils. Preliminary field observations suggest that *S. weinmannifolius* is perennial, having a subterraneous habit of growth, forming stolons that grow just

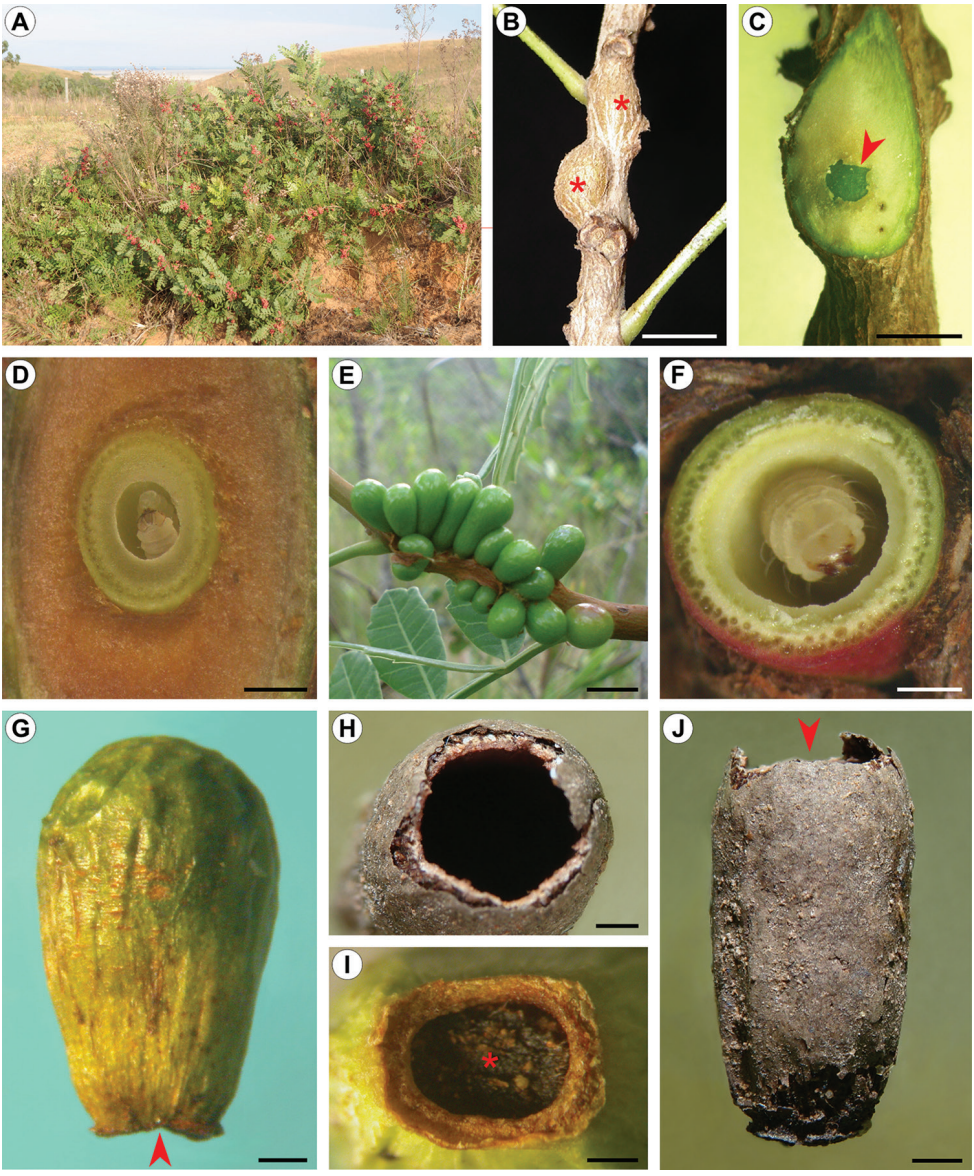

Figure 9. Natural history of *C. pampeanus* on *S. weinmannifolius*. **A** host plant patch at the type locality; **B** young developing galls within swollen stems (asterisks); **C** dissected swollen stem showing developing gall inside (indicated by arrow); **D** transversally sectioned young gall showing second instar larva inside; **E** group of external developing galls on branch; **F** transversally sectioned, full grown gall, showing last instar larva inside; **G** young dehiscent gall; **H** detail of emergence orifice left by adult on distal portion of old, empty gall (pointed by arrow in **J**); **I** detail of young dehiscent gall (arrow in **G**), showing orifice clogged by larval feces (asterisk); **J** old, empty, overwintered gall. Scale bars: 2 mm (**B**); 1 mm (**C, F, G, H, I, J**); 0.5 mm (**D**); 5 mm (**E**).

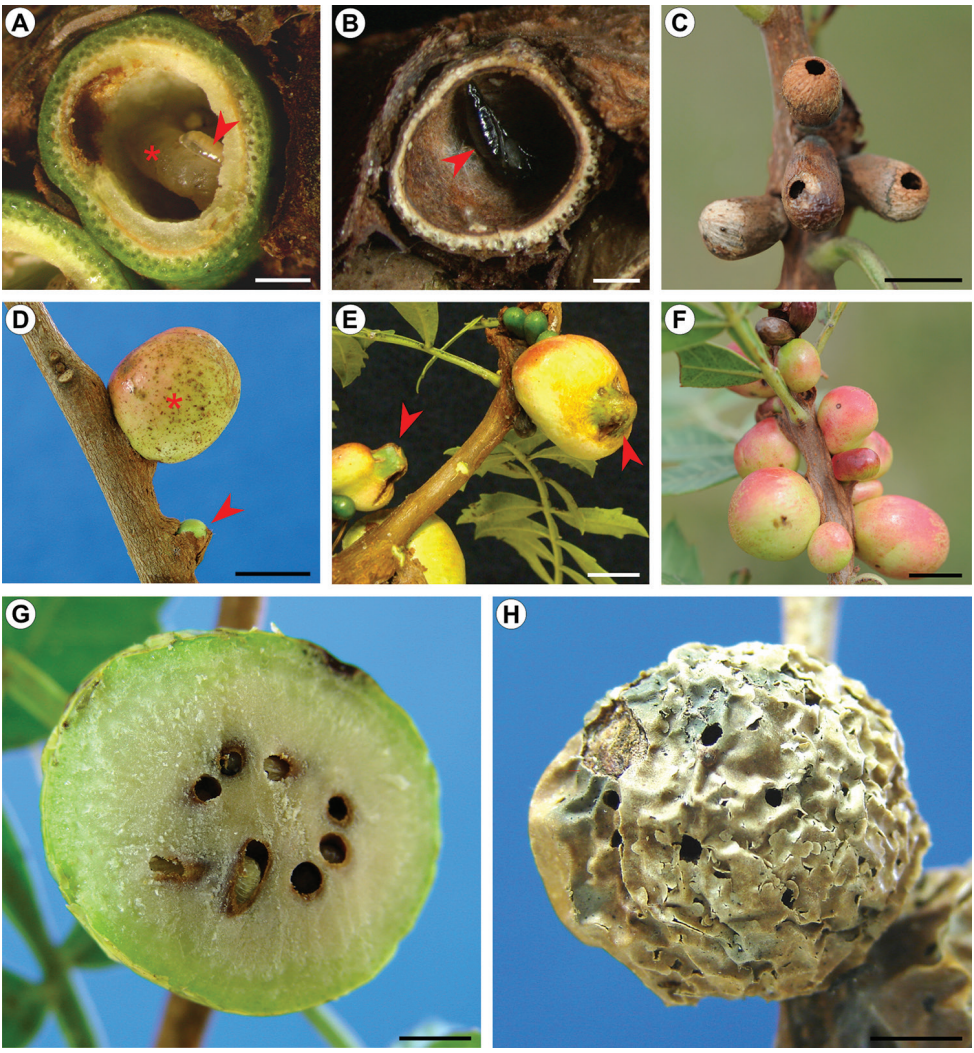

Figure 10. Hymenoptera fauna associated with *C. pampeanus* galls. **A** transversally sectioned, externally developing gall, showing inside a larva of *C. pampeanus* (asterisk) with attached larva (arrow) of *Lyrcus* sp. (Pteromalidae) **B** transversally sectioned, dried gall, with pupa of *Lyrcus* (arrow), after consumption of *C. pampeanus* larva **C** dried and empty attached galls showing orifices of emergence left by adults of *Lyrcus* **D** young, erupting, free of inquiline and adjacent inquiline attacked (*Allorhogas* sp., Braconidae) galls, indicated respectively by open arrow and asterisk **E** young galls of *C. pampeanus* (arrows) partially involved with gall tissue induced by inquilines **F** variation in size among *Allorhogas* galls early attacked **G** a full-developed inquiline-attacked gall showing larvae and pupae in cameras inside **H** senescent *Allorhogas* gall showing orifices of emergence (arrows) left by adults. Scale bars: 1 mm (**A, B, D, F**); 5 mm (**C**); 0.5 mm (**E, G, H**).

below ground and from which new sprouts emerge every year, starting in spring. At the type locality, the first flowers appear during November and the flowering season may last until March; fruits are found on plants from December to May. There is ap-

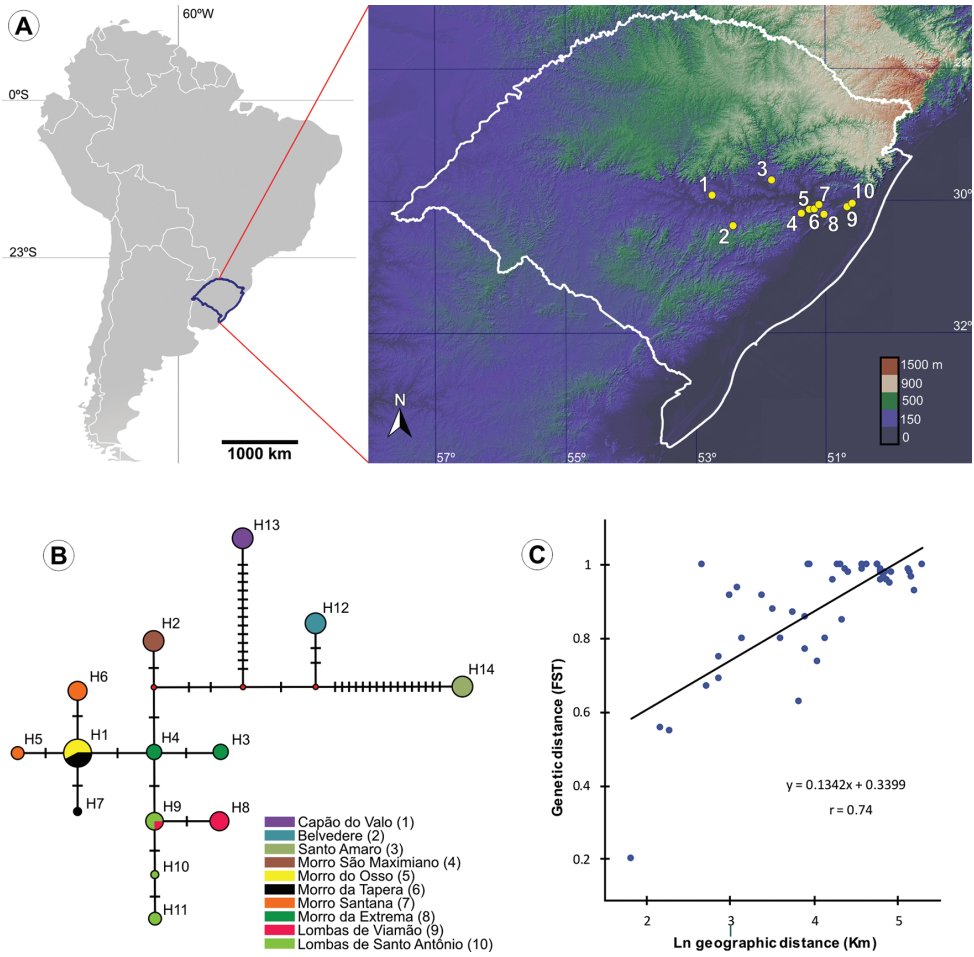

Figure 11. Geographic distribution and genetic variation among populations of *C. pampeanus* within Rio Grande do Sul State, Brazil; **A** localities of populations studied (see Suppl. material 3 for exact geographic coordinates and elevations) **B** evolutionary relationships of COI haplotypes across ten populations. The circles represent haplotypes; the diameter is proportional to the frequency in 60 analyzed individuals. Small red circles indicate intermediate vectors. Transversal bars represent mutational steps. Numbers in parentheses correspond to localities in the map (**A**) **C** correlation between pairwise geographic distance and estimates of gene flow (φST) (P<0.05).

parently little if any vegetative growth during the winter, which is also the season when the aerial parts of *S. weinmannifolius* plants may wilt and die.

