UNIVERSIDADE DE SÃO PAULO

FACULDADE DE FILOSOFIA, CIÊNCIAS E LETRAS DE RIBEIRÃO PRETO

PROGRAMA DE PÓS-GRADUAÇÃO EM ENTOMOLOGIA

Hagenulina Kluge (1994): diversity, phylogeny, and biogeography (Ephemeroptera: Leptophlebiidae)

Hagenulina Kluge (1994): diversidade, filogenia e biogeografia (Ephemeroptera: Leptophlebiidae)

(VERSÃO CORRIGIDA)

Rogério Campos de Oliveira

Tese apresentada à Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto da Universidade de São Paulo, como parte das exigências para obtenção do título de Doutor em Ciências, obtido no Programa de Pós-Graduação em Entomologia.

Ribeirão Preto-SP

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Orientador: Prof. Dr. Pitágoras C. Bispo (UNESP)

Ribeirão Preto-SP

2022

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Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto da Universidade de São Paulo

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1. Mayflies. 2. Neotropical. 3. Bayesian inference. 4. Fossils. 5. Morphological clocks. 6. Shortfalls 7. Biodiversity

Penso, existo, evoluo
analiso, pesquiso, reconstruo
insisto, resisto, e assim continuo
pra fincar raiz no ramo em que eu atuo

(Kamau, 2008)

Dedico aos meus e a todos aqueles que um dia tiveram sonhos maiores que os meus,
para assim tornar os meus sonhos possíveis.

NOTA

Essa tese é parte dos requisitos para obtenção do título de Doutor pelo Programa de Pós graduação em Entomologia da Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo. Dessa forma, não é considerada uma publicação de acordo com os requisitos do Código Internacional de Nomenclatura Zoológica (ICZN).

DISCLAIMER

This thesis is part of requirements for obtention of the Ph.D degree in the Graduate Program in Entomology of the Faculty of Philosophy, Sciences and Languages of Ribeirão Preto, University of São Paulo. Therefore, this contribution is no considered a formal publication according to the requirements by the International Commission on Zoological Nomenclature (ICZN).

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Estamos vivos!

Campos, Rogério Oliveira. **Hagenulina Kluge (1994): diversity, phylogeny, and biogeography (Ephemeroptera: Leptophlebiidae),** 2022. Tese (Doutorado em Entomologia- Progtrama de Pós-Graduação em Entomologia da Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto – Universidade de São Paulo)

Abstract

Understanding the patterns and mechanisms that drive life on Earth is an interesting and difficult task shared by biodiversity researchers. In general, biodiversity shortfalls are associated with our incomplete knowledge about: the number of species, the distribution of these species through the globe, their phylogenetic position through the tree of life, the recognition of their life stages, their interactions with biotic and abiotic factors, and so on. In this study, we study the Hagenulina subtribe (Ephemeroptera: Leptophlebiidae) aiming to reduce the biodiversity shortfalls, especially Linnean, Henninguean/Darwinian, and Haeckelian shortfalls. Here, we integrated morphological data into a Bayesian phylogenetic framework using neontological and paleontological sources, and relaxed morphological clocks to test the monophyly, estimate the timeline tree, and propose a hypothesis of relationship between the genera within Hagenulina. Given the distribution of Hagenulina (Central America, insular and mainland, and South America), we also performed a biogeographical time-sliced approach to test if the Antillean colonization occurred at once through the time and if the hypothetical GAARlandia landspan drove a central role in Antillean colonization. As result, our analyses indicate that Hagenulina is monophyletic and had an estimated South American origin around the Cretaceous, starting its diversification around the boundaries of Cretaceous-Paleogene (KPg), and dispersed to the Antilles through several independent events. The role of a possible land bridge in the colonization of Hagenulina was not clearly evidenced, but, the temporal proximity of 5 million years between the estimated period for the GAARlandia and the age of the lineages that colonized the Greater Antilles does not allow us to fully refute this hypothesis in face of our data. In addition, new species and nymphs were described based on associated semaphoronts reducing the Linnean and Haeckelian shortfalls for the subtribe.

Keywords: Mayflies, Neotropical, systematics, morphology, jump-dispersal, shortfalls,biodiversity

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Resumo

Entender os padrões e mecanismos que moldam a vida na terra é uma interessante e difícil tarefa compartilhada pelos pesquisadores da biodiversidade. Em termos gerais, os déficits da biodiversidade estão associados com o nosso conhecimento incompleto sobre: o número de espécies, a distribuição dessas espécies ao redor do globo, seu posicionamento filogenético na árvore da vida, o reconhecimento dos seus estágios de vida, suas interações com os fatores bióticos e abióticos, e assim por diante. Neste estudo, nós nos debruçamos sobre a subtribo Hagenulina (Ephemeroptera: Leptophlebiidae) com o objetivo de reduzir os déficits da biodiversidade, sobretudo os déficits Lineano, Henigueano/Darviniano e Haeckeliano. Aqui, nós integramos os dados morfológicos a uma abordagem filogenética bayesiana, utilizando informações neontológicas e paleontológicas, e relógio morfológico relaxado para testar a monofilia, estimar a cronologia e propor uma hipótese de relacionamento entre os gêneros que pertencem a Hagenulina. Dado a distribuição da subtribo (América Central, ilha e continente, e América do Sul), nós performamos uma análise biogeográfica afim de testar se a colonização das Antilhas ocorreu apenas uma vez ao longo do tempo, além de testar se a hipotética ponte GAARlandia desempenhou um papel central nessa colonização. Como resultado, nossas análises indicaram a monofilia de Hagenulina com sua provável origem na América do Sul por volta do Cretáceo. A subtribo iniciou sua diversificação durante a passagem do Cretáceo-Paleogeno (KPg), performando múltiplos eventos de dispersão para as Antilhas em sua história evolutiva. A importância de uma possível ponte de terra durante a colonização de Hagenulina não foi claramente evidenciada, mas, a proximidade temporal de 5 milhões de anos entre o período estimado para GAARlandia e a idade das linhagens que colonizaram as Grandes Antilhas não nos permite refutar totalmente essa hipótese diante dos nossos dados. Além disso, uma nova espécie e ninfas foram descritas baseada em associação dos estágios de vida, reduzindo as deficiências de Linneana e Haeckeliana para a subtribo.

Palavras-chave: Ephemeroptera, Neotropical, sistemática, morfologia, dispersão, déficits, biodiversidade

SUMÁRIO

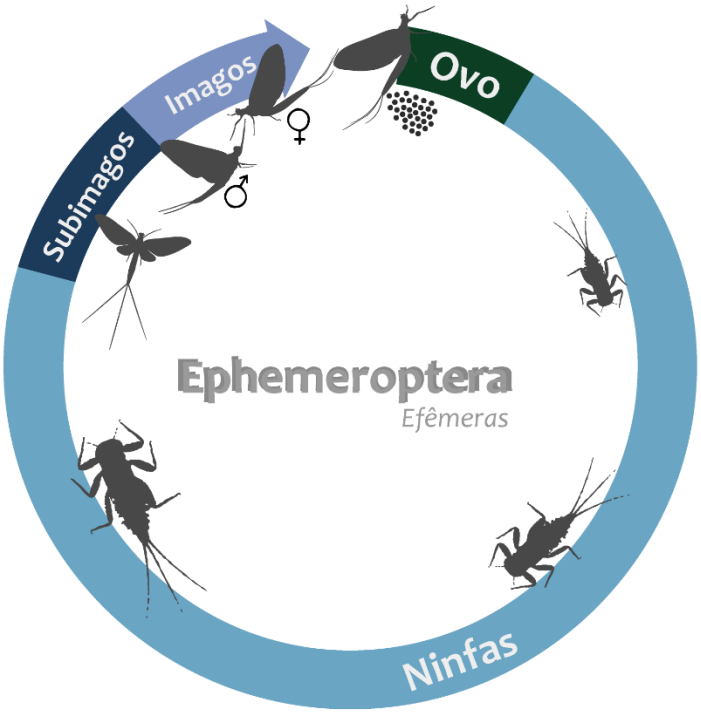
INTRODUÇÃO GERAL

EPHEMEROPTERA HYAT & ARMS, 1890

A ordem Ephemeroptera é um dos ramos de uma antiga e bem diversificada linhagem de insetos alados (Pterygota), chamada Hydropalaeoptera (Sroka et al. 2015). Estimativas apontam o surgimento dessa linhagem por volta de 360 milhões de anos (Misof et al. 2014) e, desde então, quase todos os seus representantes foram extintos, restando apenas os Ephemeroptera e os Odonata atuais. Os primeiros registros da ordem Ephemeroptera, propriamente dita, datam do Mesozoico (Jurássico–Cretáceo) (Sartori & Brittain 2015) quando indivíduos apresentando o braço costal desenvolvido na membrana alar e asas heterônimas apareceram no registro fóssil (Sroka et al. 2015).

Os efemerópteros são insetos aquáticos com desenvolvimento hemimetabólico e com ciclo de vida anfibiótico (Figura 01), cujo desenvolvimento da fase imatura (ovo e ninfa) ocorre em ambientes aquáticos dulcícolas (lóticos ou lênticos) e as fases aladas são terrestres. Durante o desenvolvimento, as ninfas dessa ordem passam por diversos instares, sendo que o processo de maturação final é marcado pelo escurecimento das tecas alares, indicando a proximidade da muda subimaginal. Após a última muda, ocorre a mudança de ambiente (aquático para terrestre), emergindo a subimago, a qual tem sido interpretada como uma condição plesiomórfica presente apenas na ordem Ephemeroptera dentre os insetos alados atuais (Sartori & Brittain 2015). Finalmente, após a muda adicional do estágio alado, temos a imago apta para reiniciar todo o ciclo.

Figura 1. Ciclo de vida da ordem Ephemeroptera.



Quando ninfa, nos ambientes aquáticos, os efemerópteros desempenham atividades cruciais para o funcionamento e manutenção da dinâmica trófica do sistema, participando principalmente como consumidores de matéria orgânica morta e algas. Nesse contexto, os efemerópteros são majoritariamente detritívoros ou herbívoros (ex. filtradores, coletores, raspadores), raramente são predadores (Domínguez, Molineri & Nieto 2009). Enquanto as fases imaturas participam ativamente da rede trófica aquática, integrando as fontes primárias e os consumidores secundários (ex. vertebrados e invertebrados), as fases aladas desempenham funções chave como fonte direta no fluxo de matéria/energia dos ambientes aquáticos para os terrestres (Jacobus et al. 2019).

Os efemerópteros estão distribuídos por todo o mundo, com exceção da Antártica e algumas ilhas oceânicas, sendo atualmente registradas cerca de 3.700 espécies classificadas em 40 famílias e 460 gêneros (Jacobus et al. 2019). Vale ressaltar que somente entre o intervalo das duas últimas listagens globais de espécies da ordem

(Sartori & Brittain 2015; Jacobus et al. 2019), cerca de 300 espécies foram descritas, reposicionando a Região Neotropical como a região com o maior número de espécies registradas até o presente momento (cerca de 900 espécies; Jacobus et al. 2019). Desta diversidade neotropical, destaca-se a família Leptophlebiidae, a qual tem uma grande diversidade funcional e compreende aproximadamente 250 espécies (Sartori & Brittain 2015).

LEPTOPHLEBIIDAE BANKS 1900

O representante mais antigo de Leptophlebiidae é de um fóssil do âmbar de Nova Jersey (*Aureophlebia sinitshenkovae* Peters & Peters, 2000) de aproximadamente 92 milhões de anos atrás, o que corresponde ao período Cretáceo Inferior (Peters & Peters 2000). A família Leptophlebiidae se posiciona na filogenia de Ephemeroptera como grupo irmão dos demais membros da subordem Furcatergalia (Ogden et al. 2009), é amplamente distribuída e possui aproximadamente 718 espécies em 147 gêneros (Jacobus et al. 2019).

A primeira classificação da família Leptophlebiidae foi proposta por Peters (1980), o qual propôs duas subfamílias: Leptophlebiinae, com origem Laurásica e distribuição no Hemisfério Norte e Ásia; e Atalophlebiinae, com origem Gondwânica e majoritariamente distribuídos no Hemisfério Sul. Desde então, a classificação interna da família tem sido modificada de forma a refletir a sua diversidade e as linhagens independentes. Atualmente, Leptophlebiidae está classificada em oito subfamílias (Monjardim et al. 2020) : Leptophlebiinae Banks, 1900, Atalophlebiinae, Peters, 1980, Habrophlebiinae Kluge, 1994a, Terpidinae Kluge, 2009, Calliarcyinae Kluge, 2009, Castanophlebiinae Kluge, 2009, Choroterpinae Kluge, 2012 and Hagenulinae Kluge, 1994.

Hagenulinae é uma linhagem Neotropical extremamente diversificada com seus representantes majoritariamente distribuídos nos escudos cristalinos Brasileiro e das Guianas (Savage 1987; Monjardim et al. 2020). Recém elevada ao status de subfamília (Monjardim et al. 2020), essa linhagem abrange duas tribos e quatro subtribos, a saber: Miroculini (Miroculina + Hermanelina) e Hagenulini (Ulmeritina + Hagenulina). Hagenulini foi inicialmente proposta por Kluge (1994; 2008) e compreendia os gêneros subordinados à *Hagenulus sensu latu* (Tabela 01) e os gêneros representantes da linhagem *Farrodes* (*Farrodes* Peters, 1971, *Homothraulus* Demoulin, 1955 and *Simothraulopsis* Demoulin, 1966).

Tabela 1. Representantes da subtribo Hagenulina Kluge 2008 (Monjardim et al. 2020) com seus respectivos números de espécies descritas. Todos os gêneros citados na tabela, com exceção de *Askola* Peters 1969, estavam subordinados ao gênero *Hagenulus* Eaton na proposta de Kluge (1994, 2008).

Gêneros	Espécies	Distribuição
Askola Peters, 1969	12 spp.	América do Sul
Borinquena Traver 1938	6 spp.	Antilhas
Ecuaphlebia Domínguez 1988	1sp.	América do Sul
Careospina Peters 1971	5 spp.	Antilhas
Hagenulopsis Ulmer 1920	10 spp.	América do Sul e Antilhas
Hagenulus *Eaton 1882*	6 spp.	América do Sul e Antilhas
Hagenulites † Staniczek 2003	1 sp.	Antilhas
Neohagenulus Traver 1938	4 spp.	Antilhas
Poecilophlebia Kluge, 1994	1 sp.	Antilhas
Traverina Peters 1921	2 sp.	Antilhas
Turquinophlebia Kluge, 1994	1 sp.	Antilhas

Hagenulina possui seus primeiros registros do Mioceno com a descrição de quatro espécies fósseis, sendo três referentes à *Borinquena* Traver, 1938 e uma referente a *Hagenulites* Staniczek, 2003 (Staniczek 2003; Staniczek, Godunko & Krzeminski 2017). Essa subtribo apresenta 49 espécies descritas, as quais estão atribuídas aos gêneros restritos às Antilhas (*Borinquena*, *Careospina*, *Neohagenulus*, *Poecilophlebia*, *Traverina* e *Turquinophlebia*), aos gêneros restritos a América do Sul (*Askola* e

Ecuaphlebia Domínguez, 1988) e aos gêneros que apresentam registro em ambas as áreas (*Hagenulus* e *Hagenulopsis*).

Embora outras espécies de Leptophlebiidae Neotropicais também apresentem registros para as Antilhas (ex. *Farrodes caribbianus* Peters, 1971), linhagens inteiras ocorrem somente em Hagenulina. Como Hagenulina possui representantes na América do Sul e nas Antilhas, ela se torna um bom modelo para testar hipóteses biogeográficas relacionadas à colonização das Antilhas. Além disso, dado a diversidade registrada para a tribo, nota-se a presença dos diversos déficits de biodiversidade listados por Hortal et al. (2015) e Faria et al. (2020), entre os quais destacamos o Linneano (conhecimento limitado das espécies existentes), Wallaceano (conhecimento limitado da distribuição geográfica), Henninguiano/Darwiniano (conhecimento limitado das relações filogenéticas) e Haeckeliano (conhecimento limitado dos semaforontes das espécies).

Diante desse contexto, nós estudamos a subtribo Hagenulina com o objetivo de propor uma filogenia (reduzindo o déficit Henninguiano/Darwiniano) e discutir a colonização do grupo nas Antilhas. Adicionalmente, nós descrevemos uma espécie (reduzindo o déficit Linneano) e as ninfas de duas espécies (reduzindo o déficit Haeckeliano).

Assim, nosso estudo tem como objetivos:

- Estimar as relações filogenéticas da subtribo Hagenulina (Hagenulinae: Leptophlebiidae: Ephemeroptera) e associar a temporalidade das divergências aos eventos geomorfológicos afim de entender o processo de colonização das Antilhas.
- Descrever uma nova espécie e a ninfa de duas espécies de Hagenulina.

A presente tese está organizada em três capítulos que estão estruturados conforme os periódicos que serão submetidos:

CAPÍTULO I. ESTIMATING PHYLOGENY AND TEMPO OF THE ANTILLEAN COLONIZATION OF NEOTROPICAL HAGENULINA (EPHEMEROPTERA: LEPTOPHLEBIIDAE): DID THE GAARLANDIA LANDSPAN DRIVE A CENTRAL ROLE? A ser submetido ao periódico **ZOOLOGICAL JOURNAL OF LINNEAN SOCIETY**.

CAPÍTULO II. *HAGENULOPSIS* ULMER (EPHEMEROPTERA: LEPTOPHLEBIIDAE): RE-DESCRIPTION, MORPHOLOGICAL NOTES AND NEW SPECIES FROM SOUTH AMERICA, BRAZIL. Submetido ao periódico **ZOOTAXA**.

CAPÍTULO III. REDUCING THE HAECKELIAN DEFICIT IN *ASKOLA* (EPHEMEROPTERA: LEPTOPHLEBIIDAE): DESCRIPTION OF A. MUCUGE CAMPOS, MARIANO & CALOR, 2019 NYMPH ASSOCIATED USING MOLECULAR TOOLS. A ser submetido ao periódico **ZOOLOGICAL STUDIES**.

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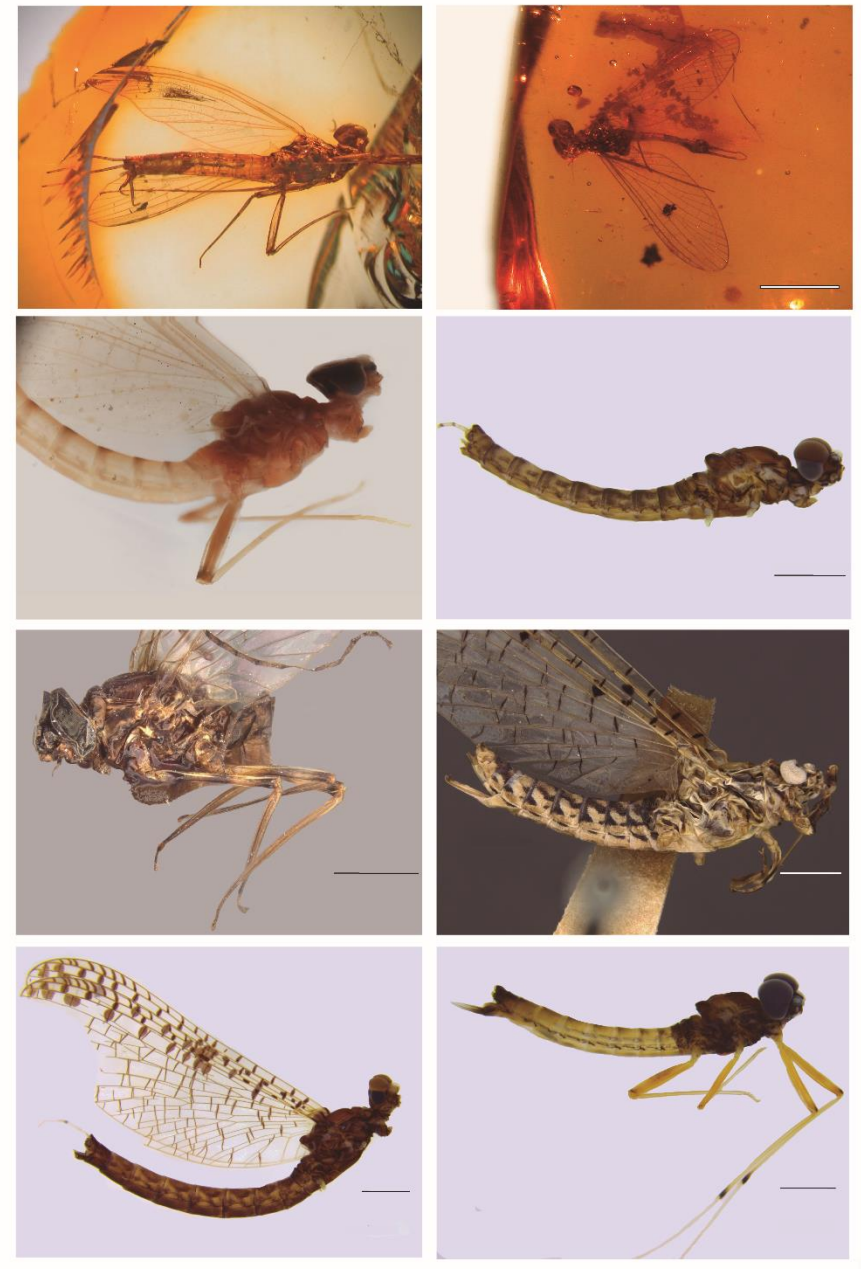
CHAPTER 1

TITLE:

ESTIMATING PHYLOGENY AND TEMPO OF THE ANTILLEAN COLONIZATION OF NEOTROPICAL HAGENULINA (EPHEMEROPTERA: LEPTOPHLEBIIDAE): DID THE GAARLANDIA LANDSPAN DRIVE A CENTRAL ROLE?



Estimating phylogeny and tempo of the Antillean colonization of Neotropical Hagenulina (Ephemeroptera: Leptophlebiidae): did the GAARlandia landspan drive a central role?

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Abstract

Hagenulina is a diversified group encompassing 49 known species arranged in 11 genera, of which seven are Antillean restricted, two are South American restricted, and the remainder are recorded in both areas. Here, we integrated data from phylogeny, morphological clocks, living and fossil species, and biogeography to test the Hagenulina monophyly, and the relationship among the genera. In addition, we test if the Antillean colonization occurred at once through the time and if the GAARlandia landspan drove a central role in Antillean colonization. Our analyses indicate that Hagenulina is monophyletic and has a South American origin around the Cretaceous, starting its diversification around the Cretaceous-Paleogene transition when *Ecuaphlebia* diverged from the other Hagenulina. Considering the biogeography, our results indicate that Hagenulina dispersed from South America to the Antilles through several independent events. The role of a possible land bridge in this colonization process was not clearly evidenced, but, the temporal proximity of 5 million years between the estimated period for the GAARlandia and the age of the lineages that colonized the Greater Antilles does not allow us to fully refute this hypothesis.

Keywords: phylogeny, mayflies, West Indies, colonization, morphological clocks, Antilles, dispersal.

Introduction

Leptophlebiidae Banks, 1900 is a worldwide family comprising around 718 species and 147 genera, being the second species-rich family within Ephemeroptera and the first in number of genera (Sartori & Brittain, 2015; Jacobus *et al*., 2019). The family has high morphological and functional diversities, occupies different habitats, and encompasses different feeding behaviors (e.g. filter, gatherers, and detritivores) (Domínguez et al., 2006). This great diversity has attracted the attention of researchers, who have proposed phylogenies and adjustments in the systematic classification, improving our understanding of the family (e.g. Kluge, 1994a; 2009; Domínguez, 2009; Monjardim *et al*., 2020).

Peters (1980) made the first internal leptophlebiid classification, circumscribing the subfamily Leptophlebiinae (Asia and North Hemisphere distributed), proposing the subfamily Atalophlebiinae (South Hemisphere distributed), and suggesting that internal classification in Atalophlebiinae should be arranged into tribes. Following Peters' (1980) suggestion, Savage (1987) assembled the Neotropical Atalophlebiinae in two faunal components, one of cold-adapted (Patagonian shield, South Andean distributed) and the other of warm-adapted (Guiana and Brazilian shields) groups. Subsequently, the circumscription of Atalophlebiinae Peters, 1980 became increasingly restricted with the proposal of four new subfamilies: Castanophlebiinae Kluge, 2009, Choroterpinae Kluge, 2012, Hagenulinae Kluge, 1994 and, Terpidinae Kluge, 2009. As a result, Leptophlebiidae is currently classified in eight subfamilies: the five aforementioned, plus Leptophlebiinae Banks, 1900, Habrophlebiinae Kluge, 1994a, and Calliarcyinae Kluge, 2009.

Hagenulini (Kluge, 1994b) was the first tribal designation for Atalophlebiinae. By that time, it included members of the following four Neotropical lineages (*sensu* Savage (1987): *Farrodes* lineage (genera *Farrodes* Peters, 1971, *Homothraulus* Demoulin, 1955 and *Simothraulopsis* Demoulin, 1966), *Hagenulopsis* lineage (genera *Hagenulopsis* Ulmer, 1920, *Borinquena* Traver, 1938 and *Askola* Peters, 1969), *Hagenulus* lineage (genera *Hagenulus* Eaton, 1882 and *Neohagenulus* Traver,1938) and *Careospina* lineage (*Careospina* Peters, 1971 and *Traverina* Peters, 1971) (Fig. 1). Savage (1987) also suggested that the last three lineages could form a superlineage, called by him as super-Hagenulopsis lineage. Kluge (1994b) described *Poecilophlebia* and *Turquinophlebia* and assigned all genera from the super-*Hagenulopsis* lineage as subgenera of *Hagenulus*, except for the genera *Askola* and *Hagenulopsis*. Later, Kluge (2008) considered *Hagenulopsis* as a subgenus of *Hagenulus* and assigned other genera to Hagenulini (Fig. 1) (for more details, see Kluge, 2008). Considering disagreements on literature and the absence of phylogenetic support to the higher-level classification of *Hagenulus* s.l. (*sensu* Kluge, 1994b, 2008), hereafter we will consider all subgenera assigned by Kluge (1994b, 2008) as non-subordinate categories of *Hagenulus*.