Population genetic structure. Inferences on the genetic variability of *C. pampeanus* resulted from 42 (3%) variable sites. Overall, haplotype (Hd) and nucleotide diversity (π) were 0.92±0.01 and 0.0007±0.0009, respectively (Table 2). Individual populations presented Hd from 0.33 to 0.73 (P9 and P10, respectively) and π from 0.002 to 0.0013 (P9 and P10, respectively). A total of 14 haplotypes were found in

ten populations (Table 2; Fig. 11B). We found only one haplotype in each in P1 to P5; therefore, standard diversity indices and neutrality tests were not performed. From P6 to P9 two haplotypes per site were observed; P10 presented three haplotypes, the highest diversity. Except for H1 and H9, which were shared between P5/P6 and P9/P10 respectively, all were unique to each locality (Fig. 11B). Characterization of pairwise gene flow based on the F_{ST} index indicated significantly high levels of genetic structure in populations of *C. pampeanus.* Overall, F_{ST} ranged from 0.55 to 1 ($P < 0.05$) (Table 3). The lowest level observed was 0.20, between P5 and P6, not significant ($P > 0.05$). Spatial genetic structure assessed by the correlation between genetic and geographic distances indicated a significant pattern of isolation by distance for the ten populations ($r = 0.74$, $P < 0.01$) (Fig. 11C). Quantitative differentiation based on two groups of comparison reinforced the structure by distance pattern (Suppl. material 2). Both analyses (Jacuí River as a barrier and geographic distance) found similar values of F_{ST} (0.97; $P < 0.001$). However, when we grouped P2 with the cluster formed by P4 to P10 the divergence among groups was lower (46.45%; $P < 0.001$) than when we grouped it with P1 and P3 (58.73%; $P < 0.001$). Similarly, the divergence among populations within groups decreased from the first to the second scenario (51%, $P < 0.01$; 39.15%, $P < 0.001$, respectively).

Finally, analysis of demographic history by mismatch distribution indicated an overall multimodal pattern for *C. pampeanus* that is not compatible with a scenario of recent demographic expansion (Suppl. material 4). Single population analysis indicated a unimodal pattern, particularly for P9 that showed a possible scenario of expansion. In addition, overall neutrality tests yielded positive and non-significant values for all indices with respect to neutral expectations (Table 2). Single populations presented positive values, except P7 that showed negative values (but non-significant) for some parameters (i.e., Tajima´s D and Fu and Li's D and F) and P9, that presented all negative (but non-significant) values.

Discussion

Taxonomy and phylogeny

Since it was proposed as a family by Brèthes (1916), the position of Cecidosidae remained for a long time uncertain until its affinity to the superfamily Adeloidea was clarified by Becker (1977), who regarded the group as endemic to South America. The affinity of *Scyrotis* with South American cecidosids was proposed later by Davis (1987). Molecular data provided here give further support to this taxonomic affinity, and show that the African *Scyrotis* are much older (*ca*. 90 Myr) than South American genera. Results also suggest there could exist more than one cecidosid lineage in Africa, since the two species we sequenced were 27% apart from each other in terms of genetic divergence in our analyses. The first studies on African cecidosids conducted by Meyrick (1909, 1913, 1928) clearly suggested the existence of at least three line-

Table 2. Summary of genetic variability of ten populations of C. pampeanus based on mitochondrial DNA sequences. Populations (Pop) are as follows: Capão do Valo (CV), Belvedere (Bel.), Santo Amaro (SA), Morro São Maximiliano (MSM), Morro do Osso (MO), Morro da Tapera (MT), Morro Santana (MS), Morro da Extrema (ME), Lombas de Viamão (LV) and Lombas de Sto. Antonio (LSA). Numbers from 1 to 14 indicate number of haplotypes found in each population. Hd, haplotype diversity; π, nucleotide diversity. Neutrality tests performed: Tajima´s (D); Fu and Li's (D and F); Fu's (Fs). Asterisks indicate significant values, P < 0.05.

Pop.	Haplotypes															π	Neutrality tests			
																	Tajima's	Fu and Li's		Fu´s
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Hd		D	D	F	Fs
CV													X		0.00	0.0000	–	–	–	–
Bel.												X			0.00	0.0000	–	–	–	–
SA														X	0.00	0.0000	–	–	–	–
MSM		X													0.00	0.0000	–	–	–	–
MO	X														0.00	0.0000	–	–	–	–
MT	X				X										0.53	0.0004	0.850	1.052	1.029	0.625
MS						X	X								0.33	0.0005	-1.131	-1.155*	-1.195	0.952
ME			X	X											0.60	0.0008	1.753	1.279	1.434	1.938
LV								X	X						0.33	0.0002	-0.933	-0.950*	-0.964	-0.003
LSA									X	X	X				0.73	0.0013	1.647	1.395	1.523	0.758
Overall															0.92	0.0068	-0.1362	1.775*	1.2683	2.886

ages of cecidosids, which can be separated by differences in the buccal apparatus of the adults. In fact, these lineages were initially grouped by him into different genera, which were later considered by Gozmány and Vári (1973) as synonyms of *Scyrotis* and treated as such ever since (for a detailed discussion, see Mey 2007). Two of these lineages are represented in our analysis by *Scyrotis* sp. and *S. granosa;* the former presents a rudimentary proboscis and maxillary palpi and the latter lacks such buccal structures. This question should be taken into account in revising the taxonomy and phylogeny of the family in the future, which is much needed. Although not linked to any *Scyrotis* species in particular, a field survey of galls conducted by van Noort et al. (2007) found them in association with several species of *Searsia* and suggested the existence not only of a wide variety of gall morphology but also considerable variation in life history styles among the African *Scyrotis*. It is unlikely that such variation will be conciliated within a single genus, which should be further explored. Unfortunately, this revision is pending upon description of the immature stages and gall morph types they induce, but as already said these aspects are still unknown for any African cecidosid species. The present study showed how valuable the inclusion of immature stages and galls is in taxonomic studies of cecidosids, whose adults in particular have relatively uniform morphology, especially regarding the genitalia (Mey 2007). In addition, our results support an accelerate evolutionary rate in all Cecidosidae lineages, as mentioned by Pellmyr and Leebens-Mack (1999) when a cecidosid (*C. eremita*) was used for the first time in a molecular phylogeny of Adeloidea. Similarly, Regier et al. (2015) in a family-level phylogenetic study based on 19 genes, found a high substitution rate in Cecidosidae when

including *C. eremita* and *D. capsulifex*, which they found in Incurvariidae as well. This faster evolution of cecidosids makes it difficult to resolve some internal evolutionary relationships within the group, generating phylogenetic uncertainties that are hard to overcome even by increasing markers, and should be further explored.

Cecidonius gen. n. resulted as a unique lineage in the present study from both morphological and molecular analyses. Also, interestingly, it appeared as one of the most recent lineages (*ca*. 24 Myr) to be evolved within the extant cecidosids. It diverged *ca*. 16% from the closest related lineage, an additional undescribed cecidosid taxon existing in Chile and Argentina, which was included in the present study for comparison. This undescribed taxon differs from *Cecidonius* by having adults that lack a rudimentary proboscis and having a three-segmented maxillary palpus, pupae bearing a gall-cutter with a different shape, larvae without long hair on thorax and galls with completely developed wall without basal orifice, and will be described elsewhere. Molecular findings also showed that although described as monotypic, there is at least one more species belonging to *Cecidonius*, associated with *Schinus terebinthifolius* Raddi, which is still awaiting description. This undescribed species diverged from *C. pampeanus* by more than 9% in DNA sequences. Its galls are conspicuous, morphologically different, and larger than those of *C. pampeanus*. They are relatively common in populations of *S. terebinthifolius* existing in southern Brazil. Unfortunately, we have no pupae or adults of this species yet, which apparently shares a similar life-history style and associated difficulties regarding rearing of *C. pampeanus*.

Life history

It took us a few years to obtain the small number of *C. pampeanus* pupae and adults used for description in this study. Although relatively abundant as young larvae when still under the bark of swollen stems, later instars of *C. pampeanus* occur at low density in the field. Collection of mature, dehiscent galls during later summer, either using cloth bags attached to the plants or picking by hand those that had naturally dropped to soil, always led to failure regarding development under laboratory conditions. Dissection of these galls demonstrated that larvae do not pupate, remaining alive in the last larval instar for months, eventually dying without any apparent cause. Interestingly, similar difficulties regarding rearing of *C. pampeanus* are also mentioned by Meyrick (1917) in relation to the African *Scyrotis*. *Cecidonius pampeanus* apparently diapauses for months in the last instar larva, which stays motionless within its dehiscent gall in the soil until pupation and adult emergence occur in the next growing season. Probably this species presents a seasonal adaptation (*sensu* Tauber, Tauber and Masaki 1986) to overcome the unfavourable low temperatures that prevent growth during winter, and also adjust its life cycle to the host plant phenology. As already mentioned, new growth shoots that are required for gall induction (Raman 1994, Yukawa 2000) start appearing on *S. weinmannifolius* plants during the spring. This time of the year coincides with adult emergence in the field, and supposedly also with oviposition in *C. pampeanus*. A group of approximately 20 galls were collected in the field by the first author during winter

Table 3. Pairwise estimates of gene flow based on φ-statistics (φ_{ST}) for cytochrome oxidase subunit I mitochondrial sequences in nine populations *C. pampeanus*. All comparisons were statistically significant (P < 0.05), except the value in bold.

	Capão do Valo	Belvedere	Santo Amaro	Morro São Maximiano	Morro do Osso	Morro da Tapera	Morro Santana	Morro da Extrema	Lombas de Viamão	Lombas de SantoAntônio
Capão do Valo	–									
Belvedere	1.0000	–								
Santo Amaro	1.0000	1.0000	–							
Morro São Maximiano	1.0000	1.0000	1.0000	–						
Morro do Osso	1.0000	1.0000	1.0000	1.0000	–					
Morro da Tapera	0.9846	0.9636	0.9875	0.9200	**0.2000**	–				
MorroSantana	0.9814	0.9583	0.9848	0.9166	0.6666	0.5500	–			
Morro da Extrema	0.9818	0.9538	0.9853	0.8800	0.8000	0.6909	0.7466	–		
Lombas de Viamão	0.9906	0.9787	0.9923	0.9565	0.9411	0.8631	0.8695	0.8000	–	
Lombas de Santo Antônio	0.9682	0.9276	0.9740	0.8521	0.8000	0.7368	0.7652	0.6285	0.5600	–

and kept under room temperature in the laboratory within plastic vials containing moist soil from the type locality. A few were dissected at fifteen-day intervals, rendering only last instar larvae. The first pupa in this case appeared in spring (October), and the adults, which were used in the present description, *ca*. one month later. The token stimuli that initially trigger and later break the diapause in *C. pampeanus* remain to be determined. We may speculate from above that the corresponding stimuli may be received during autumn by the dehiscent galls that are already in the soil.

Inquiline and parasitoid wasps

Allorhogas species are among a few braconid wasps having a phytophagous feeding habit. They are apparently relatively common and widespread in the Neotropics, all associated with galls, occurring in several plant families including Burseraceae, Fabaceae, Melastomataceae, Polygonaceae, Rubiaceae, and Solanaceae (e.g., Macedo and Monteiro 1989, Marsh et al. 2000, Marsh 2002, Penteado-Dias and Carvalho 2008, Chavarría et al. 2009, Centrella and Shaw 2010, 2013, Martínez et al. 2011, Martínez and Zaldívar-Riverón 2013). However, their biology is largely unknown, and it is still uncertain whether they are primary gall inducers or inquilines. A clear pattern always emerged during dissections of hundreds of galls from several *S. weinmannifolius* populations in the present study, demonstrating that they are inquilines. First, they were never found inside young galls that were located under swollen stem bark, where only young larvae

of *C. pampeanus* were always present. Second, erupted galls bearing either free-living *C. pampeanus* larvae or those attacked by *Lyrcus* ectoparasitoids do not change their shape, but only those bearing *Allorhogas* that turn from cylindrical into globular galls. Third, *Allorhogas* immatures were always found within older, erupted and much larger, shaped-modified galls, where larvae of *C. pampeanus* were found dead. Fourth, as already described, a progressive transition in shape between galls free from such inquilines (cylindrical) to those attacked by them (globular) is found in the field, always in association with early development of *Allorhogas* larvae. Most of the *Allorhogas* studies listed above have a taxonomic bias and are based on descriptions of adults reared during extensive surveys, without including descriptions of immature stages. They lack information on gall ontogeny, and most importantly, about identification of trophic levels of insects present within these galls. Thus, the biological status of *Allorhogas* in those gall systems should be re-examined, since some of them may not induce galls but act as inquilines, the true gall inducers being either underexplored or missed in such cases.

Similar to what was described for the *Scyrotis* galls attacked by *Rhoophilus* Ioewi (Hymenoptera: Cynipidae) inquilines (van Noort et al. 2007), space for a *C. pampeanus* larva within a given gall is progressively diminished with the development of tissues induced by *Allorhogas* larvae. In fact, in several cases in the present study during the dissections of medium-sized developing galls bearing *Allorhogas* inquilines, a dead *C. pampeanus* larva was found within a compressed space inside. From a gross morphology perspective, tissues induced to develop by *Allorhogas* are clearly different from those induced by the original inducer *C. pampeanus*, regarding thickness, consistency, and colour. In general, tissues present in insect galls are complex, and may structurally vary even within a given gall lineage (Stone and Schönrogge 2003). Specially the nutritious ones, which are absent on ungalled host plants, may also vary in complexity at a very fine scale not only among but also within galls. For example, when tissues produced by lepidopterans and hymenopterans are compared, differences between them emerge at the cell level in relation to the type, quantity, local and disposition of chemicals they store, among other characteristics (e.g., Ferreira and Isaias 2013, Vecchi et al. 2013). These tissues are used for feeding by the corresponding inducers independently, that is within their own distinctly located galls. This is not the case in the present system, where such tissues are induced by distantly related insect lineages and occur within the same gall. Thus, we suggest that tissues induced by *Allorhogas* species may inhibit feeding by *C. pampeanus*, whose larvae, by being confined in space, completely surrounded by tissues unsuitable for feeding, would be lead to death by inanition.

Additional field observations suggest that the existence of an inquiline association between *Allorhogas* species and galls of other cecidosids is common in southern Brazil. This is the case of the gall induced in *S. terebinthifolius* by the undescribed, additional species of *Cecidonius* already mentioned, as well as of those induced in *S. polygamus* by *C. eremita* and *E. minutanus*. Thus it seems that these braconid wasps parallel in the Neotropics the cynipd wasps that are inquilines of cecidosid galls in Africa (van Noort et al. 2007). Cynipids are found in South America, not acting as inquilines but as primary gall inducers, as for example in Fabaceae (e.g., Nieves-Aldrey and San Blas

2015). Unfortunately, little is known about the biology of the Neotropical species of *Lyrcus*. They are diverse and difficult to identify in the Nearctic region, where many are important parasitoids of agricultural pests, primarily belonging to Coleoptera and Diptera (Gibson GAP, Agriculture and Agri-Food Canada, pers. comm.). According to preliminary observations there are additional insect species yet to be explored in association with *C. pampeanus* galls, including cecidophages, predators, hyperparasitoids and successors that use them as shelter. The latter may include other arthropods and are common in cecidosid galls, since some of these galls may last for years after adult emergence and thus be used by other insects for shelter and even for nesting (e.g., Wille 1926, Laroca 1972). We hope this study will stimulate additional studies on this topic, thus revealing fully the hidden diversity existing in association with these galls.

Genetic diversity and conservation biology

Our study provides strong evidence that *C. pampeanus* is under threat of extinction, and protection measures should be taken to conserve its remaining populations. The reasons are based primarily on the destruction of the host plant habitat. Open savannas of southern Brazil (= Brazilian 'Campos') where populations of *S. weinmannifolius* are found have been suffering from anthropic influence for decades, mostly caused by agriculture in general and/or cattle ranching, and recently from widespread expansion of *Eucalyptus* L'Heritier, *Acacia* Martius and *Pinus* Linnaeus plantations (Overbeck et al. 2007, Cordeiro and Hasenack 2009). A search by the first author for populations of *S. weinmannifolius* of which older dried material is preserved in the main herbaria in the region (e.g., UFPR/ Curitiba, Barbosa Rodrigues/Itajai; and UFRGS/Porto Alegre), suggested that most of these have disappeared since. In Parana state, for example, extant populations seem to be restricted to a few places, including the preserved area of Parque de Vila Velha, Ponta Grossa municipality. In Rio Grande do Sul scattered populations were located on high elevation steppes, as for example in Canela and São Francisco de Paula municipalities, and particularly at low elevations in the western portions of the Pampa biome. However, as already mentioned, extant populations of *S. weinmannifolius* bearing galls of *C. pampeanus* were restricted to small patches in the latter area. More importantly, these populations are distant and isolated from each other. Most of them are located at higher elevation, such as on hilltops and hill slopes interspersed with small bushes as already mentioned, where they are relatively more protected from anthropic influence. At least two of these areas (Morro do Osso and Morro Santana) are officially protected areas already, but the remaining populations are located on private property. *Schinus weinmannifolius* is considered a pasture weed, supposedly unpalatable to livestock, the reason for which we presume it has disappeared from most low elevation areas, where agriculture and cattle ranching prevail as the main economic activities. *Schinus weinmannifolius* is apparently heliophilous, and in consequence does not grow satisfactorily within plantations such as those composed of *Eucalyptus*, *Acacia* or *Pinus*, also common in the region.

There is no indication that adults of cecidosids feed actively, last long and disperse much; oviposition supposedly occurs on the plants surrounding those where they developed as immatures (e.g., San Blas and Davis 2013). The limited dispersal together with low connected patches of *S. weinmannifolius* resulted in 'island' populations of *C. pampeanus* with reduced variability. Moreover, high genetic structure and partition of variation based on geography corroborated a pattern of isolation by distance. The restricted distribution and small population sizes are important causes of reduced genetic diversity (Hamrick and Godt 1989), since the effects of natural selection and/or demographic changes may be more pronounced in such populations (Ellstrand and Elam 1993, Gibson et al. 2008). The low variability found in *C. pampeanus* possibly makes the species vulnerable for novel selection pressure. Whether populations would be affected by stochastic processes, particularly genetic drift, depends on gene flow within and among populations, among other ecological factors. We found significantly low levels of gene flow among populations of *C. pampeanus*. Haplotypes were mainly unique to each locality, except between Morro do Osso/Morro Tapera and Lombas de Viamão/Lombas de Santo Antônio; even so, the latter presented significant high F_{ST} values.

The low number of nucleotide differences between the haplotype pairs (except for H12, H13 and H14) and a multimodal curve in the mismatch distribution analysis of *C. pampeanus* indicate that population expansion is unlikely to have occurred. In contrast, the population of Lombas de Viamão presented an expansion pattern. According to Rogers and Harpending (1992) and Tajima (1989), bottlenecks may generate waves in the distribution of pairwise nucleotide differences. However, contrary to expansion, a population contraction leads to maintenance of genetic diversity over time. In a bottleneck model individuals differ in the average number of nucleotide changes when taken randomly from a given population. Such an effect leads to multipeak nucleotide distributions, as well as large pairwise differences between them (Harpending et al. 1998, Rogers and Harpending 1992). Additionally, when estimated by median-joining, the haplotype topology also did not support a population expansion scenario, as it did not fit into a typical star-like model (Harpending et al. 1998, Slatkin and Hudson 1991). The results suggest that demographic changes in populations of *C. pampeanus* are a consequence of ancient historical processes and recent decline, likely due to landscape disturbance.

The above-mentioned higher trophic level-associated fauna may be also under threat, considering that its existence depends on the success of *C. pampeanus*, the primary gall inducer. In other words, a whole community associated with *C. pampeanus* galls may go extinct in South Brazil, even before species that integrate it have been described, in the case of extinction of the primary gall inducer. A survey should be carried out to identify the unknown fauna associated with these galls. We also suggest that additional studies should examine the degree of specificity and inter-dependence of this fauna with *C. pampeanus* and its host plant. These actions should be prioritized when planning the corresponding conservation measures, since they are prerequisite to their implantation. Protection measures have been scarcely taken in relation to the

lepidopteran species that are under threat of extinction in the Neotropical region. In Brazil, actions in this regard have involved primarily the butterflies, in total 55 species that are officially considered under threat of extinction (Freitas and Marini-Filho 2011). However, microlepidoptera and associated plants are largely unknown in this country due to a corresponding taxonomic impediment (Aguiar et al. 2009), and thus they have been completely neglected from a conservation biology perspective. Within the gall-inducer and leaf-miner micromoths there are many species that are specialists on rare and/or endemic plants, particularly in the Brazilian Atlantic Forest, most yet to be discovered and/or described (Luz et al. 2014, Moreira et al. 2017). By being dependent on endemic hosts at a regional scale, these species in particular are under comparatively greater threat, because most of such plants are restricted in distribution (Lewinsohn et al. 2005). This study is apparently the first to suggest that a micromoth and its associated fauna should be taken into account in this regard in Brazil. It is also important to emphasize that the restricted number of extant *C. pampeanus* populations are located within the southern Brazilian "Campos" (= Pampean savanna) that is considered a diverse but neglected biome from a conservation biology perspective (Overbeck et al. 2007), and where no moth has ever been targeted from a conservation biology perspective.