Although most of the leptophlebiid subfamilies were initially proposed without a modern approach of phylogenetic analysis, recently, subfamilies and tribes have been tested using morphological (e.g. Godunko *et al*., 2015) and molecular (e.g. O'Donnel & Jockusch, 2008; Monjardim *et al*., 2020) phylogenies. Monjardim *et al*. (2020) proposed a molecular phylogeny for the family based on mitochondrial *cytochrome oxidase* I (COI) and nuclear 28S rRNA (28S:D2–D5 regions) genes. As result, they elevated the status of Hagenulini Kluge (1994b, 2008) to subfamily and divided Hagenulinae in the following taxa: Hagenulini (subtribes: Ulmeritina, and Hagenulina), Miroculini (subtribes:

Miroculina and Hermanellina, and the clade R (Fig. 2 in Monjardim et al., 2020) represented by *Thraulodes* Ulmer,1920).

Hagenulina (Supplementary material 1) comprehends genera that are restricted to Antillean (*Borinquena*, *Careospina*, *Neohagenulus*, *Poecilophlebia*, *Traverina*, and *Turquinophlebia*), genera restricted to South America (*Askola* and *Ecuaphlebia* Domínguez, 1988), and genera distributed in both Antillean and South American (*Hagenulus* and *Hagenulopsis*). Moreover, Dominican amber fossils of mayflies dating from Miocene also have been assigned to the subtribe, one belonging to the fossil genus *Hagenulites* Stanickzeck, 2003 (Stanickzeck, 2003) and the others belonging to extant genus *Borinquena* (Staniczek, 2003; Godunko & Krzeminski 2009; Staniczek et al., 2017).

The first comments and tree diagram about the relationships among the genera currently belonging to Antillean Hagenulina were made respectively by Peters (1971) and Kluge (1994b). It is worthwhile to highlight that, despite the relationships among Neotropical leptophlebiid genera have been a subject longstanding addressed, few studies have shed light regarding the sister taxa relationships under a modern phylogenetic framework (Flowers & Domínguez, 1991; Monjardim et al., 2020). Therefore, proposing phylogenetic hypothesis encompassing all Hagenulina representatives can allow us to understand how they are related and how some rare and interesting characters have evolved in Leptophlebiidae, such as female egg guide, hind wings reduction, and spine-like projection on male penes. Additionally, a dated phylogenetic hypothesis would also allow us to understand the process of tempo-spatial diversification of the subtribe.

The biogeographic dynamics of the Caribbean region and its connection with South America are keys to understanding the diversification process of the subtribe Hagenulina. In fact, the arthropod fauna of the Caribbean is mainly related to that of South

American (Crews & Esposito, 2020). In the Caribbean region, Antillean landmasses have a complex geomorphological formation, dating from ~110 Ma when the Proto-Antilles became emerged and passed through for several inundation events due to the ocean expansion or due to stochastic events, such as meteor shock near to Yucatan peninsula that promoted earthquakes and tsunamis (Iturralde-Vinent & MacPhee, 1999). In addition, there is a hypothesis that, at the early of 40 Myr, Antillean landmasses became completely above the sea level as a contiguous block and afterward became separated by Mona Passage (23 Myr) splitting off Cuba + Hispaniola from Puerto Rico, and the Windward Passage splitting off Cuba from Hispaniola (15 Myr) (Iturralde-Vinent & MacPhee, 1999).

Several biological groups have been used as models for explaining the process that drove the Antillean colonization and, far from obtaining a unique pattern, they show us that the interchange with South American biota have been influenced by long dispersal, vicariance, and facilitated dispersal by a hypothetical land-bridge connection via Greater Antilles-Aves Ridge (GAARlandia) around 35–32 ±2 Myr (Matos-Maraví *et al*., 2014; Weaver *et al*., 2016; Chamberland *et al*., 2018; Massariol *et al*., 2019; Crews & Esposito 2020; Rodriguez-Silva & Schulpp, 2021). Mayflies are common habitants from island environments and its presence across these isolated regions can be explained for a myriad of process, such as short or long dispersal and vicariance (Monaghan *et al*., 2005; Sartori & Brittain 2015; Vuataz *et al*., 2013; Cozzarolo *et al*., 2019). Leptophlebiidae, in it turns, are known as a good island colonizers (McCafferty, 1992) due to its high capacity of conquering insular environments around the world, being recorded since on islands that were once connected with mainland (e.g. Zuñiga *et al*., 2015) to those that were never connected to before (e.g. Hoffman *et al*., 1999).

Here, we are presenting the first morphological phylogeny hypothesis including all representatives of Hagenulina subtribe. We performed analyses under Bayesian optimally criteria using Homoplasy-Based partitioning framework aiming to test the subtribe monophyly, to understand the relationship among the genera, as well as to estimate the time divergence of the lineages. Further, we addressed the historical biogeography of Hagenulina in order to answer the following main questions. a) Did the Antillean colonization occur at once through the time? b) Did GAARlandia landspan drive a central role in Antillean colonization?

Material and methods

Morphological terminology

General morphological terminology used in this study followed the standardization provided by Hubbard (1995) for widely used terms in Ephemeroptera taxonomy, such as egg guide in female sternum, male penes, wings' length and width, gill lamellae and so on. Terms recently proposed were also used and properly referenced, such as, comb-like row of setae, telopenes, and papillae on egg guide (see Supplementary material 2).

Taxon sampling and data matrix

In order to encompass the morphological diversity of Neotropical leptophlebiids, the taxon sampling was based on previous phylogenetic studies (e.g. Flowers & Domínguez, 1991; Domínguez, 2009; Monjardim *et al*., 2020). The terminals labeled as outgroup comprised representatives from the subfamilies of Leptophlebiidae: Habrophlebinae (1), Atalophlebiinae (6), Terpidinae (1), and Hagenulinae (9). The outgroup also included fossils, namely: oligoneuriid *Incogemina nubila* Storari,

Rodrigues, Saraiva & Salles, 2020 (tree root) (Cretaceous-Upper Apatian, 113.0–125.0 Myr), and the leptophlebiids *Leptophlebia* (*Paraleptophlebia*) *electra* Kluge, 1993 (Eocene-Priabonian, 33.9–37.8 Myr) and *Aureophlebia sinitshenkovae* Peters & Peters, 2000 (Cretaceous-Turonian, 89.8–93.9 Myr). The ingroup comprised 26 living and four fossil species dating from Miocene (Burdigalian, 20.43–15.97 ± 0.05 Myr; namely: *Hagenulites hitchingsi* Staniczek, 2003, *Borinquena maculata* Staniczek, 2003, *Borinquena parva* Staniczek, 2003, and *Borinquena schawallfussi* Staniczek, 2003). Some morphological characters were obtained from previous phylogenetic studies (e.g. Flowers & Domínguez, 1991; Domínguez, 2009; Godunko *et al*., 2015; Salles & Boldrini, 2019), while others were proposed under this study (supplementary material 2, Character list). A standardization in the character statement was applied for all characters aiming a constrained coding (Forey & Kitching, 2000; Sereno, 2007) and, due to this, some original propositions were coded into two or more characters. An amount of 91 morphological characters were scored in a matrix, which was built up on Mesquite package 3.6 (Maddison & Maddison, 2017) and then exported in an appropriate format for parsimony software analyses - Tree analysis using New Technology (TNT 1.5) (Goloboff *et al*., 2008a) and for a Bayesian software analyses - MrBayes v.3.2.7 (Ronquist et al., 2012a). Inapplicable and missing data were treated as "-" and "?", respectively.

Phylogenetic analysis

The phylogenetic analyses were carried out under Bayesian optimally criteria using Homoplasy-Based partitioning approach (Rosa *et al*., 2019). The better partition scheme was chosen through a parsimony analysis ran on TNT 1.5 (Goloboff *et al*., 2008a) with an adjusted memory to 10000 in order to accommodate the highest number of trees in the tree space. Traditional searches were carried out under equal weight (EW) and

implied weight (IW) against homoplastic characters (Goloboff, 1993; Golofoff *et al*., 2008b), with 1000 replications of tree-bisection-reconnection (TBR) branch swapping, saving 100 trees in each replication. Following Prendini (2000), we employed an analytical strategy for IW searches in which a concavity variation scheme of index K was applied (K=3–20) to better understand the topological impacts of optimization and character changes in the most parsimonious tree.

Bayesian inference was implemented in MrBayes v.3.2.7 (Ronquist *et al*., 2012a) considering the Markov model (Mkv) introduced by Lewis (2001). This model allows the modeling of morphological character through a generalization from the Jukes-Cantor model, originally applied for DNA sequence (Lewis, 2001; Wright *et al*., 2016). Criticisms have been addressed to Lewis' proposal to be a symmetrical model, which means that the transitions rates are assumed to be equal, such as parsimony equal weight (Wright et al., 2016). In order to make a more realistic Mkv model, other alternatives taking account the character change asymmetry has been proposed under distribution models (Harrison & Larsson, 2014), such as prior modeling (Wright *et al*., 2016) or homoplasy-based partitioning method (Rosa *et al*., 2019).

Homoplasy-based partitioning is a methodological strategy in which the proposers' (Rosa *et al*., 2019) used the character weighting against homoplasy (Goloboff *et al*., 2008b) to accommodate the character asymmetry, or among-character rate variation (ACRV) and then using the homoplasy scores to group characters in a partitioned data matrix. The partitions in the dataset (Table 1) were set under Concavity index as k=5, chosen subsequently to the k variation scheme, and then submitted to the Markov chains and ran for 100 x 10^6 MCMC generations with two parallel runs and four chains on MrBayes at the CIPRES gateway (Miller *et al*., 2010). Initial generations were burn-in discarded at 25%. Thereafter, the convergence among runs were accessed on MrBayes

through standard deviation of split frequencies (<0.05) and Estimated Sample Size (ESS) were checked in Tracer (Rambaut *et al*., 2014) with acceptable values > 200. Lastly, tree diagram was opened in Figtree (Rambaut, 2016) and the respective posterior probabilities were plotted on the node branches. Posterior probabilities (PP) and morphological synapomorphies respective to each clade were also provided, further unique synapomorphies were target in bold. To trace character evolution over the Bayesian topology we used the software Winclada (Nixon, 2002) choosing the delayed transformation (DELTRAN) instead non-ambiguous or accelerated optimization, assigning the changes closer to the tips.

Time divergence estimation

Time divergence allows us to access the evolutionary history taking in account macroevolutionary geological events (e.g. tectonic of plates) and past climatic changes, which can leave remarkably signatures on the evolutionary history of a lineage. Node and Tip-dating are the main competing framework to calibrate the divergence estimation. Whereas the former can have some positional bias regarding to previous attribution of relationships of fossil record to the node (Heath & Moore, 2014), the latter framework is free from this bias once it treats the fossil records as a terminal during the analysis (Pyron, 2011; Ronquist *et al*., 2012b).

To estimate the time divergence in Hagenulina we applied the Fossilized-Birth-Death model (FBD, Zhang *et al*., 2016) which considered the Tip-dating strategy. The fossil calibrations were assigned up to the corresponding geological strata in which each of them were discovered and the range ages (minimum–maximum) were set as an uniform distribution prior as follow: *Leptophlebia* (*Paraleptophlebia*) (33.9–37.8 Myr), *Aureophlebia sinitshenkovae* (89.8–93.9 Myr), *Hagenulites hitchingsi* (15.9–20.4 Myr),

Borinquena maculata (15.9–20.4 Myr), *Borinquena parva* (15.9–20.4 Myr) and *Borinquena schawallfussi* (15.9–20.4 Myr).

Clock model's usage has been increasingly changing its application, being firstly developed to accommodate molecular change rates, posteriorly it began to be used in total evidence analyses (e.g. Pyron, 2011; Ronquist *et al*., 2012b; Zhang *et al*., 2016; Lee, 2016), and recently it started to be used in analyses comprising only morphological data (Matzke & April, 2016, Canidae; King *et al*., 2017, Placoderms; Zhang & Wang, 2019, Mesozoic birds; Lucena & Almeida, 2021, Chrysididae).

Aiming to model the mutation rate, we used the independent-gamma rate (IGR) white-noise clock prior (Lepage *et al*., 2007), an uncorrelated relaxed clock which seem to perform better than correlated relaxed clocks (Drummond *et al*., 2006). The speciation, extinction, fossilization, and sampling processes were modeled using the Fossilized Birth-Death (FBD) priors that were set as: the root tree age prior (*treeagepr)* was set with *Incongemina nubila* age (113.0–125.0 Myr); for the diversity sample proportion prior (*prset sampleprob*), the total species diversity of the subtribe (n= 44) and the species sampled in this study (n= 26) were represented by a proportion of 0.5909. We carried out the strategy of sampling fossils as *prset samplestrat*, considering all fossils as a tip (r = 1). The data set (supplementary material 3) was ran under 200×10^6 generations for tip (r = 1), all of others FBD priors were assigned as default values following Zhang et al. (2016): *prset speciationpr* = exp (10), *prset extinctionpr* = beta (1,1), *prset fossilizationpr* = beta (1,1).

Biogeography analyses

In order to test the competing models No-GAARlandia (overwater dispersal) versus GAARlandia (landspan) that historically have advocated to explain the Antilles

colonization, since it (the Antilles) was completely above sea level around 40 Ma, we performed time slice analyses under two dispersal probabilities (supplementary material, table 01) based on previous studies (Matos-Maraví *et al*., 2014; Weaver *et al*., 2016; Chamberland *et al*., 2017; Crews & Esposito, 2020).

Time slice approach was preferred rather than no time slice due to the multiple events that shaped the Antilles geomorphology since proto-Antilles formation (e.g. Antilles completely above sea level, connection with northern South American land masses, and opening of the Mona and Windward passages) which could have changed the mobility capabilities of the biota (Esposito & Prendini, 2019).

No-GAARland versus GAARland competing hypotheses were integrated on the dispersal probabilities to be tested through a likelihood approach in R 4.0.2 environment using *BioGeoBEARS* package (Matzke, 2013, 2014). This package uses maximum likelihood to perform direct comparisons among three biogeographical models: Dispersal-Vicariance Analysis (DIVA) (Ronquist, 1997), Dispersal-Extinction-Cladogenesis (DEC) (Ree & Smith, 2008), and Bayesian Inference of Historical Biogeography for Discrete Areas (BAYAREA) (Landis *et al*., 2013). As we are focusing on island colonization, all models were nested with founder-event (j parameter) to direct likelihood comparisons: DIVA+J, DEC+J, BAYAREA+J (Matzke, 2014).

We adopted some premises to construct the dispersal probabilities for both hypotheses. Dispersal probabilities generally range from 0.01, when the landmasses set up a long dispersal route, or up to 1.0 when the landmasses are contiguous. Among these lower and upper probabilities bounds are: 0.1 to effective barrier, 0.5 to barrier by long dispersal, 0.7 to separated landmasses by an intermediate area, 0.8 to adjacent landmasses, and, 0.9 landmasses connected by a landspan. To landmasses not available throughout the time slices, were assigned 1e10-7 following Crews & Esposito (2020).

The definition of areas followed the regionalization of the Neotropics proposed by Morrone (2014), except for South America that was used here as a single area as it fits better for the purpose of this study. In his regionalization, Morrone (2014) treated Antilles (or West Indies) as a Neotropical sub-region and the islands of Cuba, Puerto Rico, Hispaniola (Haiti + Dominican Republic), and the Lesser Antilles as Antillean provinces. Although other provinces such as Bahamas, Jamaica and Cayman Islands also are part of Antilles, they were not considered in the present study once no species occurring in these areas were considered in our analysis.

The morphological time tree yielded for Mr. Bayes under FDB priors was pruned using the tree view software Archaeopteryx (Han & Zmasek, 2009) to exclude non-Hagenulina taxa, though we kept on tree *Miroculis niltoi* as Hagenulinae representative. Thereafter, all terminals were coded in a presence/absence matrix in according to the five (South America, Cuba, Puerto Rico, Hispaniola, and Lesser Antilles) areas and analyzed in the *BioGeoBEARS*.

Results

Hagenulinae monophyly and phylogeny of Hagenulina

The search for the best character partitioning under Parsimony criterion yielded distinct numbers of maximum parsimony trees (MPTs) related to equal (EW) and implied (IW) weights schemes that were applied during the analytical process (Supplementary material 6, Figs. 1A–D), all nodes congruence provided for parsimony are given in black squares below the nodes (Figures 2–6). Statistics related to length (L), consistency (Ci) and retention (Ri) indexes of each of them are provided in the table (Supplementary material 5, Table).

Our results based on the analysis of morphological data using Bayesian inference, including the outer groups, are shown in Figures 2–6. They held Hagenulinae *sensu* Monjardin *et al*. (2020) as monophyletic (PP, 97.91%; Figure. 3, clade A). The monophyly of the subfamily was supported by three synapomorphies: **45[1]** middle leg patelo-tibial suture absent; **46[1]** hind leg patelo-tibial suture absent; and 67[3] hind wing subcostal vein ending at wing margin, just distal to costal projection. Within Hagenulinae, Hagenulina (clade D) and the clade C (Hermanelina sensu Monjardin et al. 2020 + *Miroculis niltoi*, Miroculina sensu Monjardim et al., 2020) were recovered as closely related (PP, 65.10%), being supported by a single synapomorphy: **24 [2]** maxilla, distomedial margin, tusk large.

Hagenulina (Figure 5 and 6) was recovered as monophyletic (PP, 89.09%; Figure 5, clade D) being supported by: 47[1] abdomen, posterolateral projections on segments V or VI to IX; **53[1]** forewing, radial sector, intercalar veins IR1-2 and IRP connected; 55[1] forewing, anterior medial sector (MA), vein MA2 sagged; and 61[2] forewing, cubital sector, vein ICu1 attached to CuP. The first cladogenesis branched off *Ecuaphlebia rumignaui* from the remaining Hagenulina (PP, 97.35%; clade I), which was supported by seven synapomorphies: **31[1]** maxilla, ventral view, ventro-apical comb-like row disjunct; 32[0] mandible, outer margin curved; 58[0] forewing, MP fork, veins MP1 and MP2 originating at same time; 67[2] hind wing, subcostal vein ending on costal projection; 69[1] hind wing, vein MP unforked; 88[0] female abdomen, sternite IX strongly cleft; and 89[1] female abdomen, sternite VII with egg guide present.

Most species of *Careospina* (Figure 5, clade L), *Traverina* (Figure 5, clade M), and the monotypic genus *Turquinophlebia* were nested in a polytomy as sister group of the clade J (Figure 5). *Careospina* (Figure 5, clade L) was not recovered as monophyletic since *Careospina evanescens* Kluge, 1994 was clustered in the clade J, while the others

Careospina species were clustered together in the clade L (PP, 68.78%; Figure 5), which was supported by a single synapomorphy: 79[1] male penis, medio-ventral ridge extending laterally. *Traverina* (Figure 5, clade M) was recovered as monophyletic based on a single synapomorphy, though with a low posterior probability value (PP, 22.12%): 50[1] gill, apical emargination, present.

In it turns, the clade J (Figure 5, PP 78.05%) was comprised by *Poecilophlebia pacoi* Kluge, 1994 as sister group of clade O and was supported based on two synapomorphies: **68[1]** hind wing, veins Rs absent and 75[1] male penis, sub apical spines present. *Careospina evanescens*, *Neohagenulus* and the Clade P were nested in the clade O (PP 38.41%; Figure 5), which was supported by: 70[1] hind wing, apical third, developed. The genus *Neohagenulus* (PP 99.99%; clade N; Figure 5, clade N) was supported as a clade based on following synapomorphies: 47[0] abdomen, posterolateral projections from segment II or IV to IX; 66[2] hind wing, costal projection greater than wing length; 74[1] male penis, lobes apical 1/2 to 1/4 separated; and 80[1] constriction on apex of penes.

The clade P (PP, 75.61%; Figure 6) comprises *Hagenulites hitchingsi* †, *Hagenulus* clade, *Borinquena* clade, and Clade S. This clade was supported by a single synapomorphy: 90[1] female, abdomen, VII sternite with egg guide long, reaching segment IX. Inside Clade P, two main clades were recovered, clades S and Q (Figure 6). The clade S (PP, 82.93%), hereafter mentioned as *Hagenulopsis* sensu lato, comprises a paraphyletic *Hagenulopsis* in relation to the species of *Askola*. This clade was supported by character 62[1] hind wings absent. *Askola* species were nested together (PP, 99.99%) and was supported by **6[2]** labrum, anteromedial emargination with two denticles; 50[1] gill, apical emargination present; 61[1] forewing, cubital sector, vein ICU1 attached to

CuA; 76[1] male penis, spine orientation, laterally; and 86[0] male, styliger plate, forceps I with internal margin not displaced inward.

The clade Q (PP, 79.70%; Figure 6) encompasses the relationships of ((*Hagenulites hitchingsi* † (*Hagenulus morrisonae* + *H. caligatus*)) + (Clade R)) and was supported by **65[1]** hind wing, cross veins absent; and **91[1]** female, abdomen, sternite VII, egg guide, posterior papillae present. Internal relationships nested the fossil species *Hagenulites hitchingsi* † as *Hagenulus* sister group (PP, 59.53%), though no non-ambiguous morphological characteristic was assigned as synapomorphy in this clade. *Hagenulus* (PP, 76.95%), represented in this study by *H. calligatus* and *H. morrisonae*, was supported as monophyletic by: 11[1] labrum, third dorsal row of setae, closer to basal margin; 23[1] maxilla, distomedial margin, tusk present; 27[1] maxilla, maxillary palp, articulation basal; 32[2] mandible, outer margin, right angle; 36[1] labium, labial palp, ratio of segment I/segment II ≥1.0–1.1; 37[1] labium, labial palp II, long setae on outer margin; and 40[1] labium, labial palp II, elbowed shaped.

Lastly, *Borinquena* (PP, 90.21%; Clade R) was hold as a monophyletic group comprising fossils and living species and was supported by the characters **85[1]** male, styliger plate, forceps I elongated and 87[2] male, styliger plate, forceps I, internal margin displaced inward located on apex. Regarding its internal relationships, a clade with high support (PP, 84.64%) comprising (*B. parva*† Stanickeck, 2003, *B. carmencita* Traver, 1938 and *B. contradiscens* Traver, 1938) was supported by 66[2] costal projection on hind wings greater than the half of wing. The other species of *Borinquena* were placed in clades with low support, so we can consider them as part of a polytomy with the clade formed by *B. parva*†, *B. carmencita*, and *B. contradiscens* (Figure 6).

Time divergence estimation in Hagenulina

The phylogenetic hypothesis under FDB (Fossilized-Birth-Death model) revealed that Hagenulina (Figure 7, Clade A) originated as independent clade around 100 Myr (95% HDP - Highest prior density; median 100.47, 66.08–142.22 Myr; Figure 7), into a period covering from the Berriasian (Early Cretaceous) to Maastrichtian (Late Cretaceous). The diversification of this clade began around 61 Myr (HDP 95%, median 61.38, 37.58–92.22 Myr; Figure 7, Clade D), when *Ecuaphlebia rumignaui* diverged from other Hagenulina. This divergence is compatible with the Cretaceous-Paleogene transition (KPg) and may have extended from Turonian (Late Cretaceous) to Priabonian (Eocene).

After the divergence of *Ecuaphlebia rumignaui*, the remaining Hagenulina diverged in three main clades, all of them originated as independent lineages under higher Paleogene influence. The first diversification dates from Maastrichtian (Late Cretaceous) to Rupelian (Oligocene) around 47 Myr (95% HDP; median 46.71, 30.64–67.32 Myr) splitting the Clade P from the all other Hagenulina representatives. The stem of the clade comprising the genera *Neohagenulus*, *Careospina*, *Traverina*, *Turquinophlebia*, and *Careospina evanescens* + *Poecilophlebia pacoi* arose from the second diversification event inside Hagenulina at Maastrichtian (Late Cretaceous) to Rupelian (Oligocene) and became diversified around at Ypresian (Eocene) to Langhian (Miocene) around 29 Myr (95% HDP; median 29.4, 14.04–48.95 Myr). No further comments can be addressed regarding to the relationships among these genera within this group, but the genera *Neohagenulus*, *Careospina* and *Traverina* have begun its diversification process under Miocene influence (95% HDP; 4.92 Myr, 0.47–13.02 Myr), (95% HDP; 4.81 My , 0.45–12. 45), and (95% HDP; 1.91Myr, 0–8.46), respectively.

The clade P, which had low support (< 50%) under FDB but with a good support (PP 75.61%) under the timeless analysis, became diversified in two well supported clades

around Thanetian (Paleocene) to Chattian (Oligocene) about 40 Myr (95% HDP; median 39.94, 26.68–58.34 Myr). The daughter lineages of clade P have diversified as follow: clade Q at 36 Myr (95% HDP; median 35.64, 24.4–51.28) became diversified around Ypresian (Eocene) to Chattian (Oligocene); and clade S, which diversified around Bartonian (Eocene) to Tortonian (Miocene) at 23 Myr (95% HDP; median 23.03, 9.08–40.52). Within the clade Q, early diversification in *Borinquena* was estimated around at 26 Myr (95% HDP; median 26.56, 19.78–36.67) into Priabonian (Eocene) to Burdigalian (Miocene), whereas that the cladogenesis splitting *Hagenulites* from (*Hagenulus morrisonae* + *H. calligatus*) was estimated at 23 Myr (95% HDP; median 23.17, 16.3–35.41 Myr) into Priabonian (Eocene) to Burdigalian (Miocene).

Historical Biogeography **in Hagenulina**

The AIC comparisons retrieved DIVALIKE+J as the best model that fits our dataset (Table 2), both considering No-GAARland and GAARland hypotheses. Our data revealed that the Hagenulina ancestor likely had a South American origin, from where colonized the Antilles in different events (Figure 8). For example, between Lutetian (Eocene) and Rupelian (early Oligocene), around 47 Myr (95% HDP; median 46.71, 67.31 –30.63 Myr), in one or two events, descendants of the clade formed by current genera *Neohagenulus*, *Traverina*, *Careospina*, and *C. evanescens* + *Poecilophlebia pacoi* dispersed from South America to Antilles. As aforementioned in the section above, this clade has low support and the sister relationships among these genera are better understood as nested in a polytomy. All these genera are Antillean restricted, being *Neohagenulus* currently restricted to Puerto Rico and Hispaniola, and the others restricted to Cuba.

Other dispersal event likely occurred between the Thanetian (Paleocene) and the Chattian (Oligocene), around 40 Myr (95% HDP, median 39.94, 26.68–58.34 Myr), when

the MRCA (most recent common ancestor) of the clade (*Borinquena* (*Hagenulites* + *Hagenulus*)) colonized Hispaniola. After that, the ancestral range of MRCA of the (*Hagenulites* + *Hagenulus*) likely expanded to Cuba and a vicariance event occurred around 23 Mya (95% HDP, median 23.16, 16.3–35.41 Myr), splitting the fossil species *Hagenulites hitchingsi* (Hispaniola) from the *Hagenulus* species currently in Cuba (*Hagenulus morrisonae* + *H. calligatus*). Lastly, one or two colonization events likely also occurred within *Hagenulopsis* s.l. which had an estimated ancestral area in South America, but presents two species currently recorded in the Lesser Antilles.

Our analyses also have shown Antillean diversification into *Borinquena* crown-group, whose inherited almost the same Hispaniola range occupied by its MRCA, became diversified into Antillean landmasses around 26 Myr when the Greater Antilles used to work as contiguous landmasses (fig 8, clade R). At the first cladogenesis the sister taxa (*Borinquena maculata* † + *B. schawallfussi* †) retained the ancestral ranges in Hispaniola ranges, while the remaining *Borinquena* experienced a range expansion. Later, the remaining *Borinquena* branched off, with cladogenesis occurring at 21 Myr and splitting *B. parva* †, which inherited Hispaniola ranges, from the other living species, which inherited an estimated range composed by Cuba + Puerto Rico. After that, a subsequent cladogenesis restricting *B. sextus* to Cuba and their siblings taxa to Puerto Rico ranges. Somewhat different from FDB analysis, the timeless analysis recovered the placement of the Puerto Rico species as sister taxa to *B. parva* † (Hispaniola).

Discussion

Our major contribution is the first cladistic hypothesis proposing Hagenulina as monophyletic group incorporating fossils and living species into a calibrated time-tree based on morphological clocks. The Hagenulina clade was well supported by several synapomorphies: abdomen with posterolateral projections on segments V or VI to IX,

radial forewing sector with intercalated veins IR1-2 and IRP connected, anterior forewing medial sector (MA) with vein MA2 sagged, and cubital forewing sector with vein ICu1 attached to CuP. The Hagenulina ancestors originated into an estimated range distribution comprised by South America at about 100.47 Myr (95% HDP, 66.08–142.22 Myr). Furthermore, our findings indicate that Antillean colonization from South American lineages occurred multiples times in Hagenulina (Figure 8).

Highlighting congruences and conflicts among Phylogenetic analyses

Our results recover the subfamily Hagenulinae (sensu Monjardim *et al*., 2020). This clade was initially proposed by Kluge (1994, 2008) based on morphology and later corroborated based on molecular data (Monjardim *et al*., 2020). Here, our morphological analysis recovered this clade in all analysis strategies (i.e. Bayesian and Parsimony with or without implied weighing) reinforcing its consistency. On the other hand, our results do not recover the Hagenulini tribe (subtribes Hagenulina + Ulmeritina, sensu Monjardim et al., 2020). In contrast, our results reveal Hagenulina into different arrangements: supported as sister taxa of the clade C (*Miroculis niltoi* + Hermanelina) (Figure 3) or nested in a polytomy together with the clade C and (*Thraulodes* sp. (*Ulmeritoides* sp. + *Atopophlebia* sp.)) (Figure 7).

Our data support the monophyly of the subtribe Hagenulina (sensu Monjardim, 2020), which include the genera *Borinquena*, *Careospina*, *Ecuaphlebia*, *Hagenulopsis s.l.*, *Hagenulites* †, *Hagenulus*, *Neohagenulus*, *Traverina*, *Poecilophlebia*, and *Turquinophlebia.* The monophyly is corroborated in most morphological analysis strategies that we used (Bayesian analyses, Figures 5–7; schemes EW and IW, k = 5–20, Supplementary material 6, Figures 1A and D). However, topological conflicts were found in IW schemes (IW, schemes k = 3, 4), which suggest that *Ecuaphlebia rumignaui* does not belong to Hagenulina, instead it was placed in the earliest Hagenulinae cladogenesis

(Supplementary material 6, Figures 1B, C). Despite this, given the body of evidence, we consider *Ecuaphlebia* as belonging to the first cladogenesis inside Hagenulina.

Although the subtribe was recovered as proposed by Monjardim *et al*. (2020) in most of our phylogenetic analysis schemes, *Hermanella* and *Traverella* (Hermanellina) were depth-positioned across the Hagenulina closely related to the Clade S in the IW parsimony analysis with k = 3 (Supplementary material 6, Figure 1B). Additionally, Flowers & Domínguez (1991) found *Hagenulus calligatus* (Hagenulina) as sister group of Hermanellina, indicating *Hagenulus* as paraphyletic. Kluge (1994b) stated harsh criticisms to this hypothesis suggesting an analytical artifact due an overweight from nymph mouth characters in the analysis. Indeed, our data indicate that *Hagenulus* is monophyletic and phylogenetically positioned among Hagenulina. The hypothesis of *Hagenulus* as a sister group to Hermanellina is fragile, since it would be necessary to assume reversions of several morphological characters (e.g. **53[1]**, **31[1]**, **68[1]**, **65[1]**, and **91[1]**, Supplementary material 2, Character list) rather than those shown in Kluge (1994b). Therefore, despite the alternative results (parsimony with k=3; Flowers and Dominguez, 1991), we considered the taxonomic composition of Hagenulina as proposed by Monjardin (2020), with the monophyly of *Hagenulus* and its position within the subtribe.

Within Hagenulina, it was possible to identify some well-supported clades such as *Borinquena*, *Hagenulus*, *Hagenulopsis* s.l., and *Neohagenulus*. As well as a well-supported more inclusive clade revealing the following relationships between the genera: (*Hagenulopsis s.l.* (*Borinquena* (*Hagenulus* + *Hagenulites* †))). This large clade was recovered in all analyzes and is consistent with the previous suggestions that *Hagenulopsis*, *Borinquena*, *Hagenulites* and *Hagenulus* are closely related (Peters, 1971;

Staniczek 2003). Within *Hagenulopsis*, a well-supported *Askola* emerges, forming a more inclusive clade, *Hagenulopsis s.l.*, which is recovered in all analyses. Although shown as independent lineages in the timeline tree (Figure 8, clade S), both (*Hagenulopsis* and *Askola*) are held as a single clade supported by a high posterior probability (80–90% PP). Therefore, our data suggest that *Hagenulopsis* as currently defined is paraphyletic. Indeed, the relationship between *Hagenulopsis* and *Askola* is unstable, for example: 1) based on a morphological phylogeny, Domínguez proposes that they are sister groups (Domínguez, 2009); 2) Campos *et al*. (2019), who focused on internal relations of *Askola*, suggests its sister relationships with *Hagenulus* + *Hagenulopis*; and 3) Monjardim *et al* (2020), based on a molecular phylogeny, suggest that *Askola* is a sister group of *Ecuaphlebia* + *Hagenulopis*. All these previous phylogenies included few *Hagenulopsis* species, so the relationship between these two genera still needs to be further tested.

Although our analyzes found consistent clades and well-supported genera, our findings also indicate paraphyly of *Careospina* and low support to hold *Traverina* as monophyletic. In the case of *Careospina*, among the four species included in our analysis, three form a well-supported clade (*C. baconai* (*C. hespera* + *C. sierramestre*), but one, *C. evanescens*, is placed with another clade (Figure 5, clade J). In our study, the previous suggestions (e.g. Peters, 1971; Kluge, 1994b) and the formal hypothesis (Domínguez & Flowers, 1991) that *Careospina* and *Traverina* form a clade were not corroborated. In contrast, part of the species of paraphyletic *Careospina*, the low supported *Traverina* and *Turquinophlebia* were nested in a polytomy, being all of them considered as sister taxa of clade J (Figure 5). The results for *Careospina*, as well as for *Hagenulopsis* s.l., which includes *Askola*, reveal that taxonomic adjustments may be needed in some genera of Hagenulina. In this context, we recommend that studies based on a broader sample and

analyzing the types be carried out in order to assess the taxonomic status of these two genera.

Here, it is important to highlight two issues that may have influenced the internal relations of clade P in our phylogenetic hypotheses, the first is concerned to Bayesian vs Parsimony analyses and the second is regarding non-clock vs clock Bayesian analyses. Currently, there is a contentious question that Bayesian analyses can outperform Parsimony analyses providing more accurate results (e.g. O'Reilly *et al*., 2016 ; Puttick *et al*., 2017; Brown *et al*., 2017), mainly when the analyzed scenario include missing data (Wright & Hillis, 2014; Koch *et al*., 2021) as in the case of including fossil data. In our study, we observed that only Bayesian analyses were able to recover the *Hagenulus* monophyly and its sibling relationship with *Hagenulites hitchingsi* †, which forms a sister clade with *Borinquena*. Further, it was also only under Bayesian analyses that internal resolutions inside *Borinquena* were recovered. The other issue is related to the drop-down of the support in the Bayesian phylogeny based on clock analysis under FDB parameters (PP < 50%) compared with that based on non-clock analysis (PP = 75.61%). King (2020) argued that this kind of behavior into FDB analysis have been neglecting among the studies using this method and suggest that this kind of performance could be driven by unbalance between the FDB tree model and the dataset coding incompleteness, thereby affecting the stratigraphical fit in the absence of the strong morphological evidence (see also Koch & Parry, 2021).

In summary, our results did not recover the relationship (*Ecuaphlebia* + *Hagenulopsis*) as suggested by Domínguez (1998) neither (*Askola* (*Ecuaphlebia* + *Hagenulopsis*)) as proposed by Monjardim *et al*. (2020). Instead, we recovered *Ecuaphlebia* at the first cladogenesis within Hagenulina (Figure 5) and the clade *Hagenulopsis s.l.*, including *Askola* species, as sister taxa of the clade P (Figure 6). These

findings bring up again the outstanding discussion about the hind wing loss into Neotropical leptophlebiid, since in our study this loss supports the clade *Hagenulopsis sl*. Although, it is clear that the loss of hind wings has not happened just once through the evolutionary history of Neotropical leptophlebiid (e.g. *Perissophlebiodes* and *Bessierus*), our study agrees in part with Domínguez (2009) who also considered that this happened only once at the base of the clade *Hagenulopsis* + *Askola*. Conversely, there is also evidence that hind wing loss may have occurred independently in *Hagenulopsis* and *Askola* (Campos *et al*., 2019; Monjardim *et al*., 2020). Despite these different results, it is important to note that apparently the loss of the hind wing in Hagenulina begins with the remarkable reduction observed in clade O (Figure 5) reaching higher reductions in sister taxa of *Hagenulopsis* s.l. (Figure 6, clade P), until the total loss in that clade.

Time-space integration

Our morphological timeline tree inference and biogeographic analysis indicates that Hagenulina colonized Antilles from South American. Mayflies are a faunistic component usually found in Antillean landmasses, including genera of Leptophlebiidae (10), Baetidae (6), Caenidae (2), Euthyplocidae (1), Leptohyphidae (5), and Oligoneuriidae (1), nevertheless how they did arrive there remains a contentious question throughout decades (McCafferty, 1985; Peters, 1988; McCafferty *et al*., 1992). Peters (1988) recognized a Neotropical origin for Antillean leptophlebiids and suggested the Antillean colonization from South America lineages through dispersal driven by winds currents. Massariol *et al*. (2019), for instance, provided a dispersal hypothesis driven by range expansion of *Lachlania* (Oligoneuriidae) from South America to Central America + Antilles (Panamanian *sensu* Holt *et al*., 2013) + Nearctic regions. In fact, there are many underlying questions to answer how arthropods reached the Antilles, but it seems that

South America worked as a diversity source for many groups (Deler-Hernández *et al*., 2017; Chamberland *et al*., 2017; Esposito & Prendini, 2019; Crews & Esposito, 2020).

Neotropical leptophlebiid lineages not closely related have species recorded in the Antilles such as *Farrodes* Peters, 1971 and *Terpides* Demoulin, 1966, but restricted distributions in that region occur only in Hagenulina (e.g. *Borinquena*, *Careospina*, *Neohagenulus*, *Poecilophlebia*, and *Turquinophlebia*). Our results suggest a probable emergence of Hagenulina at Mid-Cretaceous (~ 100.5 Myr) (Fig 7, clade A) branching off *Ecuaphlebia* from the other Hagenulina still in South America at the beginning of Paleogene (~ 60.0 Myr) (Fig 7, clade D). In fact, the ancestors of several lineages of Hagenulina (e.g. ancestral of lineage I ~ 47 Myr; lineage P ~ 40 Myr; lineage S ~ 1 Myr) occurred in South America, which acted as primary source of the multiple incursions of Hagenulina to Antilles. Our results and those of other studies reinforce the important role of South America in the origin of the Antillean fauna (e.g. Weaver *et al*., 2016; Deler-Hernández *et al*., 2017; Crews & Esposito, 2020).

Our results also reveal that the Greater Antilles were colonized by Hagenulina, before (e.g. clade Q, around 40 Myr) and after (e.g. *Neohagenulus* and related genera, around 29 Myr) of the hypothesized. The hypothesis is very recent, a few years old of a land bridge with South America (35–32 ±2 Myr), which would refute the GAARlandia hypothesis as the major explanation for Antillean colonization of the subtribe. However, these results need to be cautiously interpreted, since our time estimation for colonization of the Greater Antilles by Hagenulina and the expected period of GAARlandia differ just by approximately 5 million years, close to and within the expected degree of uncertainty of the divergence time of the corresponding nodes. The GAARlandia hypothesis (Iturralde-Vinent & MacPhee, 1999) proposed that the Aves ridge connected the Greater Antilles (Cuba, Hispaniola, Puerto Rico) to northern South America during the Late

Eocene/Early Oligocene transition (35–32 ±2 Myr), which could facilitate the colonization of the Greater Antilles. On the other hand, despite our data not being able to completely refute this hypothesis, there is recent evidence, both geological and biological, against the existence of this land bridge in this time period (Ali and Heads, 2021).

In the present study, the clade that includes *Neohagenulus* and related genera (Figure 8) had low support and the relationships obtained between them were incongruent between the different approaches used (Figures. 5; 7–8). The species of these genera occur mainly in Puerto Rico (*Neohagenulus* including in this analysis), Hispaniola (*Neohagenulus* recently described by Sartori, 2021), and Cuba (*Poecilophlebia, Turquinophlebia, Traverina* and the paraphyletic *Careospina*). In view of topological incongruences between no-time (Figures 2–6) and time (Figures 7–8) trees regarding the *Neohagenulus* and its closer related Antillean genera, making difficult the test of biogeographical hypotheses. Despite this, it is possible to assert that the ancestors of these genera arrived in the Greater Antilles when it was a single landmass through one or more colonization events.

Our analysis estimates Hispaniola as an ancestral area of the clade Q (*Borinquena* (*Hagenulites* † + *Hagenulus*). The clade Q occurs in the Greater Antilles and its ancestral occupied Hispaniola at the Middle Eocene (around 40 Myr), a time when the Proto-Antilles were emerged as a single landmass (Iturralde-Vinent & McPhee, 1999). In fact, the clade Q began its diversification process when Cuba + Hispaniola + Puerto Rico were a single area, a situation that remained until 20 Myr. Inside this lineage, *Borinquena* (Greater Antilles) diverged in the first cladogenesis and the clade (*Hagenulites* † + *Hagenulus*) diverged in the second cladogenesis. Later, *Hagenulites* † (Hispaniola) diverged from the two species of *Hagenulus* (Cuba) included in our analysis around 23 Myr, when *Hagenulus* group experienced a probable range expansion to Hispaniola +

Cuba, followed by a vicariant event before the opening Windward Passage (15 Myr). However, it is important to highlight that *Hagenulus* encompases three other widespread species into Greater Antilles and a putative record to Ecuador (Peters *et al*., 2005). Therefore, to understand the biogeographic history of *Hagenulus*, it is necessary to expand the sampling of this genus and confirm the species from Ecuador, which were not possible in the present study.

In the case of curious genus *Borinquena*, which currently comprises most of its diversity as fossil species, there are topological conflicts between time (Figures 7–8) and no-time (Figure 6) Bayesian analyses regarded about who would be the sister taxa of (*B. contradiscens* + *B. carmencita*), if the living species *B. sextus* or the fossil species *B. parva*. These incongruences imply directly in two putative biogeographical scenarios that could be explained assuming or not local extinction assumptions. In the former scenario (*B. parva* (*B. contradiscens* + *B. carmencita*)), supported by the non-clock analysis (Figure 6, clade R), we would infer that the cladogenetic event branched off Puerto Rico restricted species from the Hispaniola fossil *B. parva* †. Yet in the second scenario, under FDB analysis (Figures 7–8), a tree topology assumes that the clade formed by (*B. sextus* (*B. contradiscens* + *B. carmencita*)) branched off separating *B. sextus* (Cuba restricted) from the two others species (Puerto Rico restricted) around 13 Myr, but the estimated age of this cladogenetic event does not fit with the temporality of geological events (see Iturralde-Vinent & McPhee, 1999) and, hence would be required assume an MRCA widespread distribution across Antilles with a local extinction on Hispaniola. On the other hand, an *ad hoc* dispersive event would also be required. Since there is evidence for both scenarios, they are equally possible. Whilst for the former could be held in similar patterns find for butterfly *Callisto* (Matos-Marravi *et al*. 2014) and aquatic beetle *Phaenonotum* (Deler-Hernández *et al*. 2017), for the last we can argue that the vast occurrence of

Borinquena fossil records in Hispaniola indicates that this area worked as a pivotal source of species linking Cuba and Puerto Rico.

Our data also reveal that *Hagenulopsis s.l.*, which include *Askola*, evolved and diversified in South America, but some species of the genus *Hagenulopsis* reached Central America and the Lesser Antilles (less than 23 Myr). Once more, the phylogenetic relationship between *Hagenulopsis* and *Askola* have been subject of debate as endorsed in our results, both genera are mostly distributed in South American landmass with *Hagenulopsis* species also being recorded from Northern Lesser Antilles and continental Central America.

Conclusions

Here we propose the first timeline tree for the subtribe Hagenulina aiming to answer questions related to diversification in the time-space, distributional ancestor range estimation and how the group reached the Antillean region. We conclude that Hagenulina is a monophyletic group which had a Neotropical South America origin and performed multiple incursions to Antillean landmasses. The role of a possible land bridge in the colonization of Hagenulina was not clearly evidenced, but, the temporal proximity of 5 million years between the estimated period for the GAARlandia and the age of the lineages that colonized the Greater Antilles does not allow to fully refute this hypothesis in face of our data. However, recent data reveal weak evidence that this land bridge has existed in the period proposed by GAARlandia hypothesis (Ali, 2012; Ali and Heads, 2021). Anyway, it is important to point out that we are studying winged insects and even if the existence of a continuous land mass between the Greater Antilles and South America did not exist around 35 Myr, islands resulting from the geological process in that region could act as stepping stones in the colonization process.

This is the most comprehensive biogeographic of Hagenulina in the Antilles, but some gaps still remain. For example, in some scenarios (e.g. for Borinquena), it is necessary to assume that the ancestors were distributed across the connected land mass of the Greater Antilles, with later extinction in Hispaniola. In fact, Hispaniola has many fossils (see, Poinar, 2010), indicating that several Hagenulina taxa that occurred on that island have gone extinct. On the other hand, it should be considered that we know little about the extant fauna of Leptophlebiidae from Hispaniola (Sartori, 2021) and that the conditions of fossilization in amber on the island are favorable, which may increase the representativeness of the fossils. In this context, the study of the extant fauna of Leptophlebiidae of Hispaniola and as well as from the other Antilles islands can provide additional data for improving the understanding of the evolutionary history of insular Hagenulina. Therefore, we recommend expanding the sampling effort, mainly in Antilles, and preserving the new material properly for molecular analysis, thus, it will be possible the proposition of a phylogeny better resolved by the integration of morphological and molecular data for this fauna.

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Tables

Table 01. Implemented character partitions in Bayesian inference under implied weighting K = 5.

Partitions	Characters
--	1, 2, 9, 14, 18, 21, 22, 24, 35, 38, 44, 56, 73, 78
00	6, 8, 13, 16, 19, 20, 25, 26, 29, 31, 45, 46, 52, 68, 72, 80, 84, 85, 91
0.166	3, 4, 7, 10, 11, 12, 17, 27, 28, 30, 33, 37, 39, 43, 48, 51, 53, 54, 55, 60, 62, 63, 64, 65, 71 ,76, 77, 79, 81, 90
0.285	23,34,40,57,59,66,83,88
0.375	5, 15, 36, 41, 42, 49, 58, 67, 69, 70, 82, 86, 89
0.44	32, 47, 74, 87
0.500	61, 75
0.545	50

Table 2. *BioGeoBEARS* model inference and its relative probabilities under the GAARsland and No GAARsland. All models implemented on *BioGeoBEARS* were tested allowing the founder event as a free parameter for both hypotheses. In bold is the best-fit model for each hypothesis, asterisk means the global best model. LnL, log-likelihood; d, dispersal rate; e, extinction rate; j, founder-event speciation; AIC, Akaike information criterion; AIC_w, relative likelihood normalized.

	LnL	**d**	**e**	**J**	**AIC**	**AIC_w**
GAARland						
DEC	-47.23	0.052	0.0065	0	98.47	4.6e-05
DEC+J	-37.59	0.0083	0.0018	0.74	81.18	0.26
DIVALIKE	-44.42	0.053	0.0053	0	92.85	0.0008
DIVALIKE+J	-37.01	0.010	0.0018	0.61	80.02	0.46
BAYAREALIKE	-64.31	0.052	0.026	0	132.6	1.8e-12
BAYAREALIKE+J	-37.51	0.0053	0.0018	0.50	81.03	0.28
NO GAARland						
DEC	-48.29	0.0083	0.0053	0	100.6	7.8e-06
DEC+J	-36.76	0.0012	0.0017	0.080	79.52	0.29
DIVALIKE	-44.78	0.0085	0.0033	0	93.57	0.0003
DIVALIKE+J*	-36.47	0.0016	0.0017	0.074	78.94	0.39
BAYAREALIKE	-63.21	0.0085	0.019	0	130.4	2.6e-12
BAYAREALIKE+J	-36.68	0.0010	0.0017	0.077	79.35	0.32

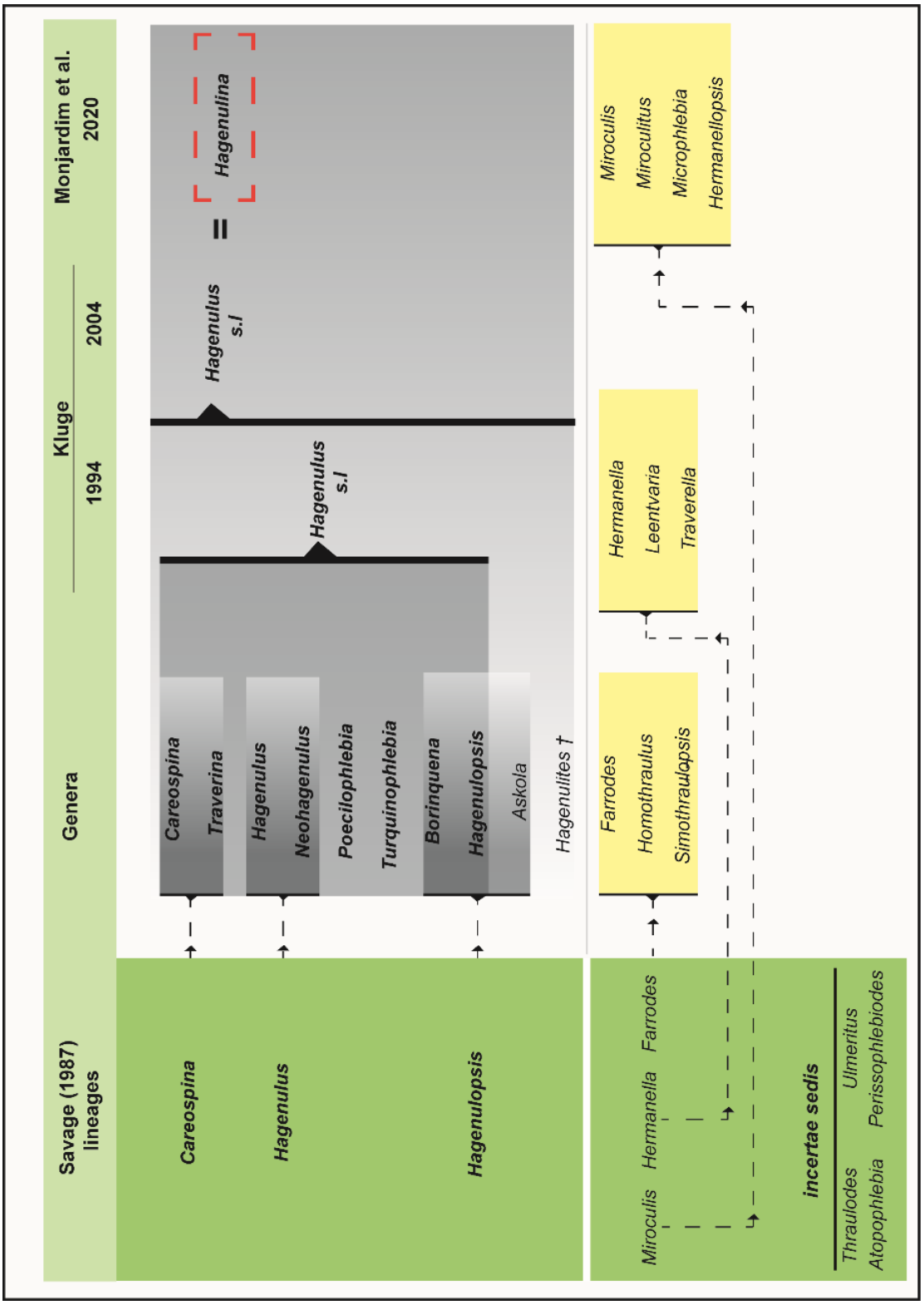


Figure 1. Retrospective classificatory scheme of the subfamily Hagenulinae (Monjardim et al., 2020), with emphasis in the classificatory system of *Hagenulus sensu latu* (Kluge, 2008) or Hagenulina (Monjardim et al., 2020). Dashed lines are linking the Savage lineages to genera that belong to each of them.