Further remarks

This study also showed how important intensive, integrative taxonomic studies are to identify accurately the role of a cecidosid species in a given gall community. *Cecidonius pampeanus* attracted our attention *ca*. 10 years ago as a cecosid lineage by comparison of DNA sequences extracted from the larval stage, dissected from under the bark of swollen stems of *S. weinmannifolius*. For several years, its identification remained provisional, tied only to DNA similarity to other cecidosids, since for this new species morphology of the last larval instar, found later in the field, was also atypical compared to any known cecidosid. Full confirmation of the existence of this new lineage came when we finally obtained their pupae and reared them to adults. We inferred that the absence of such an approach led Tavares (1909: 8) to identify the true inducers of such *S. weinmannifolius* galls as "… *probabiliter Cynips incognita*" [… probably an undescribed *Cynips* Linnaeus species]. This action has prevented unraveling not only the true gall inducer, but also the diversity of fauna associated with such galls for more than a century, since his rationale was followed without being questioned by other authors (e.g., Wille 1926, Houard 1933, Sáiz and Núnez 1997). In other words, from Tavares' original description until the present study, such galls have been treated as two trophic level systems, and their induction was erroneously associated with an unidentified species of Cynipidae (Hymenoptera). The Portuguese Jesuit priest Joaquim da Silva Tavares, also a naturalist who first described these galls, was a pioneer in the study and description of Brazilian cecidology during the first quarter of the last century. His descriptions were accurate and finely

illustrated, but most of them were based on the gall morph type only, not always being associated with precise identification of the corresponding inducers. We suppose the large and colourful *Allorhogas*- bearing galls, which appear as neat black and white photographs in his publication (Plate VIIi, figs 22, 23), called his attention to *S. weinmannifolius* plants at first sight in the field. As already mentioned, free-living external galls bearing *C. pampeanus* larvae are rarely found on *S. weinmannifolius* plants in the field, most being killed by *Lyrcus* parasitoids, and thus they may never have been encountered by him. That is, the gall phenotype that is modified by the *Allorhogas* inquilines would have misguided him and led him to suggest the primary induction of such galls to be by cynipid wasps, based on the immature stages obtained when dissecting such galls, since those dissections were also illustrated by him (Plate VIII; figs 24, 25). He apparently did not rear to the adult stage of the assumed cynipid species at that time, since later on when working with the Brazilian melastomatacean galls he made comments on his disappointment about not ever having had a Brazilian cynipid specimen in his collection (Tavares 1917, p.19). In the same publication, he indirectly admitted having erroneously thought at first that these melastomatacean galls also looked like those induced by cynipids in Europe, but that he had changed his mind after having surprisingly obtained the first adult Lepidoptera reared from them.

Acknowledgements

Thanks are due to Prefeitura Municipal de Porto Alegre (Porto Alegre city), Nelson Matzembacker (Eldorado do Sul) and Antonio Malta (Santo Antonio da Patrulha), for allowing us to collect the specimens in areas under their care, and for providing assistance with field work. Gerson Buss, Rosângela Brito, Leonardo Re Jorge and Gislane von Poser (UFRGS), Gabriel Melo, the late Lucas Beltrami and Eduardo Carneiro (UFPR) assisted with field collections. Héctor Vargas (Universidad de Arica, Chile) and Germán San Blas (CONICET- Universidad Nacional de La Pampa, Argentina) provided cecidosid specimens for comparison. André Martins and Gabriel Melo (UFPR) identified the parasitoid and inquiline wasps, respectively. We acknowledge the staff members of CME/UFRGS and Thales O. Freitas (UFRGS) for the use of facilities and assistance with scanning electron microscopy and molecular analyses, respectively. Thanks are also due to Lafayette Eaton for editing the text. We are also grateful to Erik J. van Nieukerken (Naturalis Biodiversity Center, Netherlands), Carlos Lopez-Vaamonde (INRA, France) and Germán San Blas for suggestions that improved substantially the last version of the manuscript. G. L. Gonçalves, G.T. Silva and G.R.P. Moreira were supported by fellowships from CAPES/PNPD, CAPES/PPG-Ban/UFRGS and CNPq, respectively. R.P. Eltz and R.B. Pase were supported by the undergraduate research program of PROPESQ/UFRGS.

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Supplementary material 1

Table S1.

Authors: Gilson R.P. Moreira, Rodrigo P. Eltz, Ramoim B. Pase, Gabriela T. Silva, Sérgio A.L. Bordignon, Wolfram Mey, Gislene L. Gonçalves

Data type: molecular data

Explanation note: Primers and conditions used in polymerase chain reaction (PCR) to amplify COI, 16S and Wg genes.

Link: https://doi.org/10.3897/zookeys.695.13320.suppl1

Supplementary material 2

Table S2.

Authors: Gilson R.P. Moreira, Rodrigo P. Eltz, Ramoim B. Pase, Gabriela T. Silva, Sérgio A.L. Bordignon, Wolfram Mey, Gislene L. Gonçalves

Data type: statistical data

Explanation note: Analysis of Molecular Variance (AMOVA) using φ-statistics based on cytochrome oxidase subunit I mitochondrial sequences for groups of *C. pampeanus,* defined according to different dispersal barriers.

Link: https://doi.org/10.3897/zookeys.695.13320.suppl2

Supplementary material 3

Table S3.

Authors: Gilson R.P. Moreira, Rodrigo P. Eltz, Ramoim B. Pase, Gabriela T. Silva, Sérgio A.L. Bordignon, Wolfram Mey, Gislene L. Gonçalves

Data type: specimens data

Explanation note: Specimens used in this study for phylogenetic reconstruction and genetic structure analysis of *C. pampeanus*.

Link: https://doi.org/10.3897/zookeys.695.13320.suppl3

Supplementary material 4

Figure S1.

Authors: Gilson R.P. Moreira, Rodrigo P. Eltz, Ramoim B. Pase, Gabriela T. Silva, Sérgio A.L. Bordignon, Wolfram Mey, Gislene L. Gonçalves

Data type: statistical data

Explanation note: Graphs depicting the results of the mismatch distribution analysis for the total samples (*C. pampeanus*) and populations alone (P6 to P9). The analysis was performed with 1420 bp of COI sequences (excluding all sites with missing information or gaps).

Link: https://doi.org/10.3897/zookeys.695.13320.suppl4

Supplementary material 5

Figure S2.

Authors: Gilson R.P. Moreira, Rodrigo P. Eltz, Ramoim B. Pase, Gabriela T. Silva, Sérgio A.L. Bordignon, Wolfram Mey, Gislene L. Gonçalves

Data type: statistical data

Explanation note: Neighbor-Joining tree of *Cecidonius pampeanus* with the evolutionary distances computed using the Kimura 2-parameter method based on 1.6 Kb of cytochrome oxidase sequences. The analysis involved 60 individuals from 10 populations.

Link: https://doi.org/10.3897/zookeys.695.13320.suppl5

Table S1. Primers and conditions used in polymerase chain reaction (PCR) to amplify COI, 16S and Wg genes.

Locus	Abbreviation	Fragment length (bp)	Primers (F/R)		PCR	
					Reaction	annealing temperature
Cytochrome oxidase subunit I	COI	ca.1420	K698 (F)[a]	TACAATTTATCGCCTAAACTTCAGCC		48°C
			Nancy (R)[a]	TACAATTTATCGCCTAAACTTCAGCC		48°C
			Jerry (F)[a]	CAACATTTATTTTGATTTTTTGG	94°C, 5 min; 30 cycles of	48°C
			Pat II (R)[a]	TCCATTACATATAATCTGCCATATTAG	94°C for 15 s, N°C for 30 s,	48°C
16S ribosomal RNA	16S	ca.470	16Sar1(F)[b]	CCCGCCTGTTTATCAAAAACA	and 72°C for 30 s; 72°C, 5	47°C
			Ins16Sar (R)[b]	CCCTCCGGTTTGAACTCAGAT	min	47°C
Wingless	Wg	ca. 380	LepWg1(F)[c]	GARTGYAARTGYCAYGGYATGTCTGG		45°C
			LepWg2a (R)[c]	ACT NCGCARCACCARTGGAATGTRCA		45°C

[a] Caterino and Sperling (1999); [b] Palumbi (1996); [c] Brower & DeSalle (1998)

Table S2. Analysis of Molecular Variance (AMOVA) using ϕ-statistics based on cytochrome oxidase subunit I mitochondrial sequences for groups of *C. pampeanus* defined according to different dispersal barriers.

Group definition	Populations	Va	Vb	Vc	FST	FSC	FCT	*P*
Jacuí River acting as barrier	[P1+P3] and [P2+P4+P5+P6+ P7+P8+P9+P10]	2.9698 (46.45%)	3.2674 (51%)	0.1633 (2.55%)	0.9744	0.9489	0.5873	<0.001
Major network distance	[P1+P2+P3] and [P4+P5+P6+P7+ P8+P9+P10]	4.5520 (58.73%)	3.0348 (39.16%)	0.1633 (2.11%)	0.9789	0.9523	0.4639	<0.001

Table S3. Specimens used in this study for phylogenetic reconstruction and genetic structure analysis of *Cecidonius pampeanus*.