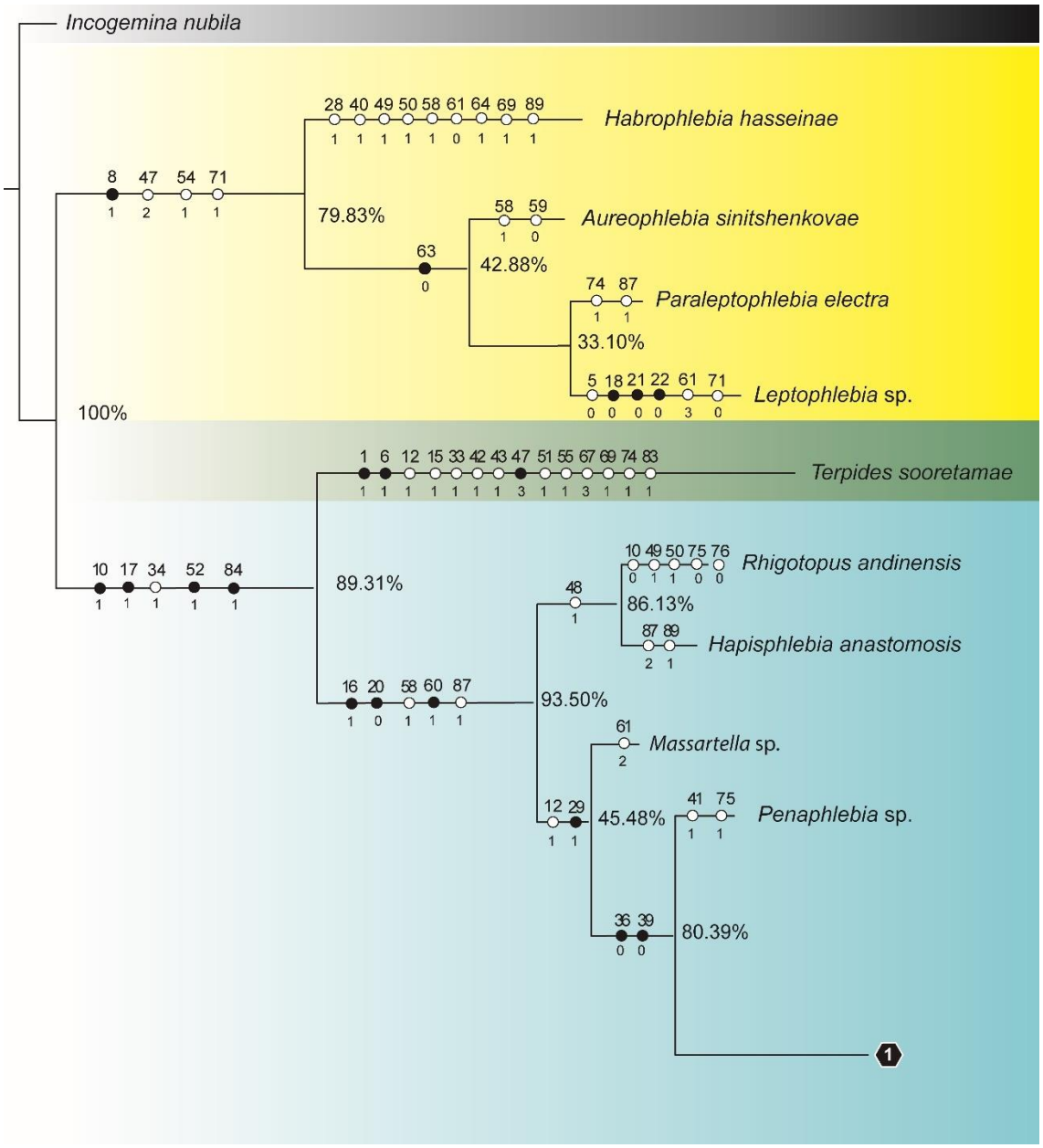


Figure 2. Consensus (all compatible) trees retrieved by Bayesian inference, posterior probability supports are assigned in each respective node. Morphological characters under slow optimization are plotted under their respective nodes. Black squares below the nodes means respective supports under parsimony schemes: equal weight EW and Implied weight, k=3; k=4 and k=5–20 (k=5-). Phylogenetic relationships among clade 1 are depicted in Figures 3–6.

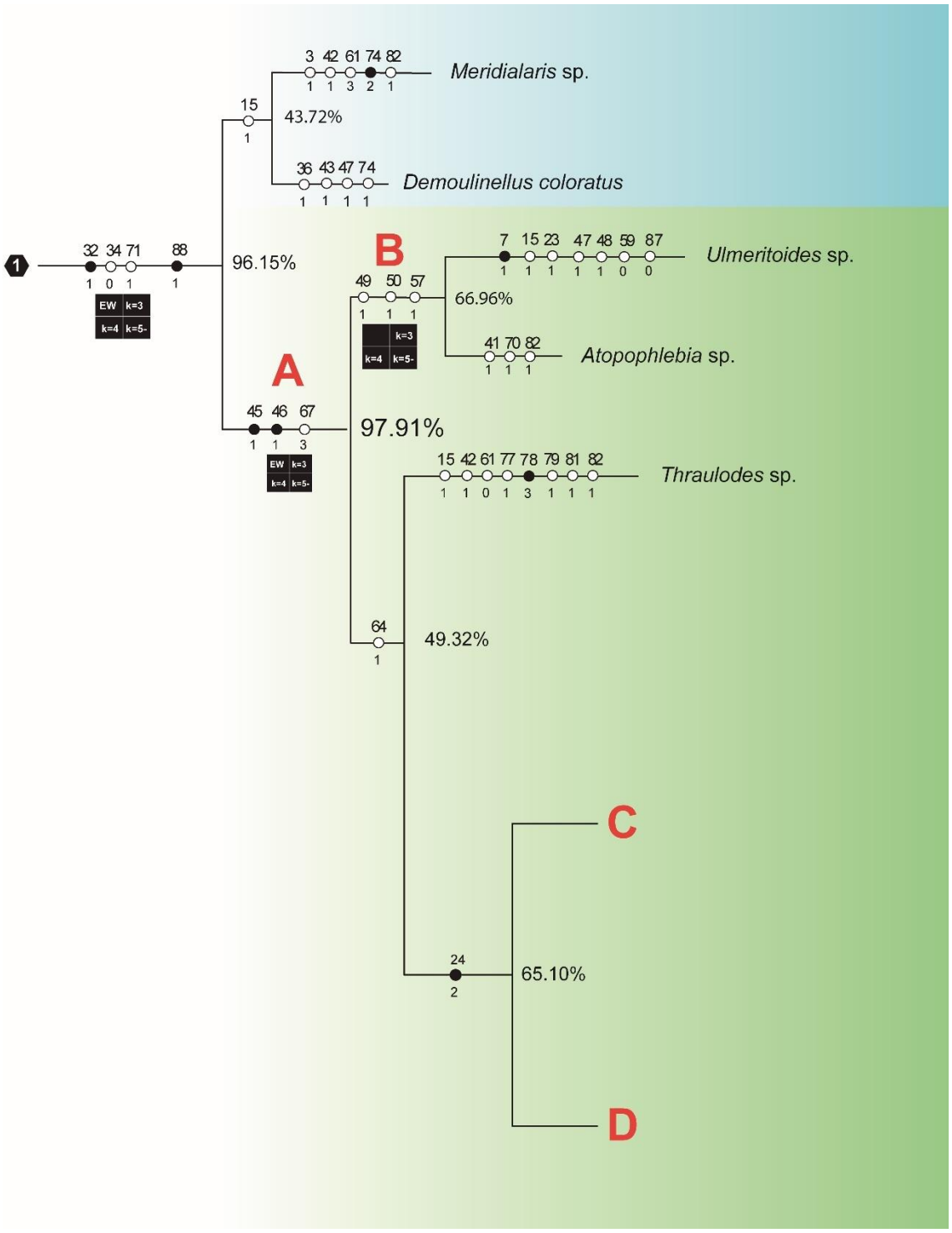


Figure 3. Part of the consensus (all compatible) tree Bayesian retrieved by Bayesian inference, posterior probability supports are assigned in each respective node. Morphological characters under slow optimization are plotted under their respective nodes. Black squares below the nodes means respective supports under parsimony schemes: equal weight EW and Implied weight, k=3; k=4 and k=5–20 (k=5-). Phylogenetic relationships among clades C and D are depicted in Figures 4–6.

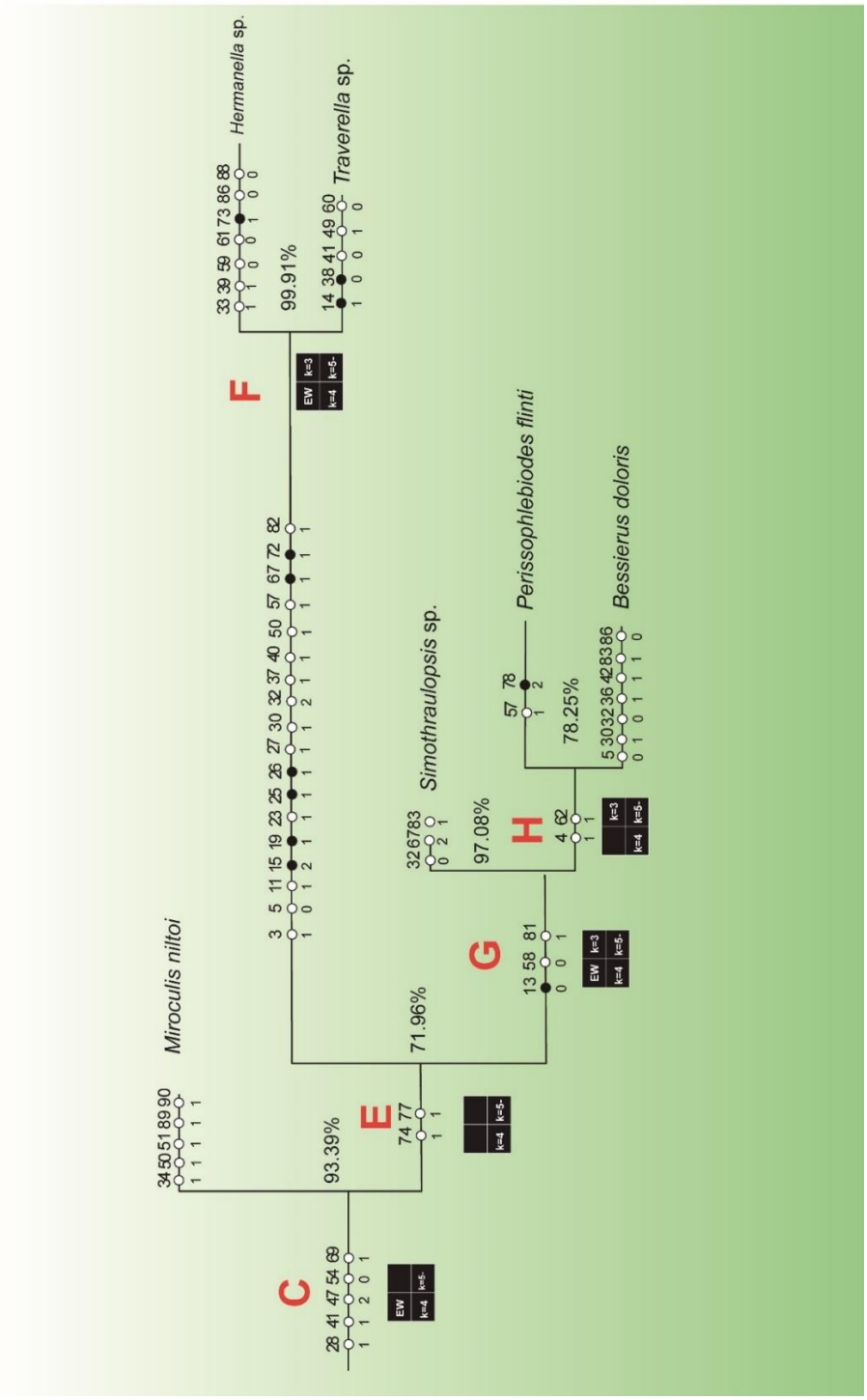


Figure 4. Part of the consensus (all compatible) tree Bayesian retrieved by Bayesian inference, posterior probability supports are assigned in each respective node. Morphological characters under slow optimization are plotted under their respective nodes. Black squares below the nodes means respective supports under parsimony schemes: equal weight EW and Implied weight, k=3; k=4 and k=5–20 (k=5-). Phylogenetic relationships among clade C.

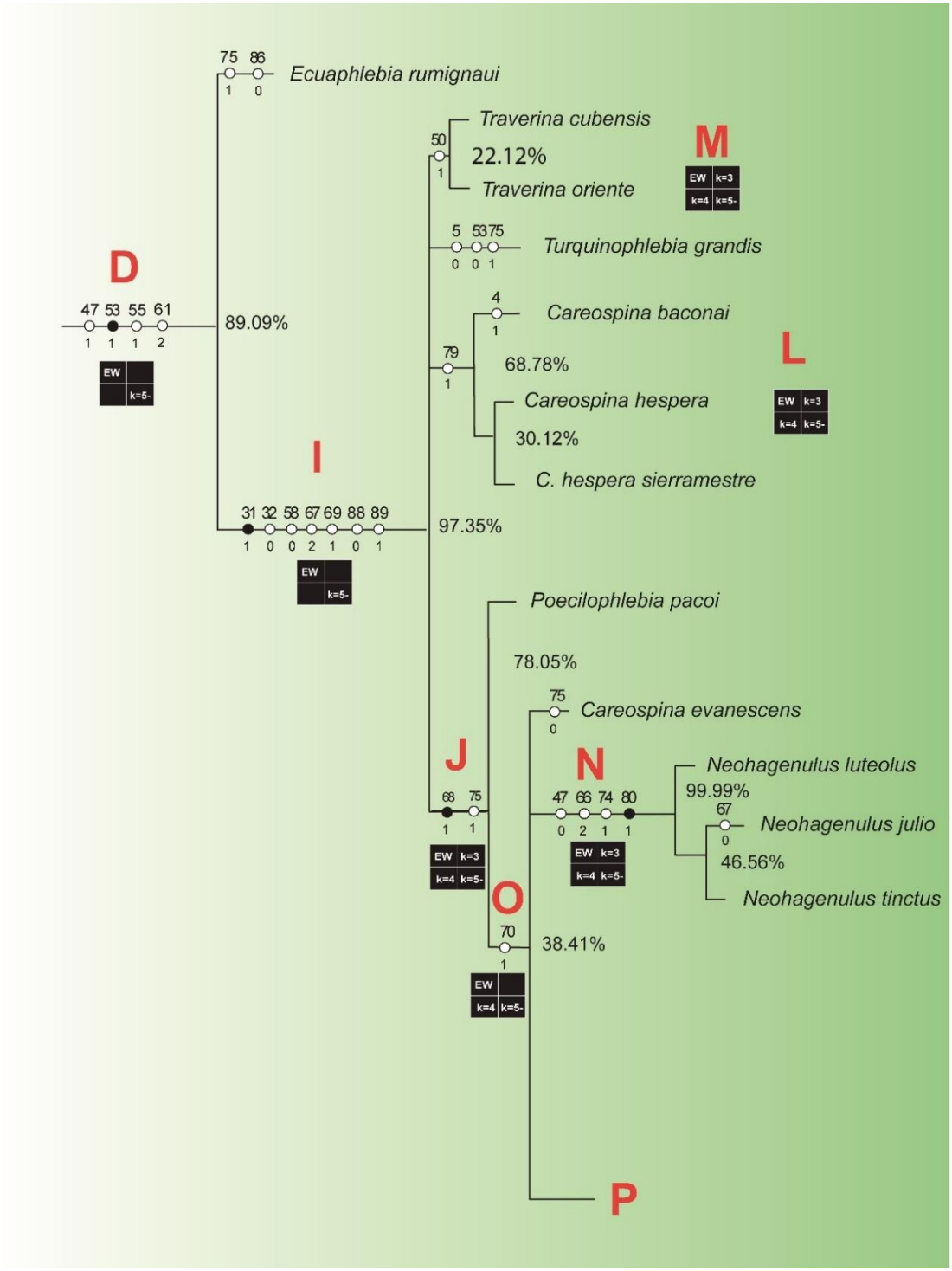


Figure 5. Part of the consensus (all compatible) tree Bayesian retrieved by Bayesian inference, posterior probability supports are assigned in each respective node. Morphological characters under slow optimization are plotted under their respective nodes. Black squares below the nodes means respective supports under parsimony schemes: equal weight EW and Implied weight, k=3; k=4 and k=5–20 (k=5-). Phylogenetic relationships among Hagenulina, Clade P is depicted in Figure 6.

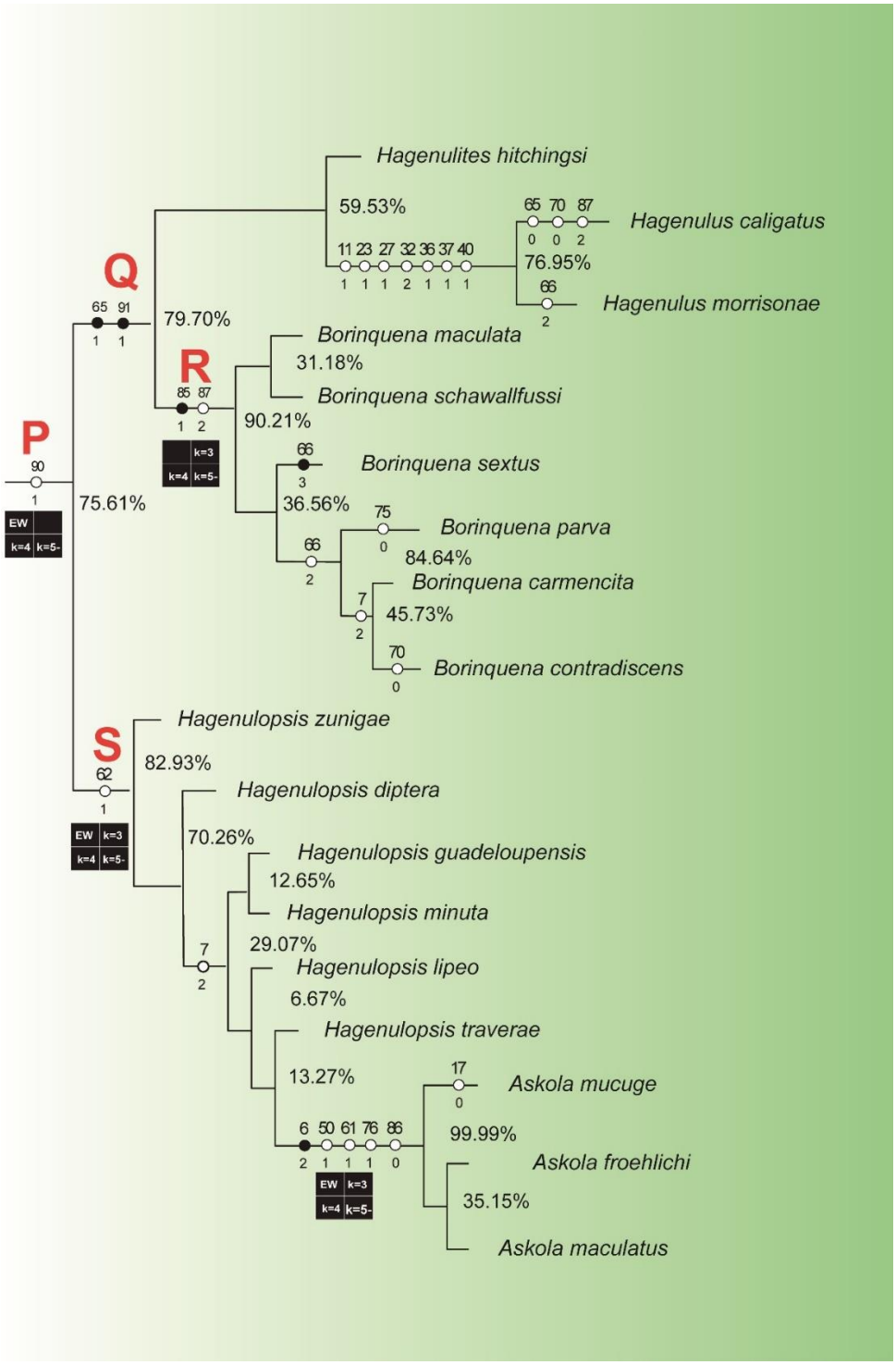


Figure 6. Part of the consensus (all compatible) tree Bayesian retrieved by Bayesian inference, posterior probability supports are assigned in each respective node. Morphological characters under slow optimization are plotted under their respective nodes. Black squares below the nodes means respective supports under parsimony schemes: equal weight EW and Implied weight, k=3; k=4 and k=5–20 (k=5-). Phylogenetic relationships among Clade P representatives.

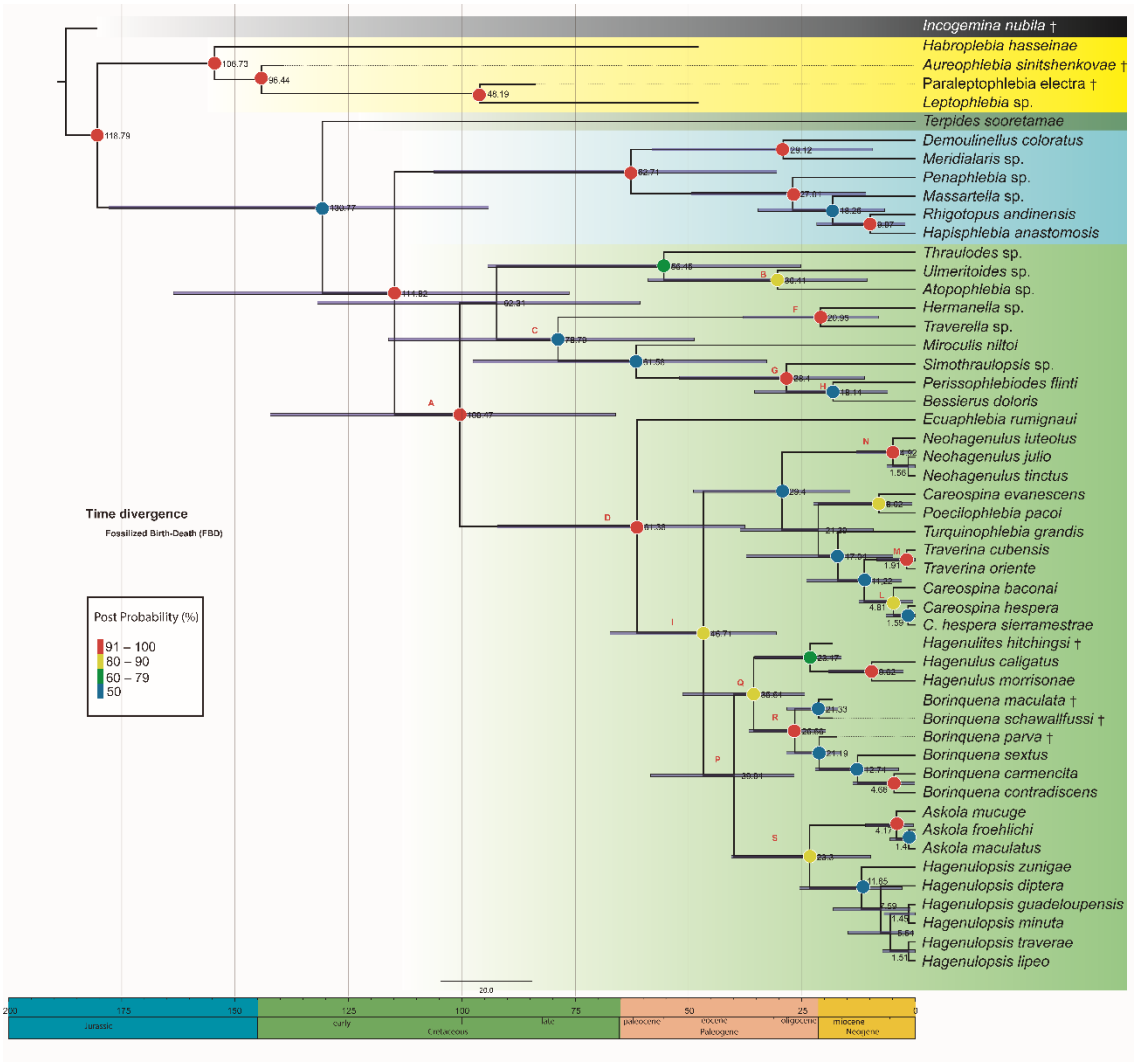


Figure 7. Chronogram estimating the divergence under Fossilized-Birth-Death model based on morphological clocks. Median ages and highest posterior density (HPD) 95% ages intervals are assigned to each respective node. Bayesian posterior probability supports are given into a gradient represented by the colored dots, nodes without colored dots retrieved posterior probability below to 50%.

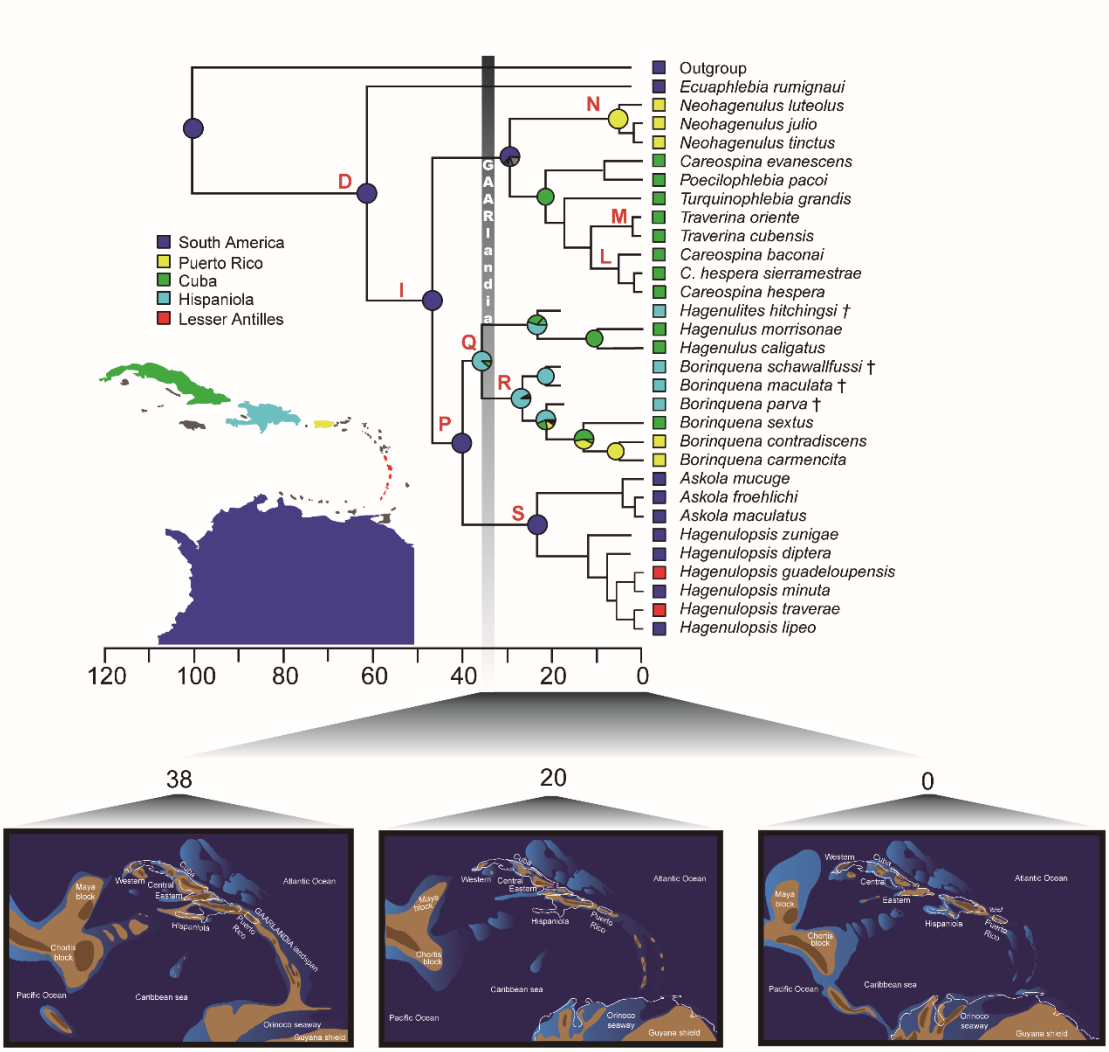


Figure 8. Maximum likelihood range estimation for Hagenulina under No-GAARsland DIVALIKE+J model. Gray scale bar crossing the chronogram indicates the timing when Antilles and South America landmasses were connected through the GAARlandia landspan availability. Below, are depicted the paleogeographic model proposed by Iturralde-Vinent & MacPhee (1999) and improvements for Iturralde-Vinent (2006).

Supplementary material 1. Hagenulina species list

Genera	Species	Distribution
Askola Peters, 1969		
	A. boiadeiro Campos, Mariano & Calor, 2019	Brazil
	A. cipoensis Domínguez, Molineri & Mariano, 2009	Brazil
	A. eduardoi Campos, Mariano & Calor, 2019	Brazil
	A. emmerichi Domínguez, Molineri & Mariano, 2009	Brazil; Colombia; Venezuela
	A. froehlichi Peters, 1969	Brazil
	A. insular Campos, Mariano & Calor, 2019	Brazil
	A. kamakan Campos, Mariano & Calor, 2019	Brazil
	A. maculatus Campos, Mariano & Calor, 2019	Brazil
	A. michelin Campos, Mariano & Calor, 2019	Brazil
	A. mucuge Campos, Mariano & Calor, 2019	Brazil
	A. paprockii Domínguez, Molineri & Mariano, 2009	Brazil
	A. yanoman Nascimento, Barcelos-Silva & Salles, 2011	Brazil
Borinquena Traver 1938		
	B. carmencita Traver	Porto Rico
	B. contradicens Traver, 1938	Porto Rico
	B. sextus Kluge, 1994	Cuba
	B. maculata † Staniczek 2003	Rep. Dominicana
	B. schawallfussi † Staniczek 2003	Rep. Dominicana
	B. parva † Staniczek 2003	Rep. Dominicana
Ecuaphlebia Domínguez 1988		
	E. rumignaui Domínguez 1988	Ecuador
Careospina Peters 1971		
	C. annulata Peters, 1971	Haiti
	C. baconai Kluge, 1994	Cuba
	C. evanescens Kluge, 1994	Cuba
	C. hespera Peters & Alayo, 1971	Cuba
	C. minuta Peters, 1971	Cuba
Hagenulopsis Ulmer 1920		
	H. diptera Ulmer, 1920	Brazil
	H. esmeralda Domínguez, Molineri & Bersoza, 2009	Ecuador; Colombia
	H. guadeloupensis Hofmann & Peters, 1999	Guadaloupe
	H. ingens Lugo-Ortiz & McCafferty, 1996	Costa Rica
	H. lipeo Domínguez, Molineri & Mariano, 2009	Argentina; Colombia
	H. traverae marginata Thomas & Boutonnet, 2004	Martinique
	H. minuta Spieth 1943	Brazil; Colombia
	H. ramosa Lugo-Ortiz & McCafferty, 1996	Costa Rica

	H. traverae Peters, 1971	Rep. Dominicana
	H. zunigae Domínguez, Molineri & Mariano, 2009	Colombia
Hagenulus *Eaton 1882*		
	H. caligatus Eaton, 1882	Cuba
	H. eatoni Banks, 1924	Haiti
	H. jamaicensis Peters & Alayo, 1971	Jamaica
	H. marshali Peters, Flowers, Hubbard, Domínguez & Savage, 2005	Ecuador
	H. morrisonae Peters & Alayo, 1971	Cuba
	H. rangelae Peters, 1971	Porto Rico
Hagenulites † Staniczek 2003		
	H. hitchingsi Staniczek 2003	
Neohagenulus Traver 1938		
	N. julio Traver, 1938	Porto Rico
	N. luteolus Traver, 1938	Porto Rico
	N. tinctus Traver, 1938	Porto Rico
Poecilophlebia Kluge, 1994		
	P. pacoi Kluge, 1994	
Traverina Peters 1921		
	T. cubensis Peters & Alayo, 1971	Cuba
	T. oriente Kluge, 1994	Cuba
Turquinophlebia Kluge, 1994		
	T. grandis Kluge, 1994	Cuba

Supplementary material 2. Character List

Character statement		
Character	**States**	**Proposers**
Nymph – Cephalic capsule		
1. Head, orientation	0. prognathous 1. hypognathous	Domínguez, 2009
2. Labrum, anteromedial emargination	0. absent 1. present	
3. Labrum, anteromedial hood	0. absent 1. present	Flowers and Domínguez 1991
4. Labrum, anteromedial emargination, shape	0. shallow 1. deep	Domínguez et al 2019
5. Labrum, anteromedial emargination, denticles	0. absent 1. present	Flowers and Domínguez 1991
6. Labrum, anteromedial emargination, number of denticles	0. 5 denticles 1. 6 denticles 2. 2 denticles	
7. Labrum, anteromedial emargination, size of denticles	0 equal or subequal 1 medial larger 2 submedial larger	Domínguez 1995 **[recoded]**
8. Labrum, first dorsal row of setae or distal row of blade-like setae	0. absent 1. present	Godunko et al. 2015
9. Labrum, second dorsal row of setae on labrum or subdistal row of simple setae	0. absent 1. present	Godunko et al. 2015
10. Labrum, third dorsal row of setae (*sensu* Godunko 2015) or median row of simple setae	0. absent 1. present	Godunko et al. 2015

11. Labrum, third dorsal row of setae or median row of simple setae, location	0 closer to distal margin 1. closer to basal margin	Domínguez, 2009; Godunko et al. 2015
12. Labrum, lateral margins	0. parallel 1. divergent	Domínguez et al 2001 **[recoded]**
13. Labrum, widest part of lateral margins	0. close to distal margin 1. in the middle	Domínguez, 1999
14. Clypeus, anteromedial projection	0. absent 1. present	Flowers and Domínguez 1991
15. Clypeus, lateral margins	0. parallel 1. divergent 2. strongly concave	Flowers and Domínguez 1991
16. Hypopharynx, lingua, apicolateral process	0. absent 1. present	Flowers and Domínguez 1991;
17. Hypopharynx, lingua, venter	0. with hairy patches 1. without hairy patches	Godunko et al. 2015
18. Hypopharynx, lobes of superlinguae, shape of flank	0. not curved 1. curved	Godunko et al 2015
19. Maxilla, apex of galea-lacinia, dentisetae,	0. present 1. absent	Kluge 1994; [phylogenetic analyzed by Godunko et al. 2015]
20. Maxilla, apex of galea-lacinia, dentisetae, number	0. one 1.two 2.three	Kluge 1994; [phylogenetic analyzed by Godunko et al. 2015]
21. Maxilla, apex of galea-lacinia, proximal dentiseta, shape	0. pectinate 1. not pectinate	Kluge 1994

		[phylogenetic analyzed by Godunko et al. 2015]
22. Maxilla, apex of galea-lacinia, setae on anterior margin	0. scattered or unevenly arranged 1. evenly arranged	Domínguez, 2009
23. Maxilla, distomedial margin, tusk	0. absent 1. present	Flowers and Domínguez, 1991
24. Maxilla, distomedial margin, tusk length	0. short 1. intermediary 2. large	Domínguez, 2009
25. Maxilla, palp, segment I, thick blunt setae on outer margin	0. absent 1. present	Domínguez, 2009
26. Maxilla, segment III of palp, ordered row of fine setae on outer margin	0. absent 1. present	Domínguez, 2009
27. Maxilla, palp, position of articulation	0. on apical 2/3 or medial 1. basal	Flowers and Domínguez, 1991
28. Labial, palp, segment III, strong setae on inner margin	0. absent 1. present	Domínguez, 2009
29. Maxilla, apical flange	0. absent 1. present	Kluge
30. Maxillae, ventral view, ventro apical comb-like row, presence	0. present 1. absent	Flowers and Domínguez, 1991
31. Maxillae, ventral view, ventro apical comb-like row, shape	0. entire 1.disjunct	Kluge, 1994 [Analyzed under phylogenetic approach for the first time in this study]
32. Mandible, outer margin, shape	0. curved 1. obtuse	Flowers and Domínguez, 1991

	2. right angled	
33. Mandible, outer margin, setae	0. absent 1. present	Flowers and Domínguez, 1991
34. Labium, glossa, ventral process	0. absent 1. present	Flowers and Domínguez, 1991
35. Labium, glossa, ventral process, orientation	0. basal 1. basolateral	Godunko
36. Labium, palp, segment I/segment II, ratio	0. sub-equal 1. 1.1 or <	Flowers and Domínguez, 1991 **[recoded]**
37. Labium, palp, segment II, long setae on outer margin	0. absent 1. present	Flowers and Domínguez, 1991 **[recoded]**
38. Labium, palp, segment II, amount of setae on outer margin	0. few, > 4 1. many	Flowers and Domínguez, 1991 **[recoded]**
39. Labium, palp, segment III/ segment II, ratio	0. menor que 0.8 1. maior que 0.8	Flowers and Domínguez, 1991 **[recoded]**
40. Labium, segment palp II, shape	0. not elbowed 1. elbowed	Flowers and Domínguez, 1991
41. Labium, paraglossa, row of subapical setae	0. absent 1. present	Flowers and Domínguez, 1991
42. Labium, submentum, setae or spine-like setae	0. absent 1. present	Flowers and Domínguez, 1991
43. Labium, submentum, anterolateral margins development	0. absent 1. present	Domínguez, 2009
Nymph – Thorax		
44. Fore tibia, patelo-tibial suture	0. absent	Kluge

	1. present	
45. Middle tibia, patelo-tibial suture	0. present 1. absent	Kluge, 1994 [phylogenetic analyzed by Godunko et al. 2015]
46. Hind tibia, patelo-tibial suture	0. present 1. absent	Kluge
Nymph – Abdomen		
47. Abdomen, posterolateral projections	0. II or IV to IX 1. V or VI to IX 2. VII or VIII to IX 3. III to VI and VIII to X	Flowers and Domínguez 1991
48. Abdomen, lateral margins, prominent setae or spine-like setae	0. absent 1. present	Domínguez 2009
49. Gills, main tracheae, long ramifications from base to apex	0. absent 1. present	Domínguez, 2009* [character statement changed]
50. Gills, apical emargination	0. absent 1. present	
Imago – Cephalic capsule		
51. Males, eyes dorsal portion on stalk	0. absent 1. present	Domínguez, 2009
52. Males, eyes, facets, shape	0. square 1. hexagonal	Peters 1980
Imago - Thorax		
53. Forewings, Radial sector, intercalary IR1-2 and IRP, connection	0. disconnected 1. connected	Monjardin et al 2020

54. Forewings, fork in vein MA originating the veins MA1 e MA2 at same time	0. absent 1. present	Flowers and Dominguez 1991 [**reinterpreted**]
55. Forewings, anterior medial sector (MA), vein MA2, shape	0. continue 1. sagged	
56. Forewings, anterior medial sector, vein MA2, attachment	0. connected at base to MA 1. free from MA	
57. Forewings, MA fork, slanting cross vein above	0. absent 1. present	Flowers and Dominguez 1991
58. Forewings, MP fork, originating the veins MP1 e MP2 at same time	0. absent 1. present	Domínguez, 2009* [**reinterpreted**]
59. Forewings, vein MP2, attachment	0. attached 1. detached	[**reinterpreted**]
60. Forewings, vein MP2, connected by cross vein in MP	0. absent 1. present	[**reinterpreted**]
61. Forewings, cubital sector, vein ICU1, attachment	0. free basally 1. attached to CuA 2. attached to CuP 3. attached to both	Flowers and Dominguez 1991
62. Hind wing, presence	0. present 1. absent	Domínguez, 2009
63. Hind wings, costal projection,	0. present 1. absent	
64. Hind wings, costal projection, shape	0. obtuse 1. acute	Flowers and Dominguez 1991 [**reinterpreted**]
65. Hind wings, cross veins, presence	0. present 1. absent	
66. Hing wing, costal projection, projection length	0. less than half	

	1. half 2. greater than half 3. as long as hind wing	
67. Hind wings, subcostal vein, extension, ending	0. in wing margin 1. in cross vein 2. costal projection 3. in wing margin, just distad to cp	Domínguez, 2009 [**reinterpreted**]
68. Hind wings, sector R, veins Rs	0. present 1. absent	
69. Hind wings, vein MP, shape	0. forked 1. unforked	Flowers and Dominguez 1991
70. Hind wing, apical third, development	0. developed 1. reduced	
71. Foreleg, pair of claws, shape	0. similar 1. dissimilar	Flowers and Dominguez 1991
Imago – Genitalia		
72. Male subgenital plate, submedial projections, paired	0. absent 1. present	Flowers and Dominguez 1991 [**reinterpreted**]
73. Male subgenital plate, paired submedial projections, shape	0. narrow 1. broad	Flowers and Dominguez 1991 [**reinterpreted**]
74. Male penis, lobes, degree of fusion	0. completely divided 1. apical 1/2 to 1/4 separated 2. fused	Flowers and Dominguez 1991
75. Male penis, subapex, spines	0. absent 1. present	Campos et al. 2019

76. Male penis, spines on subapex, orientation	0. ventrally 1. laterally	Campos et al. 2019
77. Male, telopenis	0. absent 1. present	Kluge, 2007; [phylogenetic analyzed by Salles & Boldrini, 2019]
78. Male, telopenis, orientation	0. towards base of penis 1. laterally 2. ventrally	Kluge, 2007; [phylogenetic analyzed by Salles & Boldrini, 2019]
79. Male, penes, medio-ventral ridge extends laterally	0. absent 1. present	
80. Male, penes, constriction, apex	0. absent 1. present	
81. Male, subgenital plate, sockets, disposition	0. separated 1. fused	Flowers and Dominguez 1991
82. Male, penes, base, abruptly swollen	0. absent 1. present	Flowers and Dominguez 1991
83. Male, styliger plate, posterolateral corners, development	0. not developed 1. developed 2. strongly developed	Domínguez, 2009
84. Male, styliger plate	0. deeply cleft 1. fused	Domínguez, 2009
85. Male, styliger plate, forceps I, elongation	0. not elongated 1. elongated	Staniczek, 2003
86. Male, styliger plate, forceps I, internal margin displaced inward	0. absent 1. present	

87. Male, styliger plate, forceps I, internal margin displaced inward, location	0. basal 1. medial 2. apical	
Imago – Female genitals		
88. Female, abdomen, 9th sternite, cleft	0. strongly 1. entire or shallowly	Flowers and Dominguez 1991
89. Female, abdomen, 7th sternite, egg guide	0. absent 1. present	Staniczek, 2003 Domínguez, 2009*
90. Female, abdomen, 7th sternite, egg guide, length	0. short, reaching segment VIII 1. long, reaching segment IX	Staniczek, 2003 [Analyzed under phylogenetic approach for the first time by Domínguez, 2009]
91. Female, abdomen, 7th sternite, egg guide, posterior papillae	0. absent 1. present	Kluge 1994 [Analyzed under phylogenetic approach for the first time in this study]

Supplementary material 3. Fossilized Birth death input started to run.

#NEXUS

BEGIN DATA;

DIMENSIONS NTAX=51 NCHAR=91;

FORMAT DATATYPE = STANDARD GAP = - MISSING = ? SYMBOLS = " 0 1 2 3 4 5";

MATRIX

Incogemina_nubila ??0010-00--1??????????????????????????????

Aureo_sinitshenkovae ??00-00100100-0-000?1??????????????????00??

Paralep_electra ??0000-000--?00-0-000010-10-0-000000011????

Leptophlebia 0?000??11??0-000{0 1}001000-000000?000-10-?0000100200?00?0-?00-?300-0-000000-00-0-0000000??00--

Habroplebia_hasseinae 0100100110-0-0000101110-0001000000-10-1100010020110000-00110001100001010-00-0-0000000100100

Rhigotopus_andinensis 0?001??010?0-0011100110-0000?00001?10-1000010?011101010-0111101000000000-0110-0?000101100--

Penaphlebia 01001??0110110011100110-0000100001?00-00100100??0001010-011110100?000000-0100-0?000101100--

Demoulinellus_coloratus 0100100011011011110011 0-0000100100-10-0000110?100001010-0111101000000010-10-0-00000101110--

Hapisphlebia_anastomosis 01001?001100-0011100110-0000?00001?10-1000010001 0?01?10-0111101000000000-0??0-0000010120 10?

Massartella 01001??0110110011100110-0000100001?10-1000010000000 1010-011120100?000000-0??0-00000101100--

Meridialaris 01101??0110110111100110-000010?100-00-00010100000001010-011130100?000010-20-0-00010101110--

Terpides_sooretamae 1100110011011010110111 0-0000000011?10-10011100300011011-00--101000301000-10-0-00001101000--

Hermanella 01100--011111021111--112111111-210-011110011120010100-0110-001100101011110-1000010100-00--

Traverella 01100--011111121111--112111111-200-010010001112011010-0-0111010110010101101-0-100001010??10--

Ulmeritoides 0100101011011011110011100000100100-00-00000111111101010-110-101000300010-00-0-00000101010--

Atopophlebia 0100100011011001110011-0000100100-00-00100111001101010-1111101000300110-00-0-0001010??10--

Thraulodes 01001?00110110111100110-0000100100-00-00010111000001010-01110011?300010-0--131011010??10--

Simothraulopsis 0100100011010001110011-0001100000-?0-0010011120000100-000--101100201010-10-100010110{0 1}{0 1}10--

Miroculis_niltoi 01001??0110110011100110-0001100101?00-0010011120011100-00111101100301010-00-0-0000010111110

Perissophlebiodes_flinti 01011?00110100011100110-0001???100-?0-0010011120000100-010--111-------10-10-12001001011????

Bessierus_doloris 01010--0110100011100110-0001?1-000-10-0011011?20000100-000--111-------1?-10-1000101100-????

Ecuaphlebia_rumignaui 010010?0110110011100110-0000100100-00-00000111100001111-0111201100300010-01?0-00000100-????

Borinquena_carmencita__ 01001{0 1}20110110011100110-000?10?0?0-00-00??0111100001111-00--201112211110-0100-0000011120111

Borinquena_contradiscens 01001{0 1}20110110011100110-000????0?0-00-????0111100001111-00--201112211010-0100-0000011120111

Borinquena_sextus 01001{0 1}00110110011100110-000??010?0-00-00??0111100001111-00--2011?3211110-0100-0000011120111

Borinquena_maculata__ ??01111-00--201110211110-?100-?0?001112????

Borinquena_parva__ ??01111-00--20111221?110-?0-0-?0?001112????

Borinquena_schawallfussi__ ??01111-00--201110211110-?100-?0?001112????

Hagenulites_hitchingsi___ ??01111-00--201110211?10-?100-?0?001011????

Careospina_hespera__ 0100100011011001110011O-000??010?0-00-00??0111100001111-00--201100201010-00-0-1000010110100

Careospina_baconai 0101100011011001110011O-000??010?0-00-00??0111100001?11-00--201100201010-00-0-1000010110100

Careospina_evanescens 0100100011011001110011O-000??010?0-00-00??0111100001111-00--201100211110-00-0-0000010110100

Careospina_hespera_sierramestrae 0100100011011001110011O-000??010?0-00-00??0111100001????????2011002?1010-00-0-1000010110100

Hagenulopsis_diptera__ ??01111-00--21?-------10-0100-0000010110110

Hagenulopsis_guadeloupensis_ 0100102011011001110011O-0000?010?0-00-00??0111100001111-00--21?-------10-0100-0000010110110

Hagenulopsis_minuta 0100102011011001110011O-0000?010?0-00-00??0111100001111-00--21?-------10-0100-0000010110110

Hagenulopsis_traverae 01001?2011011001110011O-0000???0?0-00-00??0111100001111-00--21?-------10-0100-0000010110110

Hagenulopsis_lipeo_ 01001?2011011001110011O-0000???0?0-?0-????0111100001111-00--21?-------10-0100-000001011????