						Genbank (accession number)		
Family	**Genus**	**Species**	**Pop.**	**Vouchers***	**Haplotype**	**COI**	**16S**	**Wg**
Cecidosidae								
	Cecidonius	*pampeanus* sp.n.	1	LMCI 75-1 to 75-5	H13	XXXXX	-	-
			2	LMCI 39-14 to 39-19	H12	XXXXX	-	-
			3	LMCI 77-13 to 77-18	H14	XXXXX	-	-
			4	LMCI 4-47, 4-48, 16-24 to 16-27	H2	XXXXX	-	-
			5	LMCI 1-1, 1-3,1-6 to 1-9	H1	XXXXX	-	-
			6	LMCI 35-17, 35-18, 35-20, 35-22, 35-24, 35-25	H1, H5*	XXXXX	-	-
			7	LMCI 36-14 to 36-16, 36-20, 36-21	H6, H7	XXXXX/ XXXXX	-	-
			8	LMCI 18-21 to 18-26	H3, H4	XXXXX/ XXXXX	-	-
			9	LMCI 37-13, 37-15, 37-18, 37-19, 37-31	H8*, H9	XXXXX	-	-
			10	LMCI 38-16 to 38-21	H9, H10*, H11*	XXXXX/ XXXXX	-	-
	Cecidonius	sp.	-	LMCI 14-72, 14-74	-	XXXXX	XXXXX	XXXXX
	Cecidosidae	sp.	-	LMCI 163-14B, 233-6	-	XXXXX	XXXXX	XXXXX
	Cecidoses	*eremita*	-	LMCI 163-1A, 16-1	-	XXXXX	XXXXX	XXXXX
	Dicranoses	*congregatella*	-	LMCI 3-1	-	XXXXX	XXXXX	XXXXX
	Eucecidoses	*minutanus*	-	LMCI 163-21	-	XXXXX	XXXXX	XXXXX
	Oliera	*argentinana*	-	LMCI 6-11	-	XXXXX	XXXXX	XXXXX
	Scyrotis	sp.	-	LMCI 228-1	-	XXXXX	-	-
		granosa	-	LMCI 228-2	-	XXXXX	-	-
Prodoxidae								
	Greya	*enchrisa*	-	-	-	EU884123	-	-
	Tegeticula	*antithetica*	-	-	-	EU585222	-	-

*Larvae preserved in 100% ethanol at -20 °C, dissected from galls induced on *S. weinmannifolius* plants, collected from RS localities, preserved in the tissue collection of Laboratório de Morfologia e Comportamento de Insetos (LMCI), as follows: **Pop. 1 (= Population 1)** > LMCI 75-1 to 29 (n = 29), Capão do Valo, Cachoeira do Sul municipality, 29°54'10"S, 52°25'11"W, 86m, 02.V.2009, S.A.L. Bordignon leg.; **Pop. 2** > LMCI 39-14 to 32 (n = 20), Belvedere, Encruzilhada do Sul municipality, 30°22'10"S, 52°25'46"W, 210m, 18.V.2008, G.R.P. Moreira leg.; **Pop. 3** > LMCI 76-12 to 28 (n = 17), Santo Amaro, General Camara municipality, 29°55'49"S, 51°53'24"W, 65m, 8.V.2009, G.R.P. Moreira, S.A.L. Bordignon & G. Von Poser leg.; **Pop. 4** > LMCI 16-24 to 43 (n = 20), Morro São Maximiano, Eldorado do Sul municipality, 30°10'46"S, 50°23'21"W, 14.VIII.2007, 56m, G.R.P. Moreira & G.L. Gonçalves leg.; **Pop. 5** > LMCI 1-1 to 10 (n = 10), Morro do Osso, Porto Alegre municipality, 30°07'05"S, 51°14'37"W, 112m, 20.VII.2007, G.R.P. Moreira & R.P. Eltz leg.; **Pop. 6** > LMCI 35-16 to 35 (n = 20), Morro da Tapera, Porto Alegre municipality, 30°06'53"S, 51°11'47"W, 156m, 13.V.2008, G.R.P. Moreira & G. Buss leg.; **Pop. 7** > LMCI 36-12 to 31 (n = 20), Morro Santana, 30°03'12"S, 51°07'14"W, 295m, Porto Alegre municipality, 13.V.2008, G.R.P. Moreira & L.R. Jorge, leg.; **Pop. 8** > LMCI 18-21 to 40 (n = 20), Morro da Extrema, Porto Alegre municipality, 30°11'44´´S, 51°02'22" W, 161, 18.III.2008, G.R.P. Moreira & G. Buss leg.; **Pop. 9** > LMCI 37-13 to 32 (n = 20), Lombas de Viamão, Viamão municipality, 30°04'30"S; 50°41'08"W, 49m, 14.V.2008, G.R.P. Moreira & S.A.L.Bordignon leg.; **Pop. 10** > LMCI 38-15 to 34 (n = 20), Lombas de Santo Antônio, Santo Antônio da Patrulha municipality, 30°01'36"S, 50°36'49"W, 78m, 14.V.2008, G.R.P. Moreira & S.A.L Bordignon leg.

Additional larvae preserved in 100% ethanol at -20 °C, used for DNA extraction for comparison: from ***Cecidonius* sp**., dissected from galls induced on *Schinus therebinthifolius* Raddi, LMCI 14-71 to 74 (n = 4), Parque Passaúna, Campo Comprido municipality, Paraná State, Brazil, 25°27'35"S, 49°22'50"W, 905m, 22.II.2008, G.R.P. Moreira, O.S. Ribas, E. Carneiro & L. Beltrami legs.; from **Cecidosidae sp**., LMCI 163-14 (n = 3), Rungue/Tiltil, Chile, 12.10.2011, G.San Blas leg.; LMCI 233-6 (n =1), Cuesta La Dormida, 28.11.2013, H.A Vargas & G.R.P. Moreira legs.; from ***Cecidoses eremita***, LMCI16-1 to 20 (n=20), Morro Maximiano, Eldorado do Sul municipality, RS, Brazil, 14.03.2007, G. R.P.Moreira & G.L. Gonçalves legs.; from ***Dicranoses congregatella***, LMCI 3-1 to 10 (n =10), Rincão da Ronda, Canguçú municipality, RS, Brazil, 20.07.2007, G.R.P. Moreira leg.; from ***Eucecidoses minutanus***, LMC163-21(n =4), Las Heras, Mendoza, Argentina, 26.10.2011, G. San Blas & G.R.P. Moreira legs.; from ***Oliera argentinana***, LMC 6-1 to 15 (n=15), Rincão da Ronda, Canguçú municipality, RS, Brazil, 15.10.2007, G.R.P. Moreira leg.; LMCI 163-1 (n = 2), Luján de Cuyo, Mendoza, Argentina, 14.11.2009. G. San Blas leg.;.

Also used for DNa extraction for comparison, three pine-dried specimens from South Africa, all collected by Wolfram Mey: ***Scyrotis* sp.**, female, RSA, Eastern Cape, Asante Sana, leg. 2012 (LMCI 228-1); ***Scyrotis granosa*** Meyrick, male, RSA, Tsitsikamma, 2011 (LMCI 228-2); ***Scyrotis pulleni*** Mey, paratype, RSA, Mpumalanga, 2005 (LMCI228-3) that failured regarding DNA extraction.

Figure S1. Graphs depicting the results of the mismatch distribution analysis for the total of *C. pampeanus* samples and populations alone (P6 to P10). The analysis was performed with 1420 bp of COI sequences (excluding all sites with missing information or gaps).

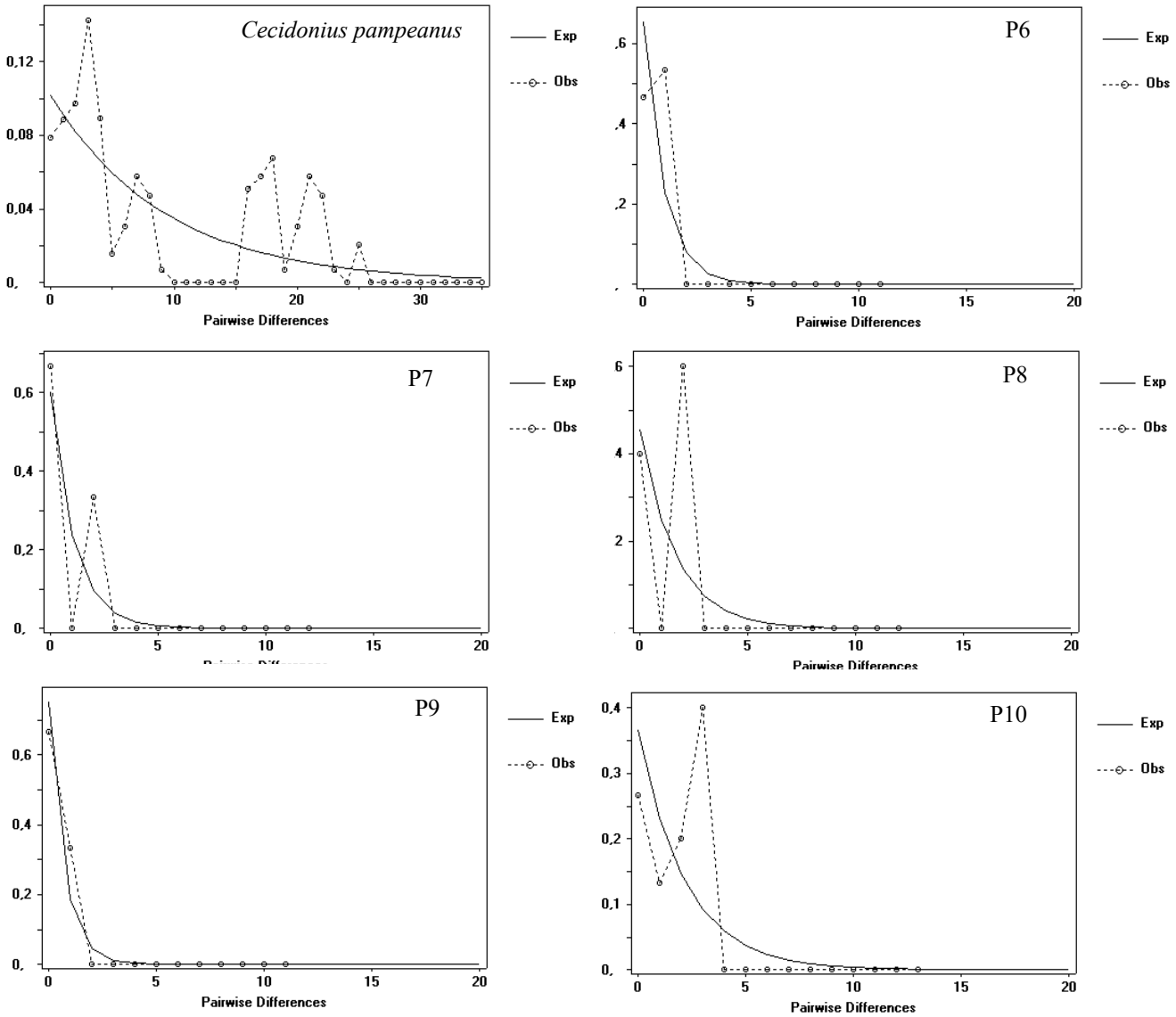
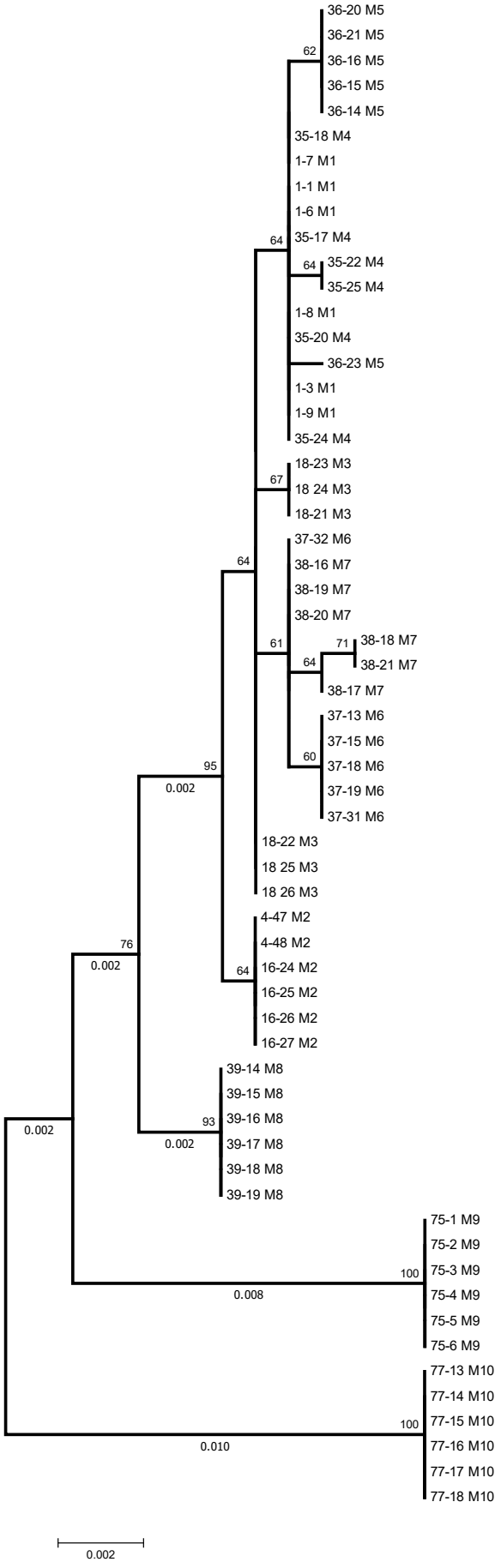


Figure S2. Neighbor-Joining tree of *Cecidonius pampeanus* with the evolutionary distances computed using the Kimura 2-parameter method based on 1.6 Kb of cytochrome oxidase sequences. The analysis involved 60 individuals from 10 populations.