Hagenulopsis_zunigae 01001?0011011001110011O-0000???0?0-?0-????0111100001111-00--21?-------10-0100-00000101??110

Hagenulus_caligatus__ 01001?0011111001110011120012??012?0-11101??0111100?01?11-00--201100211010-0100-0000010120111

Hagenulus_morrisonae 01001?0011111001110011120012??012?0-11101??0111100001?11-00--201112211110-0100-000001011?111

Neohagenulus_julio__ 0100100011011001110011O-000??010?0-00-00??01110?0001111-00--2011020?1110-1100-0100010110100

Neohagenulus_luteolus 0100100011011001110011O-000????0?0-00-00??01110?0001111-00--2011022?1110-1100-0100010?10100

Neohagenulus_tinctus 0100100011011001110011O-000????0?0-00-00??01110?0001111-00--20110???1110-1100-0100010110100

Poecilophlebia_pacoi__ 0100100011011001110011O-000??010?0-00-00??0111100001111-00--201100211010-0100-?00001011?100

Traverina_cubensis__ 0100100011011001110011O-000??010?0-00-00??0111100101111-00--201100201010-00-0-0000010110100

Traverina_oriente 0100100011011001110011O-000??010?0-00-00??0111100101?11-00--201100201010-00-0-000001011?100

Turquinophlebia_grandis__ 01000--0110110011100110-000??010?0-00-00??0111??0001011-00--201100201010-0100-?00001011?100

Askola_froehlichi__ 0100122011011001110011O-000010?0?0-00-00??0111100101111-00--11?-------10-0110-00000100-0110

Askola_maculatus 0100122011011001110011O-0000?0?0?0-00-00??0111100101111-00--11?-------10-0110-00000100-0110

Askola_mucuge 010012201101100101O?110-0000?0?0?0-00-00???111100101111-00--11?-------10-0110-00000100-0110

;

END;

begin mrbayes;

set autoclose=yes nowarn=yes;

[currently specified groups if you wish to use these]

charset Particao_1 = 1- 2 9 14 18-21\3 22 24 35-38\3 44 56 73 78;

charset Particao_3 = 3- 4 7-10\3 11- 12 17 27- 28 30-33\3 37 39 43 48-51\3 53-55 60 62- 65 71 76- 77 79 81 90;

charset Particao_5 = 5 15 36 41- 42 49 58 67 69- 70 82 86-89\3;

charset Particao_2 = 6 8 13-19\3 20 25- 26 29 31 45- 46 52 68 72 80 84- 85 91;

charset Particao_6 = 32 47 74 87;

charset Particao_4 = 23 34 40 57 59 66 83 88;

charset Particao_8 = 50;

charset Particao_7 = 61 75;

partition currentPartition = 8: Particao_1, Particao_3, Particao_5, Particao_2, Particao_6, Particao_4, Particao_8, Particao_7;

set partition = currentPartition;

lset applyto=(1, 2, 3, 4, 5, 6, 7, 8);

lset nst=1;

prset ratepr=variable;

unlink igrvar = (all);

```
outgroup Incogemina_nubila;

[relaxed clock model]
prset clockvarpr = igr;
prset clockratepr = gamma(1, 1);
prset igrvarpr = exp(10);

[tip dating]
calibrate
    Incogemina_nubila = unif(113.0, 125.0)
    Paralep_electra = unif(33.9, 37.8)
    Aureo_sinitshenkovae = unif(89.8, 93.9)
    Borinquena_maculata__ = unif(15.9, 20.4)
    Borinquena_parva__ = unif(15.9, 20.4)
    Borinquena_schawallfussi__ = unif(15.9, 20.4)
    Hagenulites_hitchingsi___ = unif(15.9, 20.4)
;
prset nodeagepr = calibrated;

[Topology constraints]
constraint Leptophlebiinae = Aureo_sinitshenkovae Leptophlebia
Paralep_electra;
constraint Atalophlebolinguata = Rhigotopus_andinensis Penaphlebia
Demoulinellus_coloratus Hapisphlebia_anastomosis Massartella Meridialaris
Hermanella Traverella Ulmeritoides Atopophlebia Thraulodes Simothraulopsis
Miroculis_niltoi Perissophlebiodes_flinti Bessierus_doloris Ecuaphlebia_rumignaui
Borinquena_carmencita__ Borinquena_contradiscens Borinquena_sextus
Borinquena_maculata__ Borinquena_parva__ Borinquena_schawallfussi__
Hagenulites_hitchingsi___ Careospina_hespera__ Careospina_baconai
Careospina_baconai Careospina_evanescens Careospina_hespera_sierramestrae
Hagenulopsis_diptera__ Hagenulopsis_guadeloupensis_ Hagenulopsis_minuta
Hagenulopsis_traverae Hagenulopsis_lipeo_ Hagenulopsis_zunigae
Hagenulus_caligatus__ Hagenulus_morrisonae Neohagenulus_julio__
Neohagenulus_luteolus Neohagenulus_tinctus Poecilophlebia_pacoi__
```

Traverina_cubensis__ Traverina_oriente Turquinophlebia_grandis__ Askola_froehlichi__ Askola_maculatus Askola_mucuge;

constraint Hagenulinae = Hermanella Traverella Ulmeritoides Atopophlebia Thraulodes Simothraulopsis Miroculis_niltoi Perissophlebiodes_flinti Bessierus_doloris Ecuaphlebia_rumignaui Borinquena_carmencita__ Borinquena_contradiscens Borinquena_sextus Borinquena_maculata__ Borinquena_parva__ Borinquena_schawallfussi__ Hagenulites_hitchingsi___ Careospina_hespera__ Careospina_baconai Careospina_baconai Careospina_evanescens Careospina_hespera_sierramestrae Hagenulopsis_diptera__ Hagenulopsis_guadeloupensis_ Hagenulopsis_minuta Hagenulopsis_traverae Hagenulopsis_lipeo_ Hagenulopsis_zunigae Hagenulus_caligatus__ Hagenulus_morrisonae Neohagenulus_julio__ Neohagenulus_luteolus Neohagenulus_tinctus Poecilophlebia_pacoi__ Traverina_cubensis__ Traverina_oriente Turquinophlebia_grandis__ Askola_froehlichi__ Askola_maculatus Askola_mucuge;

constraint Hagenulina = Ecuaphlebia_rumignaui Borinquena_carmencita__ Borinquena_contradiscens Borinquena_sextus Borinquena_maculata__ Borinquena_parva__ Borinquena_schawallfussi__ Hagenulites_hitchingsi___ Careospina_hespera__ Careospina_baconai Careospina_baconai Careospina_evanescens Careospina_hespera_sierramestrae Hagenulopsis_diptera__ Hagenulopsis_guadeloupensis_ Hagenulopsis_minuta Hagenulopsis_traverae Hagenulopsis_lipeo_ Hagenulopsis_zunigae Hagenulus_caligatus__ Hagenulus_morrisonae Neohagenulus_julio__ Neohagenulus_luteolus Neohagenulus_tinctus Poecilophlebia_pacoi__ Traverina_cubensis__ Traverina_oriente Turquinophlebia_grandis__ Askola_froehlichi__ Askola_maculatus Askola_mucuge;

prset topologypr = constraint(Leptophlebiinae, Atalophlebolinguata, Hagenulinae, Hagenulina);

[fossilized birth-death prior]

prset brlenspr = clock:fossilization;

prset samplestrat = fossiltip; [r=1]

[prset samplestrat = randon;] [r = 0 sample ancestors]

prset sampleprob = 0.5909; [26/44]

prset speciationpr = exp(100);

prset extinctionpr = beta(1, 1);

prset fossilizationpr = beta(1,1);

prset treeagepr = offsetexp(113.0, 125.0);

```
        mcmcp ngen= 200000000 relburnin=yes burninfrac=0.25 printfreq=1000
samplefreq=1000 nchains=4 savebrlens=yes;

        mcmc;

        sump relburnin=yes burninfrac=0.25;

        sumt relburnin=yes burninfrac=0.25 contype=allcompat;

end;
```

Supplementary material 4. Dispersal probabilities: connected areas, 1; connected by a landspan 0.9; separated by adjacent landmasses, 0.8; separated by an intermediary landmass 0.7; barrier, long dispersal, 0.5; effective barrier, 0.1; long distance barrier, 0.01; area not yet emergent, 1.00E-07. SA: South America; HI: Hispaniola; CU: Cuba; PR: Puerto Rico; LA: Lesser Antilles.

Time slices

GAARlandia

80-40

	SA	HI	CU	PR	LA
SA	1	1,00E-07	1,00E-07	1,00E-07	1,00E-07
HI	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07
CU	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07
PR	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07
LA	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07

40-35

	SA	HI	CU	PR	LA
SA	1	0,01	0,01	0,01	1,00E-07
HI	0,01	1	0,1	0,1	1,00E-07
CU	0,01	0,1	1	0,1	1,00E-07
PR	0,01	1	0,1	1	1,00E-07
LA	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07

35-30 - Graalandia

	SA	HI	CU	PR	LA
SA	1	0,9	0,9	0,9	1,00E-07
HI	0,9	1	1	1	1,00E-07
CU	0,9	1	1	1	1,00E-07

No GAARlandia

80-40

	SA	HI	CU	PR	LA
SA	1	1,00E-07	1,00E-07	1,00E-07	1,00E-07
HI	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07
CU	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07
PR	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07
LA	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07

40-35

	SA	HI	CU	PR	LA
SA	1	0,5	0,5	0,5	1,00E-07
HI	0,5	1	0,8	0,8	1,00E-07
CU	0,5	0,8	1	0,7	1,00E-07
PR	0,5	0,8	0,7	1	1,00E-07
LA	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07

35-23

	SA	HI	CU	PR	LA

	PR	0,9	1	1	1	1,00E-07
	LA	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07

	SA	1	0,5	0,5	0,5	1,00E-07
	HI	0,5	1	1	1	1,00E-07
	CU	0,5	1	1	0,7	1,00E-07
	PR	0,5	1	0,7	1	1,00E-07
	LA	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07

30-23		SA	HI	CU	PR	LA
	SA	1	0,01	0,01	0,01	1,00E-07
	HI	0,01	1	1	1	1,00E-07
	CU	0,01	1	1	0,1	1,00E-07
	PR	0,01	1	0,1	1	1,00E-07
	LA	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07

23-20		SA	HI	CU	PR	LA
	SA	1	0,01	0,01	0,01	1,00E-07
	HI	0,01	1	1	0,1	1,00E-07
	CU	0,01	1	1	0,1	1,00E-07
	PR	0,01	0,1	0,1	1	1,00E-07
	LA	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07

	SA	1	0,5	0,5	0,5	1,00E-07
	HI	0,5	1	1	0,8	1,00E-07
	CU	0,5	1	1	0,8	1,00E-07
	PR	0,5	0,8	0,8	1	1,00E-07
	LA	1,00E-07	1,00E-07	1,00E-07	1,00E-07	1,00E-07

20-15		SA	HI	CU	PR	LA
	SA	1	0,01	0,01	0,01	0,01
	HI	0,01	1	0,1	0,1	0,01
	CU	0,01	0,1	1	0,1	0,01
	PR	0,01	0,1	0,1	1	0,01
	LA	0,1	0,01	0,01	0,01	1

	SA	1	0,5	0,5	0,5	0,7
	HI	0,5	1	1	0,8	0,5
	CU	0,5	1	1	0,7	0,5
	PR	0,5	0,8	0,7	1	0,5
	LA	0,7	0,5	0,5	0,5	1

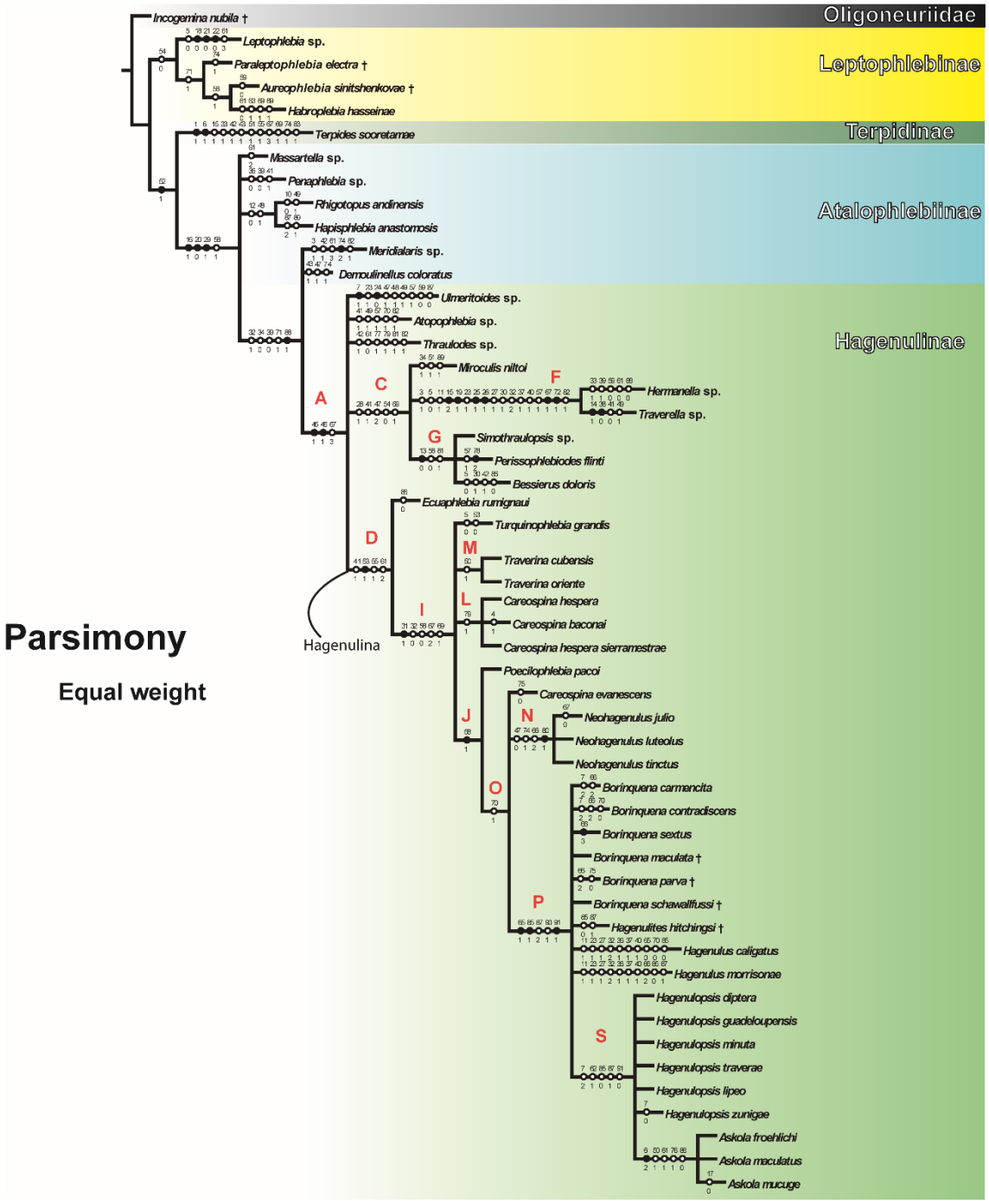
15-0 (2)		SA	HI	CU	PR	LA
	SA	1	0,01	0,01	0,01	0,01
	HI	0,01	1	0,1	0,1	0,01
	CU	0,01	0,1	1	0,1	0,01

	SA	1	0,5	0,5	0,5	0,7
	HI	0,5	1	0,8	0,8	0,5
	CU	0,5	0,8	1	0,7	0,5

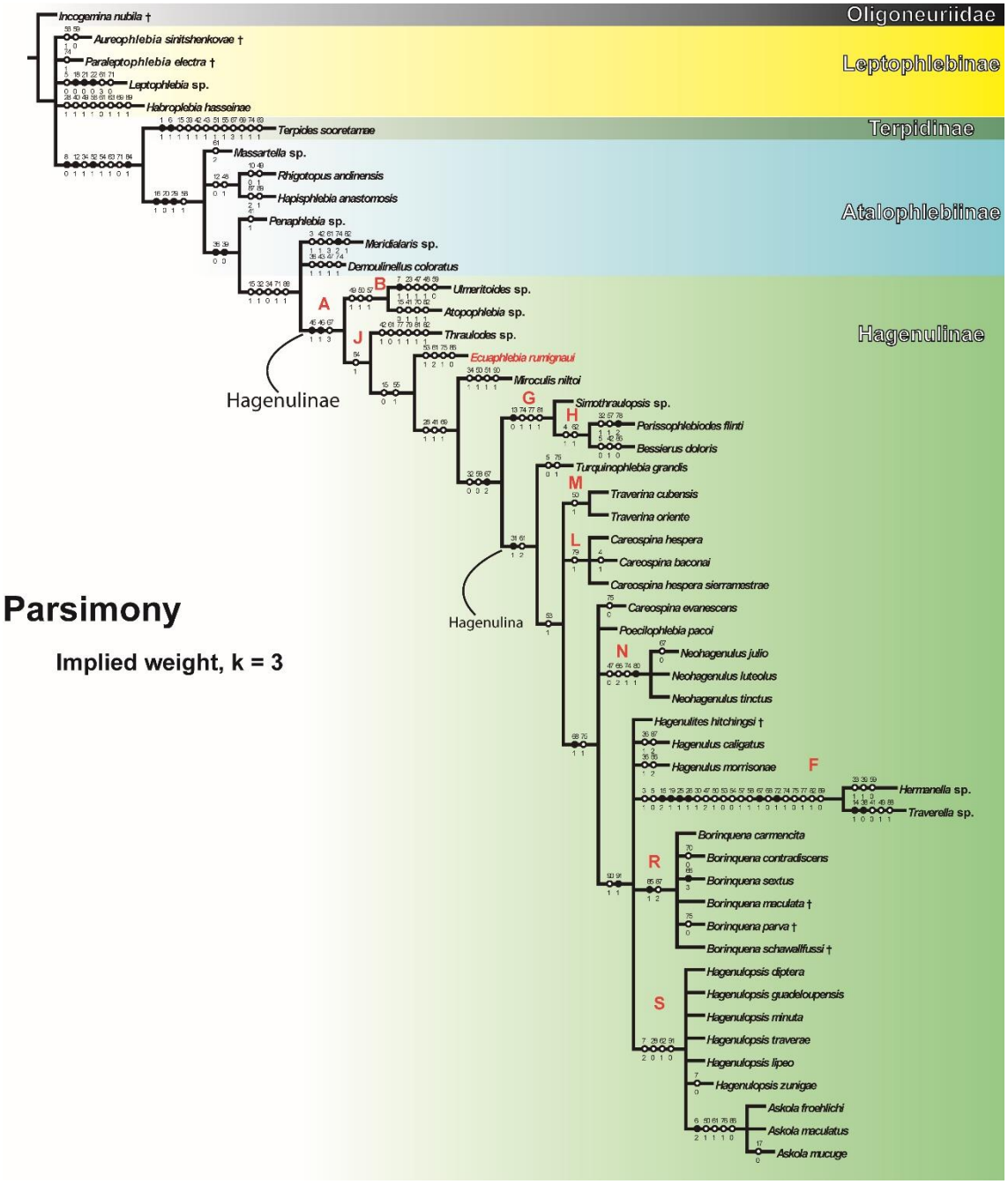
PR	0,01	0,1	0,1	1	0,01	PR	0,5	0,8	0,7	1	0,5
LA	0,1	0,01	0,01	0,01	1	LA	0,7	0,5	0,5	0,5	1

Supplementary material 5. Statistics summary from consensus tree yielded by Parsimony searches. Maximum Parsimonious tree (MTPs), Length (L), Consistence index (CI) and Retention index (RI).

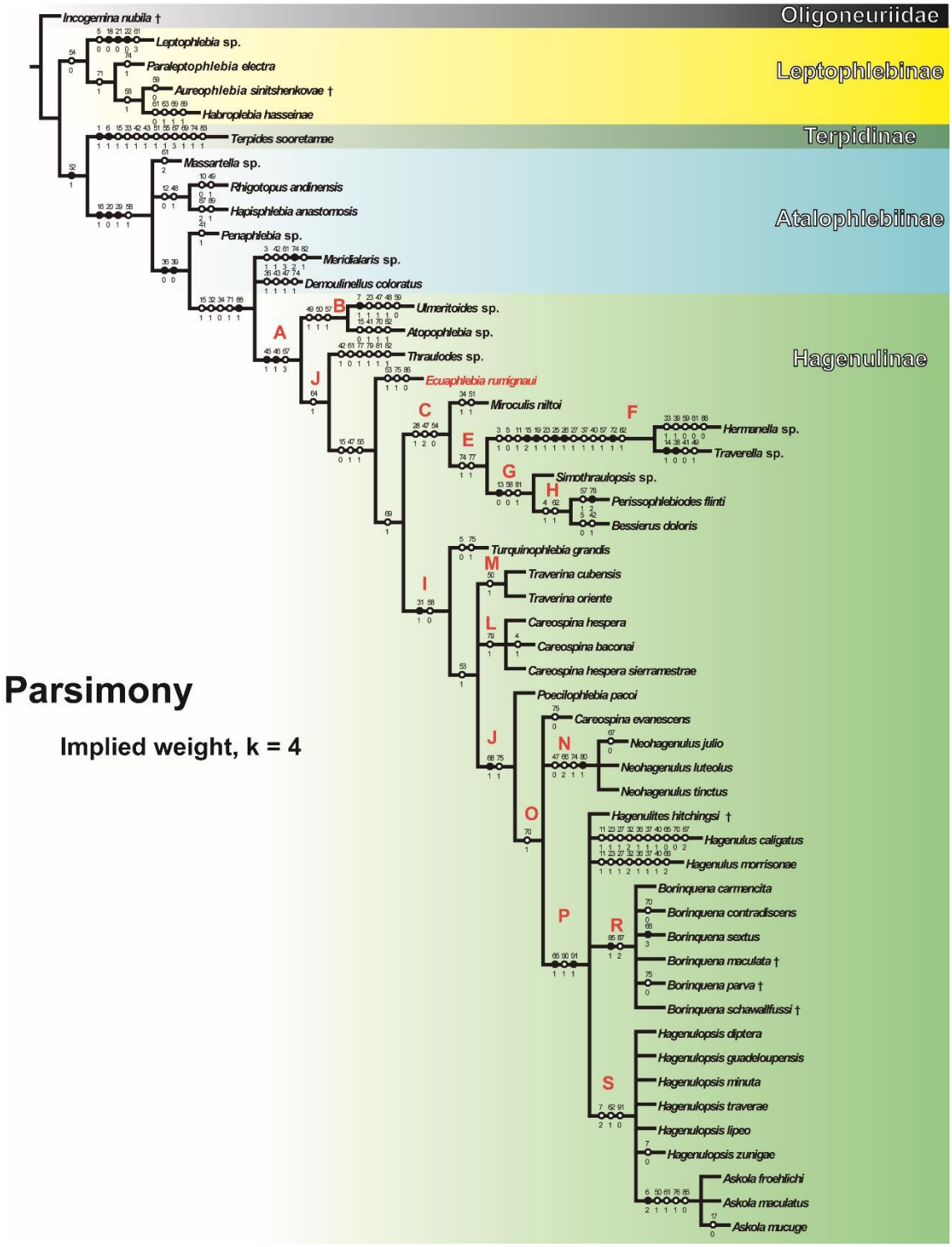
Tree scheme	MTPs	L	CI	RI
Equal weight	6750 MPTs	248 steps	40	66
IW, k=3	240 MPTs	240 steps	40	66
IW, k=4	66 MPTs	230 steps	43	70
IW, k=5–20	594 MPTs	231 steps	43	70



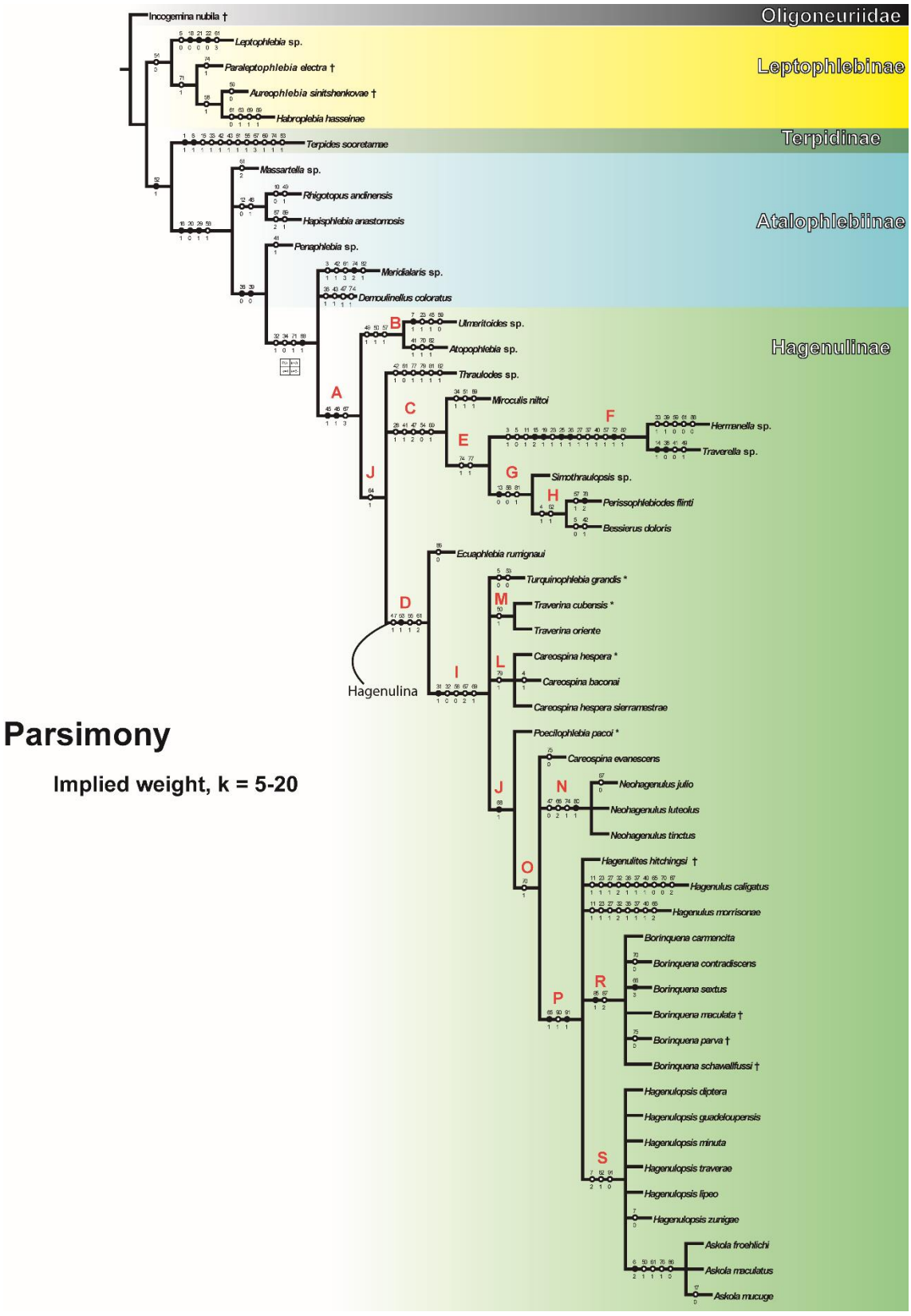
Supplementary material 6. Figure 1A. Consensus tree yielded from 6750 maximum parsimonious tree under equal weight analysis



Supplementary material 6. Figure 1B. Consensus tree yielded from 240 maximum parsimonious tree under Implied Weight analysis, k=3



Supplementary material 6. Figure 1C. Consensus tree yielded from 66 maximum parsimonious tree under Implied Weight analysis, k=4



Supplementary material 6. Figure 1D. Consensus tree yielded from 594 maximum parsimonious tree under Implied Weight analysis, k=5–20. Boxes below the nodes are representing clade consensus among all parsimony analyses.

CHAPTER 2

TITLE:

HAGENULOPSIS ULMER (EPHEMEROPTERA: LEPTOPHLEBIIDAE): RE-DESCRIPTION, MORPHOLOGICAL NOTES AND NEW SPECIES FROM SOUTH AMERICA, BRAZIL

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Abstract

Hagenulopsis diptera Ulmer, type species of the genus *Hagenulopsis*, was originally described based on imagos from Santa Catarina State, Southern Brazil. Misconceptions of *H. diptera* circumscription led to erroneous attribution of material from Minas Gerais and Espírito Santo, Southeastern Brazil, to *H. diptera*. Despite the increase in the number of species attributed to *Hagenulopsis*, little attention has been given to the type species. After comparative examination of photographs of the holotype and fresh

material of *H. diptera* from Southeastern Brazil, we conclude that many specimens previously assigned to *H. diptera* represent a new species. Thus, we redescribe *H. diptera* and describe a new species *Hagenulopsis perere* sp. nov. based on nymphs and imagos. Diagnostic features of *Hagenulopsis perere* sp. nov. include cross veins between C and RP_1 strongly clouded with brown and outer surface of mid femur with a brown spot at midlength. Finally, comments and new records are presented for *Hagenulopsis minuta* Spieth.

Key words. Hagenulinae, Hagenulina, diversity, taxonomy, Neotropical.

Introduction

The genus *Hagenulopsis* Ulmer, 1920 was described about a century ago for a new species of Neotropical Leptophlebiidae (Ephemeroptera) with unique characteristics: the absence of hind wings and the presence of an egg guide (Ulmer 1920). The type species *Hagenulopsis diptera* Ulmer, 1920 was described based on male and female specimens collected at Humboldt District (now known as municipality of Corupá), Santa Catarina, Southern Brazil. A second species, *H. minuta* Spieth, 1943 was described based on a female imago from Surinam (Spieth 1943). Traver (1944) described the nymph of the genus for the first time based on two undetermined specimens from Minas Gerais, Southeastern Brazil.

The distribution of the genus was expanded to the Antilles and to Central America based on additional new species from Dominica (Peters 1971), Costa Rica and Nicaragua (Lugo-Ortiz & McCafferty 1996), and Guadeloupe (Hoffman *et al*. 1999). Peters & Domínguez (2001) described the nymph of *H. minuta* based on reared material and provided the male description. In addition, they also transferred the Dominican species *Borinquena* (*Australophlebia*) *traverae* Peters, 1971 to *Hagenulopsis*, and stated that the

nymphs described by Traver (1944) from Minas Gerais State were probably those of *H. diptera* based on their distribution in Southern Brazil and to the clouded cross veins in the fore wing pads. More recently, Domínguez *et al*. (2009) described three new species from South America (Argentina, Bolivia, Ecuador, and Colombia). Currently, *Hagenulopsis* ranges from Central to South America and presently comprises nine species.

Despite the gradual description of new species, and the improvements on the taxonomy of some of them, little is known regarding the type species. Imagos were never redescribed or illustrated following the original publication (Ulmer 1920), and the clouded cross veins in wing pads used by Peters & Domínguez (2001) to suppose that the nymphs described by Traver (1944) were those of *H. diptera* is a misunderstanding on the circumscription of the species; this was followed by Salles *et al*. (2010) who reported this species from Espírito Santo State. After examining fresh material from Santa Catarina, photographs of the type material of *H. diptera*, and dozens of adults and nymphs from Minas Gerais and Espírito Santo, we conclude that the species with clouded cross veins represents a new species. The aim of this paper, therefore, is to describe this new species, to redescribe *H. diptera*, and to provide additional comments and records for *H. minuta*.

Material and methods

The specimens collected in this study were sampled in several sites across Brazil (Fig. 1). The nymphs were captured using an aquatic net and reared, whenever possible, in order to obtain the adult stage. Winged specimens were caught up using Malaise traps, light attraction at dusk (Calor & Mariano 2012) and entomological nets. All material collected was preserved in 80–100% ethanol. Wings, legs and male genitalia from

imagos were dissected and mounted on slides using a dry technique for the wings and Euparal® for genitalia and legs.

Morphological terminology used in descriptions follows Kluge (1994) for sutures on thorax. Photographs of type specimens were provided by the Museum of Hamburg where the holotype is deposited; the remaining photographs were taken using Leica M205A stereomicroscope with subsequent improvements made on the software Adobe Photoshop®. Drawings sketches were made using a camera lucida and then vectorized on Adobe Illustrator®. For SEM analyses of eggs, dissected parts of a female abdomen containing eggs were dehydrated in a graded ethanol series, dried by the critical point-method and then mounted on stubs, sputter-coated with gold, and finally observed with a JSM 6610 LV scanning electron microscope. We use the terminology proposed by Koss (1968) and Koss & Edmunds (1974) in the egg descriptions complemented by descriptions published in the literature (Campos *et al*. 2019).

Analyzed specimens include female imagos (♀), female subimagos (s♀), male imagos (♂), male subimagos (s♂), and nymphs (N). Biological collections acronyms are used as follows: Instituto Nacional de Pesquisas da Amazônia (INPA), Museu de Zoologia, Universidade de São Paulo (MZUSP), Museu de Entomologia Universidade Federal de Viçosa, Minas Gerais (UFVB), Museu de História Natural da Bahia (MNHBA), Museu de Zoologia, Universidade Estadual de Santa Cruz (MZUESC), Coleção do Laboratório de Biologia Aquática, Universidade Estadual Paulista Júlio de Mesquita Filho, Assis-São Paulo (CLBA), Coleção Entomológica Heróis do Jenipapo, Universidade Estadual do Piauí (CEHJ).

Taxonomy

Hagenulopsis perere sp. nov. Campos, Costa & Salles

(Figs 2A–D, 3A–J, 4G, 5F, 6A–H, 7A–D)

Diagnosis. *Hagenulopsis perere* can be separated from its congeners by the following combination of characters. Nymph: (i), outer surface of fore and mid femur with a brown spot nearly at apex; (ii), 14 denticles on fore claw. Male Imago: (i), general coloration dark brown (Figs 3A, B); (ii), dorsal portion of eyes meeting dorsally on head; (iii), 21–33 facets on the longest row; (iv), outer surface of mid femur with a brown spot at midlength; (v), cross veins between C and RP_1 clouded with brown. Female imago: (i), general coloration dark brown; (ii), cross veins between C and RP_1 clouded with brown; (iii), egg guide dark brown, apically acute.

Male imago. Body length: 7.0–7.8 mm (n = 7). General coloration: dark brown (Fig 2A–D).

Head: orange brown (Fig 3A–B). Upper portion of compound eye orange, area surrounding facets dark orange, lower portion black. Eyes meeting dorsally on head and with around 21–33 facets on the longest row. Ocelli white surrounded with orange, lateral ocelli larger than the median ocellus (Fig 3A). Antenna: scape and pedicel brown washed black, flagellum pale brown.

Thorax (Fig 3B): pronotum brown, dark brown submedially and on lateral margins. Mesonotum brown, median longitudinal and medioparapsidal sutures brown, lateroparapsidal suture and anterolateral corner of scutellum pale. Metanotum brown, dark brown on posterior margin. Pleural sclerites brown, membranous area washed with gray (Fig 3A). Sterna brown. Legs yellowish-brown (Fig 3F–I). Foreleg (Fig 3F): coxa and trochanter brown; femur yellowish-brown, washed with brown on basal half and with a brown band at midlength; tibia light yellow, dark brown on apex; tarsi light

yellow, dark brown on apex of each tarsomere. Mid leg (Fig 3G): coxa and trochanter brown; femur yellowish-brown with outer surface with a brown spot at midlength and an apical brown band; tarsi yellowish-brown. Hind leg (Fig 3H): yellowish-brown; femur with median and apical brown bands. Claws yellowish-brown (Fig 3I), both similar.

Wings (Fig 4G–J): membrane hyaline, longitudinal and cross veins yellowish tinged with brown, area between costal brace dark brown; cross veins between C and RP_1 clouded with brown, clouds more extensive between Sc and RP_1; six cross veins basal to bulla, 14 distal to bulla.

Abdomen (Fig 3C–D): terga hyaline gray washed with brown. Tergum I entirely washed with brown; terga II–IX with a hyaline longitudinal stripe; terga VII, VIII and IX with hyaline anteromedial spot; tergum X with a hyaline median oblong mark. Sterna translucent gray washed with brown. Sterna II–VIII with hyaline anteromedial mark.

Genitalia (Figs 3E, 5F). Styliger plate brown and quadrangular; forceps segment I brown, curved medially; forceps segment II brown washed with white; forceps segment III white; penes pale, acuminating towards apex and not covered by styliger plate; spine ventrally oriented on subapex of each penis lobe. Caudal filaments white with black annulations basally and on apex of each annulation.

Male subimago (Fig 2B). General coloration brown, similar to male imagos. Wings opaque, covered by microtrichia; longitudinal veins yellowish opaque tinged with brown, cross veins dark brown; cross veins between C and RP_1 clouded.

Female imago (Fig 3J–K). General coloration: dark brown (n = 5).

Head: Brown, lighter brown around eyes and medially. Eyes black. Thorax: brown, pleural sclerites brown with membranes whitish. Sterna brown. Wings: membrane

hyaline, longitudinal and cross veins yellowish-brown, costal brace dark brown; cross veins between longitudinal veins C and RP_1 clouded with brown, clouds more extensive on cross veins between Sc and RP_1; 4–5 cross veins basal to bulla, 15–16 distal to bulla. Abdomen: dark brown with light brown lines U shaped, terga IX similar to male; egg guide dark brown, apically acute (Fig 3J–K). Caudal filaments white with black annulations on basal fourth and apex of each annulation.

Female subimago (Fig 2C) General coloration brown, similar to female imago. Wings opaque, covered by microtrichia; longitudinal veins yellowish opaque tinged with brown, cross veins dark brown; cross veins between C and RP_1 clouded. Egg guide brown, whitish brown toward apex.

Nymph (Figs 6A–F, 7A–F). Body length: 5.3–5.7 mm (n = 3). General coloration (Fig 6A–B): yellowish-brown.

Head: yellowish-brown, frons and vertex washed with black, and with vertical black stripes. Eyes black. Ocelli white surrounded with black. Antenna broken off and lost. Labrum (Fig 7A) almost as wide as clypeus; anteromedial emargination with 5 denticles, submedial denticles larger. Mandible (Fig 7E) yellowish-brown, dark brown on incisors and molar; outer margin with 9–10 filiform long setae. Hypopharynx translucent yellow (Fig 7D). Maxilla (Fig 7B–C) yellowish-brown, washed with black on base of stipe; maxillary palp, segment I (0.18–0.24 mm), segment II (0.27–0.30 mm), segment III (0.14–0.15 mm). Labium (Fig 7F) translucent yellow, postmentum washed with black; labial palp translucent yellow, segment I (0.25–0.29 mm) covered by strong setae, segment II (0.27–0.34 mm) covered by filiform setae, and segment III (0.12–0.15 mm) with dorsal spine-like setae.

Thorax: pronotum yellowish-brown with middle and lateral margins washed with black. Mesothorax yellowish-brown, washed with black on lateral margins. Fore wing pad

yellowish-brown, with clouded cross veins on mature nymphs (Fig 6B). Legs yellowish-brown (Fig 6C–F); outer surface of fore (Fig 6E) and mid (Fig 6D) femora with a brown spot nearly at apex; mid femur washed brown at midlength; hind femur with two dark brown bands (Fig 6C). Claws yellowish-brown; about 14 denticles progressively larger toward apex (Fig 6F).

Abdomen (Fig 6A): yellowish-brown washed with black, darker on lateral margins; posterolateral spines on segments V–IX progressively larger posteriorly (Fig 6A). Gills translucent gray, tracheae black. Caudal filaments yellowish-brown.

Eggs (Figs 8A–D). Length 161–9 µm (n = 5). Elliptic in shape (Fig 8A). Chorionic surface perforated; sucker-like discs irregularly distributed on surface, each located in a small concavity (Fig 8B–D). One micropylar area located in the equatorial region (Fig 8B).

Etymology. This species name is given in apposition after "Saci-Pererê", a remarkable character from the Brazilian folklore. The word "pererê" means leaping in the Tupi Guarani language.

Material examined. *Holotype*, ♂ imago. BRAZIL, **Espírito Santo**, Santa Teresa, Reserva Biológica Augusto Ruschi, Córrego Bragacho (19°52′2.53″S, 40°33′34.27″W; 830 m a.s.l), 18.xii.2017–17.i.2018, Malaise trap, Salles FF. & Costa V. leg. (UFVB). *Paratypes*. BRAZIL, **Espírito Santo**, Santa Teresa, Reserva Biológica Augusto Ruschi, Córrego Bragacho, (19°52′2.53″S, 40°33′34.27″W; 830 m a.s.l), 19.xii.2017, D-net Salles FF & Costa V. leg., 3 N, 2 ♀♀ (UFVB); same data as for preceding except for: 30.ix.2017,

3 ♂♂ (INPA); **Minas Gerais**, Araponga, Serra do brigadeiro, Vale das Luas (20°39′37″S, 42°26′55″W; 990 m a.s.l), 24.i.2014, Entomological net, Salles FF. leg., 2 ♂♂ (MZUSP).

Remarks. Pigments in forewings are usual in *Hagenulopsis* species and it may occur on cross veins, longitudinal veins, on the membrane or surrounding longitudinal and cross vein (Fig 4). The males of *H. perere* sp. nov. are somewhat similar to males of *H. diptera* sharing with them the general color, the upper portion of compound eye meeting dorsally, and the brown bands on middle and apex of hind femur. However, the males of *H. perere* sp. nov. can be differentiated from the males of *H. diptera* by the remarkable brown clouds surrounding the cross veins located between longitudinal veins C to RP_1 and by a brown spot at the midlength on the outer surface of the middle femur. In addition, mature nymphs and females of *H. perere* sp. nov. can also be distinguished from all other species by the presence of clouds surrounding the cross veins between longitudinal veins C and RP_1.

Hagenulopsis diptera Ulmer 1920

(Figs 4A–F, 5A–D, 9A–B, 10A–H).

Hagenulopsis diptera Ulmer, 1920: 34 (description); Lestage 1922:33 (note); Da-Silva et al. 2009: 389 (new record); Domínguez *et al*. 2009:43 (diagnosis and new record).

Redescription

Diagnosis. *Hagenulopsis diptera* can be separated from its congeners by the following combination of characteristics. Male imago. (i), general coloration yellowish-brown to dark brown (Fig. 8A); (ii), dorsal portion of eyes meeting dorsally on the head; (iii), cross

veins between C and RP_1 slightly tinged with brown; (iv), cross veins posterior to C and RP_1 light brown (v), hind femora with medial and apical brown bands.

Male imago. Body size: 5.9–7.6 mm (n = 11). General coloration, brown (Fig 10A–H). Head: orangish brown (Figs 10A–B, E–H) to dark brown (Fig 10C–D). Upper portion of compound eye light orangish brown (Figs 10A–B, E–F), chestnut (Fig 10G–H) or dark brown (Fig 10C–D), lower portion black. Eyes meeting dorsally on the head and with around 18–25 facets on the longest row. Ocelli white surrounded with dark brown, lateral ocelli larger than median ocellus. Antenna: scape brown, pedicel washed with dark brown, flagellum pale brown.

Thorax: pronotum yellowish brown (Figs 10A–B, E–F) to dark brown (Figs 10C–D, G–H), posterior margin and submedian areas of pronotum dark brown. Mesonotum, median longitudinal, medioparapsidal and lateroparaspsidal sutures brown (Figs 10A–B, E–F) to dark brown (Figs 10C–D, G–H). Metanotum yellowish-brown (Figs 10A–A, E–F) to dark brown (Figs 10C–D, G–H), darker on posterior margin. Pleura. Sclerites yellowish brown (Figs. 10A–B, E–F) to dark brown (Figs 10C–D, G–H), membranous area white. Sterna brown. Legs yellowish brown. Femora with apical brown band; fore and hind femora with subapical brown band, missing in mid femur; fore tibia dark brown at apex. Wings (Figs 9B and 4A–F): membrane hyaline, longitudinal and cross veins yellowish brown, costal area dark brown; cross veins between C and RP_1 slightly tinged with brown; five to six cross veins basal to bulla, 13–17 apical to bulla; pterostigma brown.

Abdomen: terga translucent yellowish brown (Figs 9A, 10A–B, E–F) to dark brown (Figs 10C–D, G–H). Tergum I brown; terga II–VIII yellowish brown (Figs 10A–B, E–F) to dark brown (Figs 10C–D, G–H) with longitudinal pale yellowish-brown stripe and lateral margins washed with gray, oval whitish marks surrounding abdominal spiracles; terga IX

and X darker than preceding terga. Sterna translucent brown, dark brown on posterior margin; sterna II–VIII with a pair of light brown submedian marks on anterior margin.

Genitalia (Figs 5A–D). Styliger plate brown and quadrangular; forceps segment I completely brown, inner margin curved medially; forceps segment II brown washed with white; forceps segment III entirely white; penis pale yellowish-brown acuminating towards apex and not covered by styliger plate; spine ventrally oriented on subapex of penis. Caudal filaments yellowish white with blackish annulations at articulations.

Material examined. *Holotype*, ♂ male imago. BRAZIL; **Santa Catarina**, Corupá, 01.xi.1910, Wilh. Ehrhardt leg. (Images of ♂ *Holotype* deposited in Hamburg Museum). **Other material.** BRAZIL, **Bahia**, Camacan, Reserva Particular do Patrimônio Natural Serra Bonita (15°25′16″S, 39°33′57″W; 300 m a.s.l), ii.2011, Malaise trap, LEAq leg., 1 ♂ (MHNBA); same data as for preceding, except for: ii.2012, 2 ♂♂ (MHNBA); same data as for preceding, except for: ii.2013,1, ♂ MHNBA; same data as for preceding, except for: Trilha da 2ª Cachoeira; 09.viii.2015; Calor A. & Campos R., leg, 1 ♂ (MHNBA); same data as for preceding, except for:; Córrego Bapeba; 07.viii, 2017; Entomological net, Dias E., Campos R., Laurindo F. & Gudin F., leg., 1 s♂ (CLBA); same data as for preceding, except for: **Rio de Janeiro**, Parque Nacional Itatiaia, Rio Campo Belo (22°27′37.66″S, 44°36′08″W; 745 m a.s.l), 06.x.2017, Light attraction, Dias E., Campos R. & Laurindo F. leg., 1 ♂ (CLBA); same data as for preceding, except for: **São Paulo**, Campos do Jordão, Cachoeira Galharada (22°41′33″S, 45°27′54.4″W), 11–17. ii.2017, Malaise trap, Almeida L. leg., 1 ♂ (CLBA); same data as for preceding, except for: **Santa Catarina**, Grão Pará, Parque Estadual da Serra Furada (28°09′07″S, 49°23′18″W), 16.xi.2013, Malaise trap, Pinho LC., Novaes MC. & Haddad MF. leg., 2 ♂♂, 2 ♀♀ (MZUESC).

Remarks. *Hagenulopsis diptera* was described for a handful of specimens from Santa Catarina State and currently is recorded from other Brazilian states, such as São Paulo (Dominguez *et al*. 2009) and Rio de Janeiro (Da-Silva *et al*. 2009). Among its congeners, the male imago of *H. diptera* resembles those of *H. perere* sp. nov., sharing with it similarities on general coloration, marked cross veins between C and RP_1, and by the presence of two brown bands on hind femur (which are also present on *H. minuta*). However, *H. diptera* can be distinguished from *H. minuta* by the eyes meeting dorsally on the head without a bridge and differs from *H. perere* sp. nov. mainly because the cross veins between C and RP_1 are not clouded as in *H. perere* sp. nov. (Fig. 4A–F). In addition, the females of *H. diptera* can also be distinguished from all other species by wing coloration similar to that in males.

Distribution. Brazil **(**Bahia [new record], Rio de Janeiro, São Paulo, and Santa Catarina).

NEW RECORDS

Hagenulopsis minuta Spieth, 1943

(Figs 2E, 11A–F)

Hagenulopsis minuta Spieth, 1943: 10 (description); Traver 1946: 247 (note); Peters & Domínguez 2001: 354 (revision).

Material examined. BRAZIL, **Bahia**, Uruçuca, Serra Grande, Parque Estadual Serra do Conduru, Cachoeira da trilha principal, (14°29′48.5″S, 39°07′53.1″ W; 227 m a.s.l) 18.i.2014, Light pan trap, Dias E. & Pereira T. leg., 5 s♂♂, 2 ♂♂ (MHNBA); same data

as for preceding, except for: Chapada diamantina, Capão, Riacho do Morro Branco; (19°39′13.8″S, 41°29′14.3″W; 917 m a.s.l.), 12.v.2014, Entomological net, Salles FF. & Nascimento J. leg., 3 ♂♂ (UFVB); same data as for preceding, except for: Varzedo, Serra da Jiboia Fazenda Baixa Grande, Propriedade do Sr. Getúlio, Córrego Cai Camarão, (12°57′45.3″S, 39°27′13.1″W; 280 m a.s.l.), 09.iv.2015, Malaise trap, Dias E. & Campos R. leg., 1 ♂ (MHNBA); same data as for preceding, except for: **Piauí**, Piripiri municipality, Cachoeira do Bota-Fora (04°12′51.1″S, 41°40′01.7″ W; 162m a.s.l.), 01.vi.2019, Entomological net, Lima LRC. & Rodrigues JAO. leg., 11 ♂♂ (CEHJ) and 6 ♂♂ (CLBA); same data as preceding, except for: 10.II.2018, 2 ♂♂ (CEHJ); same data as preceding, except for: 15.XII.2018, 1 ♂ (CEHJ); same data as preceding, except for: **Espírito Santo**, Santa Nova Lombardia, Capitel de Santo Antônio, Córrego Grande, (19°52′30.8″ S, 40°31′49.1″ W), 19.ii.2009, CEUNES, leg., 1♂, 1 ♀ (UFVB); same data as preceding, except for: **Minas Gerais**, Conceição do Mato Dentro, Peixe tolo, (19°00′05″S, 43°36′04″W), 30.xi.2020; Entomological net, Salles FF. leg., 3 ♂♂ (UFVB); same data as preceding, except for: **Mato Grosso**, Bonito, Fazenda da ONG Brasil Bonito, Rio Taquaral (20°52′14″S, 56°35′19″W; 466m a.s.l.), 27.vi.2009, Light Pan trap, Lecci L., Schulz G. & Stefan G. leg., 1 ♂ (MZUESC-Eph0034). COLOMBIA, **Putumayo**, Puerto Asís, Quebrada Água Negras (0°31′36.3″N, 76°31′38.3″W), 19.xii.2015, light trap, Dias L. leg., 12 ♂♂, 2♀♀ (UFVB).

Remarks. The males imagos sampled in Piauí State (Fig. 11A–D) were caught up in the evening at the riparian environment under a dense canopy cover. Instead of a well-developed bridge between the stalks of the compound eyes, as reported in the literature or based on examined material from Bahia state (Fig. 11E) and Colombia (Fig. 11F), some of these imagos from Piaui state (n = 4) present only a small protuberance.

According to Peters & Domínguez (2001), the presence of these protuberances is a subimaginal character, which in imagos develop to the bridge characteristic of the species. In order to complement the description of the species, herein we report the absence of this bridge in some of the examined imagos (Fig. 11C–D).