CONSIDERAÇÕES FINAIS

A história evolutiva do cecidosídeo Sul Americano, *Eucecidoses minutanus* Brèthes foi explorada neste estudo a partir de um contexto genético, filogeográfico e de especiação. Populações correspondentes foram analisadas quanto à distribuição geográfica, associação com planta hospedeira, estrutura genética, tempo de divergência e o papel da vicariância e dispersão atuando na diversificação deste. A íntima relação entre *Eucecidoses* e sua planta hospedeira [*Schinus polygamus* (Cav.) Cabrera], sugeria previamente uma especiação ecológica que poderia implicar em um processo de co-evolução entre os grupos; entretanto, os registros geográficos encontrados para *S. polygamus*, se extenderam geograficamente muito além daqueles para *Eucecidoses*, descartando assim esse processo com causa principal da diversificação correspondente. O padrão atual de distribuição encontrado para *E. minutanus* é coincidente com as áreas montanhosas à leste dos Andes e com àquelas da costa Atlântica; essas regiões sendo conectadas na região do Pampa no passado, formam um arco orogênico Peripampásico, no sul do continente. Pela primeira vez para um grupo de insetos, a nível específico, com os dados filogeográficos calcados na biologia molecular, datação e biogeografia, corroboram este cenário quanto ao padrão de distribuição e especiação, com base principalmente nos eventos de vicariância. Os resultados apontam para existência de algumas espécies a serem descritas dentro deste táxon até então monoespecífico, com distribuição alopátrica ao longo deste arco orogênico. Assim, os resultados deram suporte para a elucidação do caminho evolutivo deste grupo de microlepidópteros em termos de diversificação,a partir de uma perspectiva cladogênica, associada a eventos orogênicos.

Dois gêneros novos e três novas espécies de microlepidópteros da região Neotropical foram descritas nesta tese, contribuindo assim para o conhecimento da família Cecidosidae que ainda carece de estudos a cerca de diversidade e biologia de seus representantes. Para o bioma Pampa, no sul do Brasil, *Cecidonius pampeanus* gen. nov. et sp. nov. foi descrita como novo gênero e espécie, induzindo pequenas galhas sob ramos de *Schinus weinmannifolius* Mart. ex Engl. As análises mostraram que o gênero é monofilético, e um dos mais recentes na família; e o isolamento por distância das populações foi corroborado pela distribuição geográfica e alta estrutura genética que apresentam atualmente. As galhas de *C. pampeanus* são severamente atacadas por

diferentes parasitóides ainda no início do seu desenvolvimento. Inquilinos modificam as galhas induzidas por *C. pampeanus*, tornado-as mais vistosas e duradouras. Supostamente devido a isso, o real indutor das galhas ficou desconhecido por muitos anos na literatura. O sucesso de *C. pampeanus* está associado à fenologia de sua planta hospedeira, adaptação à sazonalidade, incluindo possivelmente diapausa larval no inverno e, à fauna associada às galhas, que ainda é desconhecida em nível de espécie. Com a antropização dos Campos, as populações com presença dessas galhas estão cada vez mais restritas no espaço, e assim correndo o risco de extinção, não somente *C. pampeanus* em si, mas toda comunidade à ela associada. Apenas duas áreas localizadas na região de Porto Alegre, que abrangem a distribuição da espécie são protegidas oficialmente. Considerando que no Brasil ainda não há estudos de biologia da conservação relacionados à microlepidoptera, torna este, o primeiro estudo a sugerir medidas de proteção para um microlepidóptero e fauna associada no país.

Um gênero novo e duas espécies novas de Cecidosidae foram descritas para a região central do Chile. Uma delas, *Andescecidium parrai*, foi por muito tempo também negligenciada. Nesse caso, uma espécie de coleóptero era tida até então como a indutora das galhas correspondentes. Este coleóptero. supostamente um cleptoparasita, usurpa a galha induzida por *A. parroi*, o que levou a tal confusão taxonômica. Além disso, os estágios imaturos de *A. parrai* ainda não haviam sido explorados, sendo então aqui descritos. O presente trabalho fortalece o valor que as descrições dos estágios imaturos apresentam para a taxonomia deste grupo. A outra espécie (*Oliera saizi*) descrita em conjunto também é indutora de galhas em *S. polygamus*. Os adultos, estágios imaturos, história de vida e distribuição geográfica foram investigados para ambas as espécies. À semelhança do estudo anteriormente citado, uma análise filogenética com base em sequências de DNA foi realizada incluindo estas duas novas espécies. De acordo com os respectivos resultados, *Andescecidium* consta da linhagem mais recente de Cecidosidade, proximamente relacionada com *Cecidonius*.

Os dois últimos estudos de cunho taxonômico apontam também para a existência de uma maior diversidade específica em relação a esses três gêneros de cecidosídeos (*Cecidonius*, *Andescecidium* e *Oliera*) na América do Sul, o que deve ser melhor investigado.

Apêndice 1

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The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
- Abbreviations, if possible

Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

Protocol documents for clinical trials, observational studies, and other **non-laboratory** investigations may be uploaded as supporting information. Read the supporting information guidelines for formatting instructions. We recommend depositing **laboratory protocols** at protocols.io. Read detailed instructions for depositing and sharing your laboratory protocols.

Human or animal subjects and/or tissue or field sampling

Methods sections describing research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the reporting guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

Data

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

Large data sets, including raw data, may be deposited in an appropriate public repository. See our list of recommended repositories.

For smaller data sets and certain data types, authors may provide their data within supporting information files accompanying the manuscript. Authors should take care to maximize the accessibility and reusability of the data by selecting a file format from which data can be efficiently extracted (for example, spreadsheets or flat files should be provided rather than PDFs when providing tabulated data).

For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to "data not shown."

Cell lines

Methods sections describing research using cell lines must state the origin of the cell lines used. See the reporting guidelines for cell line research for more information.

Laboratory Protocols

To enhance the reproducibility of your results, we recommend and encourage you to deposit laboratory protocols in protocols.io, where protocols can be assigned their own persistent digital object identifiers (DOIs).

To include a link to a protocol in your article:

1. Describe your step-by-step protocol on protocols.io
2. Select **Get DOI** to issue your protocol a persistent digital object identifier (DOI)
3. Include the DOI link in the Methods section of your manuscript using the following format provided by protocols.io: http://dx.doi.org/10.17504/protocols.io.[PROTOCOL DOI]

At this stage, your protocol is only visible to those with the link. This allows editors and reviewers to consult your protocol when evaluating the manuscript. You can make your protocols public at any time by selecting **Publish** on the protocols.io site. Any referenced protocol(s) will automatically be made public when your article is published.

New taxon names

Methods sections of manuscripts adding new taxon names to the literature must follow the reporting guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Criteria for Publication for more information.

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, providing the manuscript has a citable DOI or arXiv URL.

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., "unpublished work," "data not shown"). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., "We used the techniques developed by our colleagues [19] to analyze the data"). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts or author summaries.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

Formatting references

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the "Vancouver" style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source	Format
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence

Source	Format
	cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005. *Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers. When providing a DOI, adhere to the format in the example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.*
Accepted, unpublished articles	Same as published articles, but substitute "Forthcoming" for page numbers or DOI.
Online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available from: arXiv:1403.3301v1. Cited 17 March 2014.
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html Cited 17 March 2014.
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

Supporting Information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 10 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an "S" and number. For example, "S1 Appendix" and "S2 Appendix," "S1 Table" and "S2 Table," and so forth.

Supporting information files are published exactly as provided, and are not copyedited.

Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

Figures and Tables

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order upon first appearance in the manuscript file.

Read the guidelines for figures.

Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- A figure label with Arabic numerals, and "Figure" abbreviated to "Fig" (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of "Fig 1" must refer to a figure file named "Fig1.tif").
- A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

Tables

Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., "Table 1") and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Read the guidelines for tables.

Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

Read our policy on data availability.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

See our list of recommended repositories.

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Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers of DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at submission.

Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

Striking image

You can choose to upload a “Striking Image” that we may use to represent your article online in places like the journal homepage or in search results.

The striking image must be derived from a figure or supporting information file from the submission, i.e., a cropped portion of an image or the entire image. Striking images

should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows.

If no striking image is uploaded, we will designate a figure from the submission as the striking image.

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Additional Information Requested at Submission

Funding Statement

This information should not be in your manuscript file; you will provide it via our submission system.

This information will be published with the final manuscript, if accepted, so please make sure that this is accurate and as detailed as possible. You should not include this information in your manuscript file, but it is important to gather it prior to submission, because your financial disclosure statement cannot be changed after initial submission**.**

Your statement should include relevant grant numbers and the URL of any funder's web site. Please also state whether any individuals employed or contracted by the funders (other than the named authors) played any role in: study design, data collection and analysis, decision to publish, or preparation of the manuscript. If so, please name the individual and describe their role.

Read our policy on disclosure of funding sources.

Competing Interests

This information should not be in your manuscript file; you will provide it via our submission system.

All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full.

Read our policy on competing interests.

Manuscripts disputing published work

For manuscripts disputing previously published work, it is *PLOS ONE* policy to invite a signed review by the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process.

If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.

Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

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Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to *PLOS ONE* or elsewhere, authors must include a copy with the submitted article. Reviewers will be asked to comment on the overlap between related submissions.

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Read our policies on related manuscripts.

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PLOS encourages authors to post preprints as a way to accelerate the dissemination of research and supports authors who wish to share their work early and receive feedback before formal peer review. Deposition of manuscripts with preprint servers does not impact consideration of the manuscript at any PLOS journal.

Authors posting on bioRxiv may concurrently submit directly to select PLOS journals through bioRxiv's direct transfer to journal service.

Authors submitting manuscripts in the life sciences to *PLOS ONE* may opt-in to post their work on bioRxiv during the *PLOS ONE* initial submission process.

Apêndice 2

Normas para publicação na revista Zookeys:

Authors Guidelines

Main Text

Title: The title should be in a sentence case (only scientific, geographic or person names should be with a first capital letter, i.e. *Elater ferrugineus* L., Germany, etc.), and should include an accurate, clear and concise description of the reported work, avoiding abbreviations. The higher taxa within the title should be separated with commas and not with a semicolon, e.g.: (Coleoptera, Elateridae, Elaterini).

Authors and Affiliations: Provide the complete names of all authors, and their addresses for correspondence, including e.g., institutional affiliation (e.g. university, institute), location (street, boulevard), city, state/province (if applicable), and country. One of the authors should be designated as the corresponding author. It is the corresponding author's responsibility to ensure that the author list, and the individual contributions to the study are accurate and complete. If the article has been submitted on behalf of a consortium, all consortium members and their affiliations should be listed after the **Acknowledgements section**.

Abstract and Keywords: Please have your abstract and keywords ready for input into the submission module. Keywords should be in alphabetical order and ideally differ from the words used in the title.

Body Text: All papers should be in grammatically correct English. Non-native English speaking authors are required to have their manuscripts checked by a native English speaker prior to submission. Use either British/Commonwealth or American English provided that the language is consistent within the paper. A manuscript must be written with precision, clarity, and economy. The voice - active or passive - and the tense used should be consistent throughout the manuscript. Avoid the use of parenthetical comments and italics or bold for emphasis. This journal discourages the use of quotation marks except for direct quotations, words defined by the author, and words used in unusual contexts. Short quotations should be embedded in the text and enclosed in double quotation marks ("). Long quotations should be on a separate line, italicized, but without quotation marks. Single quotation marks are to be used only for a quotation that occurs within another quotation.

Spacing, Fonts, and Page Numbering: Single-space all material (text, quotations, figure legends, tables, references, etc.). Separate paragraphs with a blank line. Use a 12-point font (preferably Times New Roman or Arial).

Capitals: First capital letters should be used only in the beginning of a sentence, in proper names and in headings and subheadings, as well as to indicate tables, graphs and

figure/s within the text. Software programmes should be written with capital letters (e.g., ANOVA, MANOVA, PAUP).

Italicization/Underlining: Scientific names of species and genera, long direct quotations and symbols for variables and constants (except for Greek letters), such as p, F, U, T, N, r, but not for SD (standard deviation), SE (standard error), DF (degrees of freedom) and NS (non significant) should be italicized. These symbols in illustrations and equations should be in italics to match the text. Italics should not be used for emphasis, and not in abbreviations such as e.g., i.e., et al., etc., cf. Underlining of any text is not acceptable.

Abbreviations: Abbreviations should be followed by '.' (full stop or period; for instance: i.e., e.g., cf., etc.). Note that you shouldn't add a full stop at the end of abbreviated words if the last letter of the abbreviation is the same as the last letter of the full word. For example, you should abbreviate "Eds", "Dr", "Mr" without full stop at the end. All measures, for instance mm, cm, m, s, L, should be written without full stop.

On the use of dashes: (1) Hyphens are used to link words such as personal names, some prefixes and compound adjectives (the last of which vary depending on the style manual in use) **(2)** En-dash or en-rule (the length of an 'n') is used to link spans. In the context of our journal en-dash should be used to link numerals, sizes, dates and page numbers (e.g., 1977–1981; figs 5–7; pp. 237–258); geographic or name associations (Murray–Darling River; a Federal–State agreement); and character states combinations such as long–pubescent or red–purple. **(3)** Em-dash or em-rule (the length of an 'm') should be used rarely, only for introducing a subordinate clause in the text that is often used much as we use parentheses. In contrast to parentheses an em-dash can be used alone. En-dashes and em-dashes should not be spaced.

Footnotes: Avoid footnotes in the body text of the manuscript. It is always possible to incorporate the footnote into the main text by rewording the sentences, which greatly facilitates reading. Additionally, footnotes are not always handled well by the journal software, and their usage may cause a failure of submission. Footnotes are acceptable only below tables; instead of numbers, please use (in order): †, ‡, §, |, ¶, #, ††, ‡‡, §§, ||, ¶, ##.

Geographical coordinates: It is strongly recommended to list geographical coordinates as taken from GPS or online gazetteer, or georeferencer. Geographical coordinates must be listed in one of the following formats:
Definition: The locality consists of a point represented by coordinate information in the form of latitude and longitude. Information may be in the form of

- Degrees, Minutes and Seconds (DMS),
- Degrees and Decimal Minutes (DDM), or
- Decimal Degrees (DD).

Records should also contain a hemisphere (E or W and N or S) or, with Decimal Degrees, minus (–) signs to indicate western and/or southern hemispheres.
Examples:

- Example 1: 36° 31' 21" N; 114° 09' 50" W (DMS)
- Example 2: 36° 31.46’N; 114° 09.84’W (DDM)
- Example 3: 36.5243° S; 114.1641° W (DD)
- Example 4: −36.5243; −114.1641 (DD using minus signs to indicate southern and western hemispheres)

Note on accuracy: Because GPS units are very commonly used today to record latitude/longitude, many authors simply give the GPS readings for their localities. However, these readings are much too accurate. For example, a GPS unit might give the latitude in decimal seconds as 28°16'55.87"N. Since one second of latitude is about 30 m on the ground, the second figure after the decimal in 55.87 represents 30 cm, yet a typical handheld GPS unit is only accurate at best to a few metres.

We therefore recommend two ways to report GPS-based locations. If you give the GPS reading without rounding off, make sure you include an uncertainty figure as a context for the over-accurate GPS reading. We recommend the Darwin Core definition of uncertainty (http://rs.tdwg.org/dwc/terms/index.htm#coordinateUncertaintyInMeters):

"The horizontal distance (in meters) from the given decimalLatitude and decimalLongitude describing the smallest circle containing the whole of the Location."

If you only give the GPS reading, please round it off to an implied precision appropriate to the error in the measurement, or to the extent of the area sampled. We suggest rounding off

- to the nearest second in degree-minute-second format (28°16'56"N), which implies roughly ± 25-30 m at middle latitudes
- to four decimal places in decimal degree format (28.2822°N), which implies roughly ± 10-15 m at middle latitudes
- to two decimal places in decimal minute format (28°16.93'N), which implies roughly 15-20 m at middle latitudes

Altitude: Many GPS users simply record the elevation given by their GPS unit. However, GPS elevation is NOT the same as elevation above sea level. GPS units record the elevation above a mathematical model of the earth's surface. The difference between this elevation and elevation above sea level can be tens of metres. In any case, the accuracy of a GPS elevation is often the same as the usual accuracy in horizontal position, so a GPS elevation such as '753 m' is much too accurate and should be rounded off to 'ca 750 m'.

We **strongly recommend** the use of Example 2 (the DDM format). The other three are also possible but will be recalculated to DDM during the process of online mapping from the HTML version of the paper.

The only restriction on format is in creating a KML (Keyhole Markup Language) file. KML latitudes and longitudes must be in the DD format shown above in Example 4.

Please also consider submitting a **table of localities** with your manuscript, either as a spreadsheet or in CSV text format. By doing so you will make your specimen localities much more easily available for use in biodiversity databases and geospatial investigations. The geospatial table will be put online as supplementary material for your paper. A minimum table will have three fields: species (or subspecies) name, latitude and longitude. A full table will have the same data for each specimen lot as appears in the text of your paper. Please check latitude/longitude carefully for each entry.

Units: Use the International System of Units (SI) for measurements. *Consult Standard Practice for Use of the International System of Units*(ASTM Standard E−380−93) for guidance on unit conversions, style, and usage.

Statistics: Use leading zeroes with all numbers, including probability values (e.g., $P < 0.001$). For every significant F−statistic reported, provide two df values (numerator and denominator). Whenever possible, indicate the year and version of the statistical software used.

Web (HTML) links: Authors are encouraged to include links to other Internet resources in their article. This is especially encouraged in the reference section. When inserting a reference to a web-page, please include the **http://** portion of the web address.

Supplementary files: Larger datasets can be uploaded separately as Supplementary Files. Tabular data provided as supplementary files can be uploaded as an Excel spreadsheet (.xls), as an OpenOffice spreadsheets (.ods) or comma separated values file (.csv). As with all uploaded files, please use the standard file extensions.

Headings and subheadings: Main headings: The body text should be subdivided into different sections with appropriate headings. Where possible, the following standard headings should be used:

Introduction, **Methods**, **Results**, **Discussion**, **Conclusions**, **Acknowledgements**, **References**. These headings need to be in bold font on a separate line and start with a first capital letter. Please do not number headings or subheadings.

- **Introduction** − The motivation or purpose of your research should appear in the Introduction, where you state the questions you sought to answer, and then provide some of the historical basis for those questions.
- **Methods** − Provide sufficient information to allow someone to repeat your work. A clear description of your experimental design, sampling procedures, and statistical procedures is especially important in papers describing field studies, simulations, or experiments. If you list a product (e.g., animal food, analytical device), supply the name and location of the manufacturer. Give the model number for equipment used. Supply complete citations, including author (or editor), title, year, publisher, and version number, for computer software mentioned in your article.
- **Results** − Results should be stated concisely and without interpretation.
- **Discussion** − Focus on the rigorously supported aspects of your study. Carefully differentiate the results of your study from data obtained from other sources. Interpret your results, relate them to the results of previous research, and discuss the implications of your results or interpretations. Point out results that do not support speculations or the findings of previous research, or that are counter-intuitive. You may choose to include a Speculation subsection in which you pursue new ideas suggested by your research, compare and contrast your research with findings from other systems or other disciplines, pose new

questions that are suggested by the results of your study, and suggest ways of answering these new questions.

- **Conclusion** −This should state clearly the main conclusions of the research and give a clear explanation of their importance and relevance. Summary illustrations may be included.
- **References** − The list of References should be included after the final section of the main article body. A blank line should be inserted between single-spaced entries in the list. Authors are requested to include links to online sources of articles, whenever possible!

Where possible, the standard headings should be used in the order given above. Additional headings and modifications are permissible.

Subordinate headings: Subordinate headings (e.g. *Field study and Simulation model or Counts, Measurements and Molecular analysis*), should be left-justified, italicized, and in a regular sentence case. All subordinate headings should be on a separate line.

English Language Editing

This journal has well-defined policies for English language editing. Involving mandatory outsourced language editing services would considerably increase the price of the Article Processing Charges, which would become an additional obstacle for persons and institutions to publish in the journal. Therefore we rely both on the conscience of our authors to provide stylistically written texts and our editors and reviewers to filter out badly written manuscripts.

Authors are required to have their manuscripts edited by a native English speaker **BEFORE** submission. Authors have to confirm by checking a tick box in the submission process that they have followed the above requirement:

The text is checked by a native English speaker, duly acknowledged in the manuscript. I am aware that non-edited manuscripts could be rejected prior to peer-review.

The submission process includes an option to request a professional linguistic and copy editing at a price of **EURO 15 per 1800 characters**:

The text has not been checked by a native speaker and I request thorough editing prior to peer review at a price. I agree to cover the costs even if my manuscript is not accepted for publication.

The authors are **NOT** obliged to use our linguistic services, but they must ensure that their manuscripts have been checked by a native speaker.

Citations and References

Citations within the text: Before submitting the manuscript, please check each citation in the text against the References and vice-versa to ensure that they match exactly. Citations in the text should be formatted as follows: Smith (1990) or (Smith 1990), Smith et al. (1998) or (Smith et al. 1998) and (Smith et al. 1998, 2000, Brock and Gunderson 2001, Felt 2006).

References: It is important to format the references properly, because all references will be linked electronically as completely as possible to the papers cited. It is desirable to add a DOI (digital object identifier) number for either the full-text or title and abstract of the article as an addition to traditional volume and page numbers. If a DOI is lacking, it is recommended to add a link to any online source of an article. Please use the following style for the reference list (or download the *Pensoft EndNote style*): here

Published Papers:
Polaszek A, Alonso-Zarazaga M, Bouchet P, Brothers DJ, Evenhuis NL, Krell FT, Lyal CHC, Minelli A, Pyle RL, Robinson N, Thompson FC, van Tol J (2005) ZooBank: the open-access register for zoological taxonomy: Technical Discussion Paper. Bulletin of Zoological Nomenclature 62: 210-220.

Accepted Papers:
Same as above, but "in press" appears instead the year in parentheses.

Electronic Journal Articles:
Mallet J, Willmott K (2002) Taxonomy: renaissance or Tower of Babel? Trends in Ecology and Evolution 18 (2): 57-59. doi: 10.1016/S0169-5347(02)00061-7.

Paper within conference proceedings:
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Link/URL:
BBC News: Island leopard deemed new species http://news.bbc.co.uk/
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General guidelines

By publishing in this journal you are already creating a modern taxonomic product that is more accessible than previous print only works. The following guidelines are provided to ensure that other elements of the work follow modern standards and enable the full advantage of the ARPHA platform.

- Include unique specimen identifiers for type material. Unique identifiers are for example museum collections specimen IDs. Unique identifiers can be provided also by international taxon-based databases that do not indicate ownership, such as AntWeb.org for ants, for example.
- Holotype should not deposited in private collections.
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- Include latitude, longitude, elevation, habitat, microhabitat information of primary type material. For format of geographical coordinates see section "Main text" above.
- Provide dichotomous key of taxa or related taxa (i.e. species group) or links to online-based keys.
- Single species descriptions should be clearly justified with regard as to why a more detailed larger scale, comparative revision was not conducted. For descriptions of single species see also section "Focus and Scope".

Sequence data

Manuscripts containing novel amino acid sequences (e.g. primer sequences) will only be accepted if they carry an International Nucleotide Sequence Databases (INSD) accession number from the European Biology Laboratory (EMBL), GenBank Data Libraries (GenBank) or DNA Data Bank of Japan (DDBJ). We strongly recommend that authors include institutional catalog numbers for specimens preserved in collections, and information identifying sequences that are derived from type specimens (see below) when they deposit data in genetic databanks. A summary table with the INSD accession [catalog] numbers should be included in either Materials and Methods or Data Resources section of the paper. If specimens were not vouchered (tissued specimens should be vouchered whenever possible!), collection locality data and possibly photographs of tissued specimens must be provided. A nomenclature for genetic sequences for types and confidently identified nontype specimens has been proposed by Chakrabarty et al. (2013); a sequence from a holotype is identified as genseq-1, one from a paratype is identified as genseq-2, one from a topotype is genseq-3, etc. The genetic marker(s) used should also be incorporated into the nomenclature (e.g. genseq-2 COI).

Examples

Table 1. Ranking Sequence Reliability. Ranking of source materials of genetic sequences based on reliability of taxonomic identification. Examples of the source material are listed in the third column with the last column providing the corresponding GenSeq nomenclature (after Chakrabarty et al. (2013)).

Reliability Ranking	**Source Materials**	**Examples**	**Corresponding GenSeq Nomenclature**
Highest 1st	Primary Types	Holotype, Lectotype, Syntype, Isosyntype, Neotype, Isotype	genseq-1
2nd	Secondary Types	Paratype, Paralectotypes, etc.	genseq-2
3rd	Topotypes (vouchered), or non-type specimens listed in original description or redescription	Topotype, Non-type specimen listed in original description or redescription	genseq-3
4th	Collections-vouchered non-types (not from original description or redescription)	Vouchered specimen	genseq-4
5th	Photo voucher only	No specimen voucher but photo voucher available	genseq-5
Lowest	No voucher	Non-vouchered	No classification

Table 2. Example Reporting Table. Examples of how links between genetic sequences and vouchers in institutional collections could be displayed as a table in publications reporting new sequences.

Species	**Specimen Catalog #**	**GenBank #**		**GenSeq Nomenclature**
		COI	**ND1**	
Typhleotris mararybe	LSUMZ 13636 (holotype)	HM590594	HM590606	genseq-1 COI, ND1
Paretroplus tsimoly	AMNH 229558 (paratype)	JZ590596	NA	genseq-2 COI

Nandopsis haitiensis	UMMZ 236321 (topotype)	BK590595	BK590607	genseq-3 COI, ND1
Halieutichthys intermedius	FMNH 96353 (non-type specimen voucher)	AY722169	AY722306	genseq-4 COI, ND1
Equulites absconditus	NMNH 12345PV2 (photo voucher)	NA	BG34621	genseq-5 ND1

International Code of Zoological Nomenclature

This journal will publish papers that strictly adhere to the rules of the last edition of the International Code of Zoological Nomenclatureand its amendment. Authors are also advised to follow all recommendations of the Code and to consult the guidelines below, as well as ICZN's manual Best practice in the use of the scientific names of animals prior to submitting the manuscript.

General: Each **first mentioning** of an animal species name within the text must be provided with author(s)' name(s). **Year of publication** of an animal species should be given in taxonomic revisions with quotation of the work providing the original species' description in the list of references.

New names: When new taxonomic acts are proposed, they should be explicitly indicated as being new by adding the respective abbreviation after the taxon name i.e., sp. n., comb. n., nomen n. Authors of newly described taxa should be given any time the taxon is mentioned, if different from the publication authors.

Examples:

- Genus *X-us* Smith, new genus (author(s) of the publication and authority (-ies) of the taxon is/are identical);
- *X-us albus* Jones & Peters, new species (the publication is authored by persons different in composition or combination from the authority (-ies) of the taxon itself, e.g. Smith, Jones & Peters or Peters & Jones).

We highly recommend that authors of new species are also included as co-authors of the work where the taxa are described. If the authors of the work do not want to include the authors of the taxonomic name then to be absolutely certain that the authority for the name is unequivocal there should be a statement in the work saying that these authors (of the name) are responsible for making the name available under the code (Article 50.1.2, etc.) i.e. they are responsible for coining the name and for satisfying all other criteria for availability.

New family-group names: Although all family group names are derived/based on their type genus, the type genus is to be compulsorily designated in any description of a family-group name published after 31st December 1999 (Article 16.2). It is not sufficient that the type genus is mentioned as belonging to the new family-group name; it must be stated that this is the type genus. We recommend a single type line as: Type-genus: *Musca* Linnaeus, 1758.

New genus-group names: The origin ("etymology", or "derivatio nominum") of name and its gender should be indicated. The type-species and the character of the proposed taxonomic act should be specified for new genus-group names. The type species name should be given in its original combination with an author and year. If the type species is now considered a junior synonym there need to be a clear mention of that. The fixation type should derive from the International Code of Zoological Nomenclature (see Articles 68 & 69; original designation, monotypy, absolute tautonymy, Linnaean tautonymy, subsequent monotypy, subsequent designation).
Example:

- *Sympycnus* Loew

Type-species: *Porphyrops annulipes* Meigen, 1824 by subsequent designation of Coquillett (1910: 610) =*pulicarius* Fallen, 1823.

New species-group names: According to the ICZN Art. 11.9, but also Art. 11.3 the origin "etymology", or "derivatio nominum") new species-group names should be supplemented by information on whether the epithet is an 1) adjective or participle in the nominative singular; 2) noun in the nominative singular; 3) a noun in the genitive case; 4) an adjective used a substative in the genitive case; or 5) an arbitrary combination of letters (ICZN Art. 11.3). For **species-group names**, there are two separate statements of type information that are needed:

- the **statement of species' type locality** – that is the exact place whence the primary type origins, including exact collecting dataplace with geographical coordinates, geographical or political unit (Area/ District/ State) and country;also, if possible, supplementary locality information should be included – habitat type, method of collecting, date, collector's names, host name (for parasites), etc.
- there should be a separate statement about the **type specimen**, exact quotation of its original label, condition of specimen (dry pinned, in alcohol, slide, fossil, etc.) and repository (organization's name and city).

Examples:
For a **new species**:

- **Type-locality:** USA, Viriginia: Fairfax County, Kingstowne, 38°46'N, 77°07'W, broad-leaf forest, under bark, 10 July 2000, J. Smith leg.
- **Type-specimen:** Holotype male, pinned, with genitalia in a separate microvial. Original label: "USA, VA, Fairfax, Kingstowne, 38°46'N, 77°07'W, 12 Oct 2003, BJ & FC Thompson" "USNM ENT 00033805" [Code 49 barcode], "HOLOTYPE / Xylota / x-us / Thompson [red handwritten label].

For a **previously described species**:

Lectotype male, pinned … [details] here designated to fix the concept of *X-us albus* Jones and to ensure the universal and consistent interpretation of the same. Or … [details then] by designation of Smith (1976: 999).

Previously published names: For a **previously published name**, please provide the year of description. Also use the parentheses convention for subsequent new combinations.

[Etymology]

Authors of new species name should state exactly what the epithet is in terms of the ICZN, as outlined in Article 11.9.1.1 to 11.9.1.4 as well as 11.3. A name may be a word in or derived from Latin, Greek or any other language (even one with no alphabet), or be formed from such a word. In short, a name can be declared as arbitrary combination (the best solution) or must be or be treated as:

I) a word of two or more letters, or a compound word, and, if a Latin or latinized word must be, or be treated as:

1. an adjective or participle in the nominative singular (as in *Echinus esculentus*, *Felis marmorata*, *Seioptera vibrans*), or
2. a noun in the nominative singular standing in apposition to the generic name (as in *Struthio camelus*, *Cercopithecus diana*), or
3. a noun in the genitive case (e.g. *rosae*, *sturionis*, *thermopylarum*, *galliae*, *sanctipauli*, *sanctaehelenae*, *cuvieri*, *merianae*, *smithorum*), or
4. an adjective used as a substantive in the genitive case and derived from the specific name of an organism with which the animal in question is associated (as in *Lernaeocera lusci*, a copepod parasitic on *Trisopterus luscus*).

II) An adjectival species-group name proposed in Latin text but written otherwise than in the nominative singular because of the requirements of Latin grammar is available provided that it meets the other requirements of availability, but it is to be corrected to the nominative singular if necessary.

Arranging sections within species treatments (sections in square brackets are requested for new descriptions only!):

[Name]

[Material]

- [Type material]
- Other material

[Diagnosis]

[Description]

[Etymology]

Distribution

Ecology (including phenology)

Conservation status (optional, we encourage authors to follow the IUCN categories and criteria, please see http://www.iucnredlist.org/static/categories_criteria_3_1#critical))

Discussion (optional, but very desirable)

Materials and Methods

In line with responsible and reproducible research, as well as FAIR (Findability, Accessibility, Interoperability and Reusability) data principles, we highly recommend

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