Distribution. Brazil (Amazonas, Bahia, Espírito Santo, Mato Grosso [new record], Minas Gerais [new record], Roraima, and Piauí [new record]), Colombia, Guyana, Suriname, Venezuela.

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We are grateful to Dr. Adolfo Calor (Universidade Federal da Bahia), Dr. Luíz Carlos Pinho (Universidade Federal de Santa Catarina), and Dr. Lucimar Dias (Universidade of Caldas, Colombia) for assistance in specimens access. We specially would like to thank Martin Husemann and Thure Dalsgaard, both from Hamburg Museum, for providing us images of *Hagenulopsis diptera* holotype. This study was supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, procs. 1432793/2014; 88882.328342/2019-01) and Conselho Nacional de Desenvolvimento Científico e tecnológico (CNPq, productivity grant #309666/2019-8 to FFS). We thank the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) and Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA) (Processes number 75174-1, 12777-1 and 56881-1) for collection permissions in Brazil, Reserva Biológica Augusto Ruschi and Instituto Uiraçu for providing facilities during our surveys of aquatic insects, and the Laboratório de Biologia Aquática (LABIA), Universidade Estadual Paulista Júlio de Mesquita Filho, Assis-São Paulo and Dr. Og DeSouza (Universidade Federal de Viçosa) for the use of optic equipment.

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Figures.

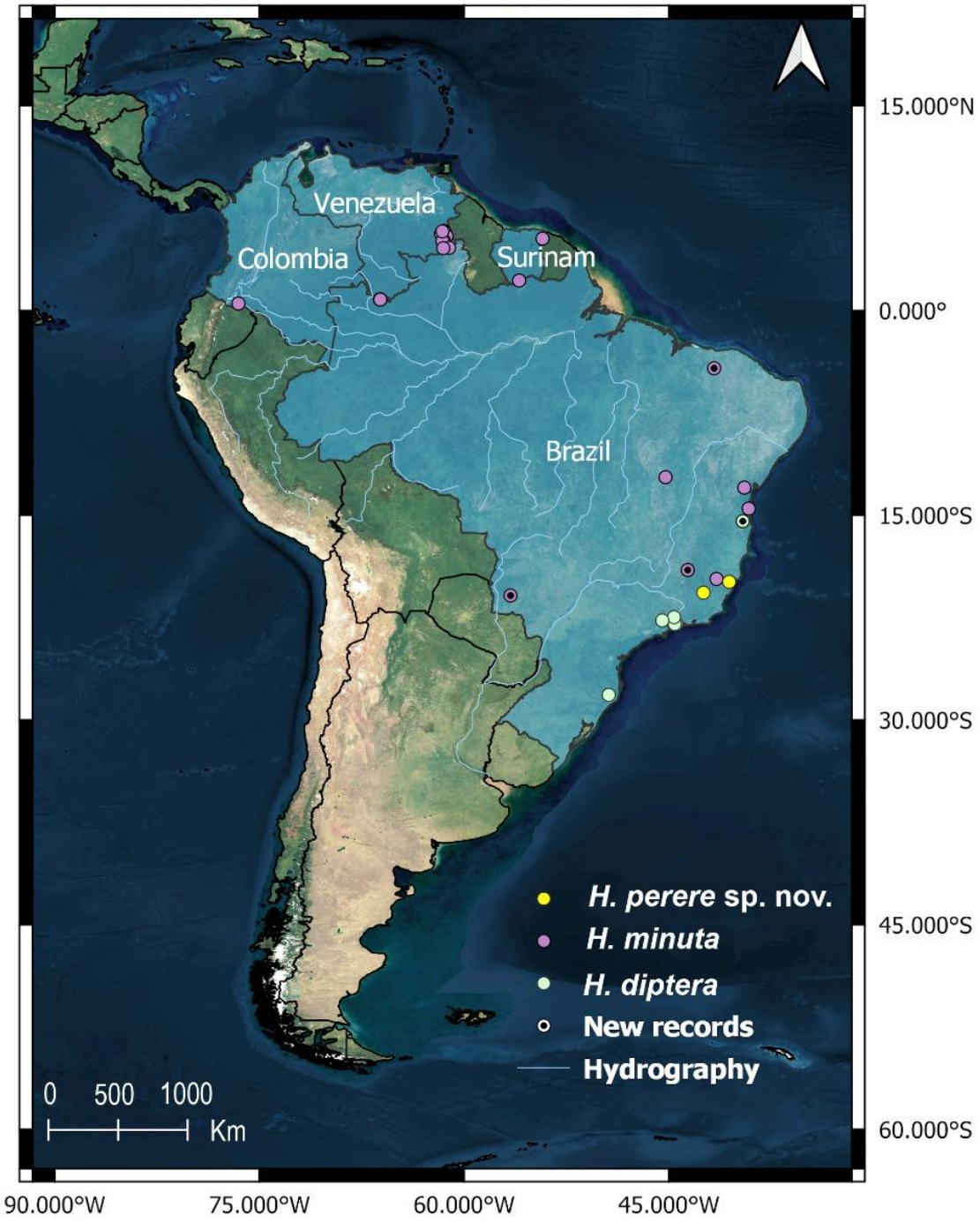

Figure 1. Distributional map highlighting the records of *Hagenulopsis diptera* Ulmer, 1920, *Hagenulopsis minuta* Spieth, 1943 and *Hagenulopsis perere* sp. nov.

Figure 2. *Hagenulopsis* spp. *in vivo*. A–D, *Hagenulopsis perere* sp. nov. (A) male imago; (B) male subimago; (C) female subimago; (D) nymph; (E) *Hagenulopsis minuta* Spieth, 1943, male imago

Figure 3 *Hagenulopsis perere* Campos, Costa & Salles sp. nov. (holotype). Male imago: (A) head and thorax in lateral view ; (B) head and thorax in dorsal view; (C) abdominal coloration, dorsal view; (D) abdominal coloration, lateral view; (E) genitalia,

ventral view; (F) foreleg; (G) midleg; (H) hind leg; (I) mid tarsi. Female imago: (J) egg guide, ventral view; (K) abdominal segments VI–X in lateral view, highlighting the egg guide. Scales: A–B, 0.5 mm; C, 1 mm; D, 2 mm; E, 0.2 mm; F–H, 1 mm.

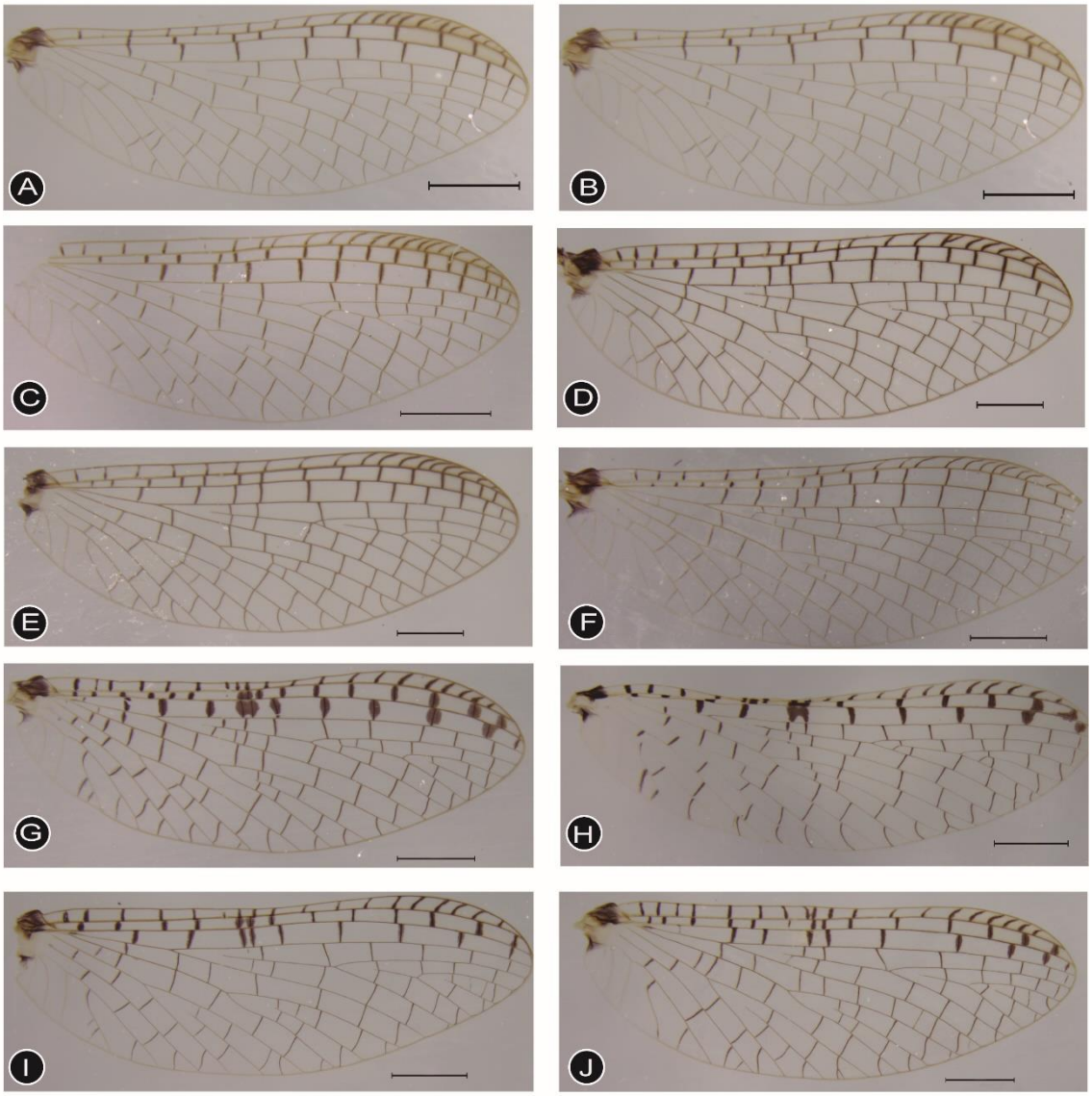

Figure 4. *Hagenulopsis* male forewings. *Hagenulopsis diptera* Ulmer, 1920. (A–C) Camacan (Bahia); (D) Itatiaia (Rio de Janeiro); (E) Campos do Jordão (São Paulo); (F) Grão Pará (Santa Catarina). *Hagenulopsis perere* sp. nov. (G–H) Santa Teresa (Espírito Santo); (I–J) Araponga (Minas gerais). Scales: A–J, 1 mm.

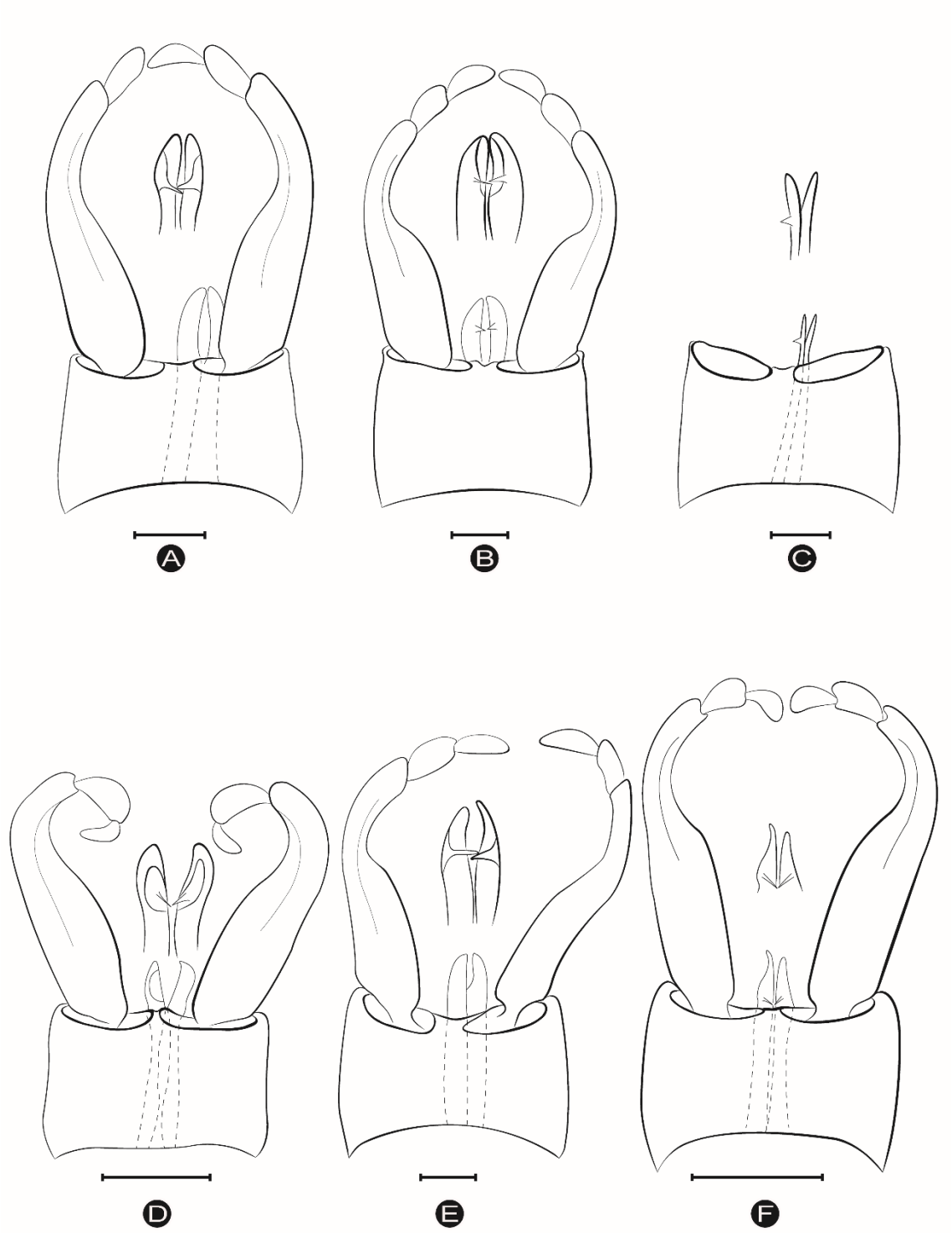

Figure 5. *Hagenulopsis* genitals. *Hagenulopsis diptera* Ulmer, 1920. (A) Camacan (Bahia); (B), Itatiaia (Rio de Janeiro); (C) Campos de Jordão (São Paulo); (D) Grão Pará (Santa Catarina). *Hagenulopsis perere* sp. nov. (E) Holotype, Santa Teresa (Espírito Santo); (F) Araponga (Minas Gerais). Scales: A–C, F, 0.1 mm; D–E 0.2 mm.

Figure 6. *Hagenulopsis perere* sp. nov., nymph. (A) Female in dorsal habitus; (B) wings pad; (C) hind leg; (D) mid leg; (E), foreleg; (F) hind claw. Scales: A, 1 mm; B, 0.5 mm; C–E, 0.5 mm; F, 0.2 mm.

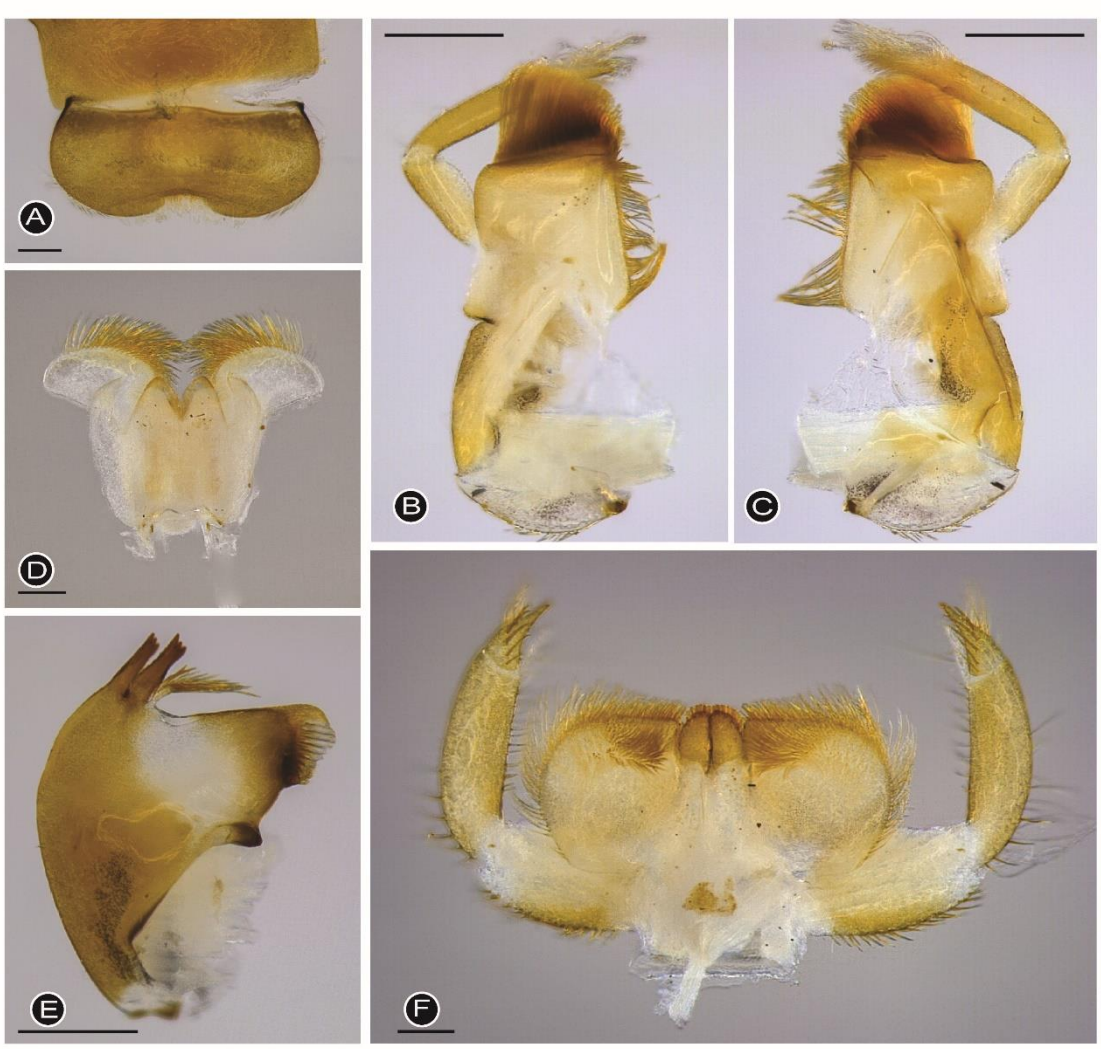

Figure 7. *Hagenulopsis perere* sp. nov., mouthparts. (A) labrum; (B) left maxilla; (C) right maxilla; (D) hypopharynx; (E) left mandible; (F) labium. Scales: A, 1 mm; B, 0.5 mm; C–E, 0.5 mm; F, 0.2 mm.

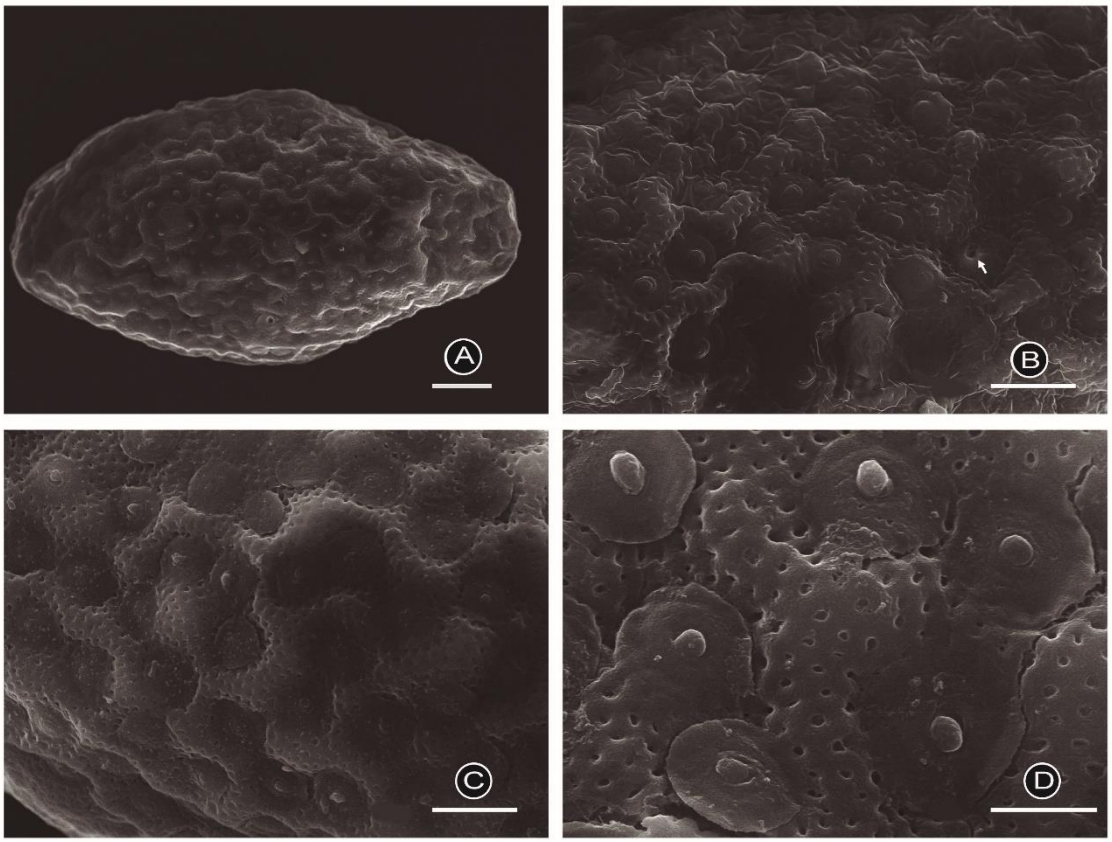

Figure 8. *Hagenulopsis perere* sp. nov., eggs. (A) egg in general view; (B) micropyle indicated by White arrow; (C–D) Chorionic surface of egg in detail; Scales: A, 20 µm; B–C, 10 µm; D, 5 µm.

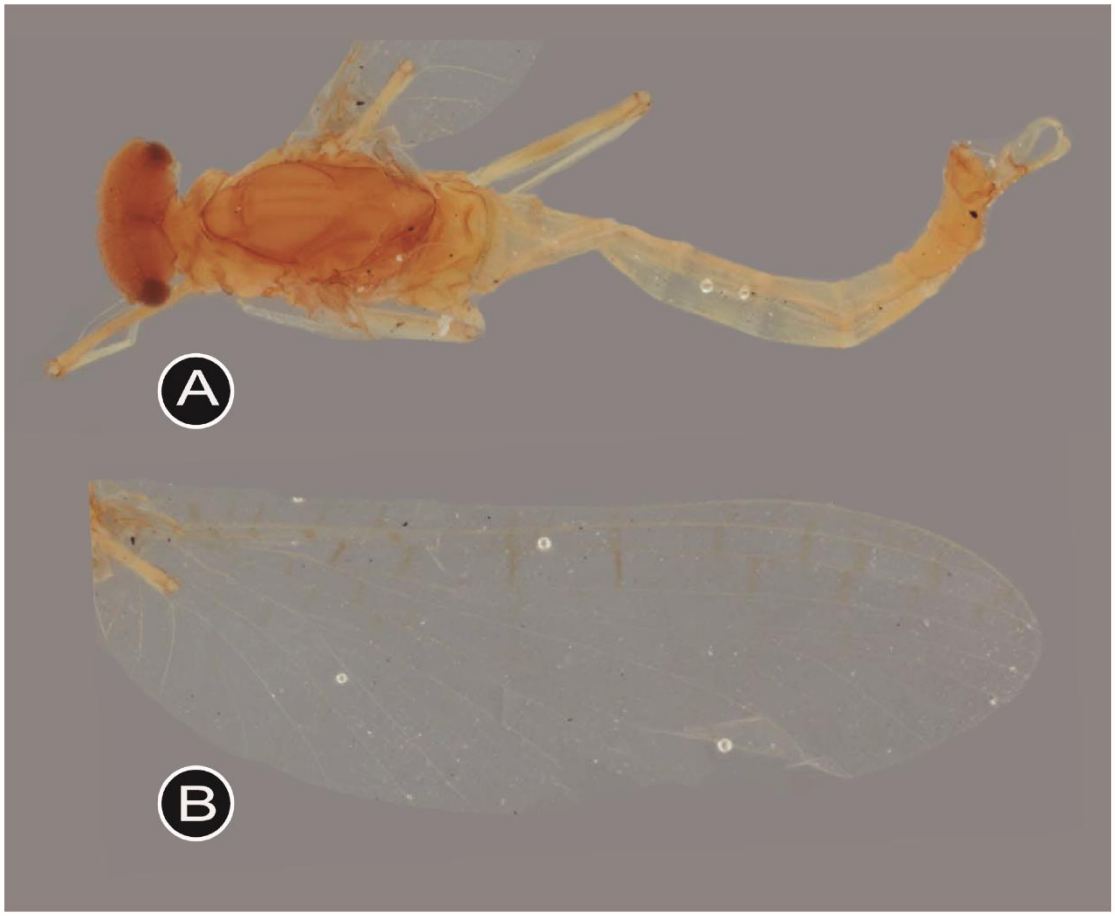

Figure 9. *Hagenulopsis diptera* Ulmer, 1920 type specimen. (A) male imago in dorsal habitus; (B), forewing.

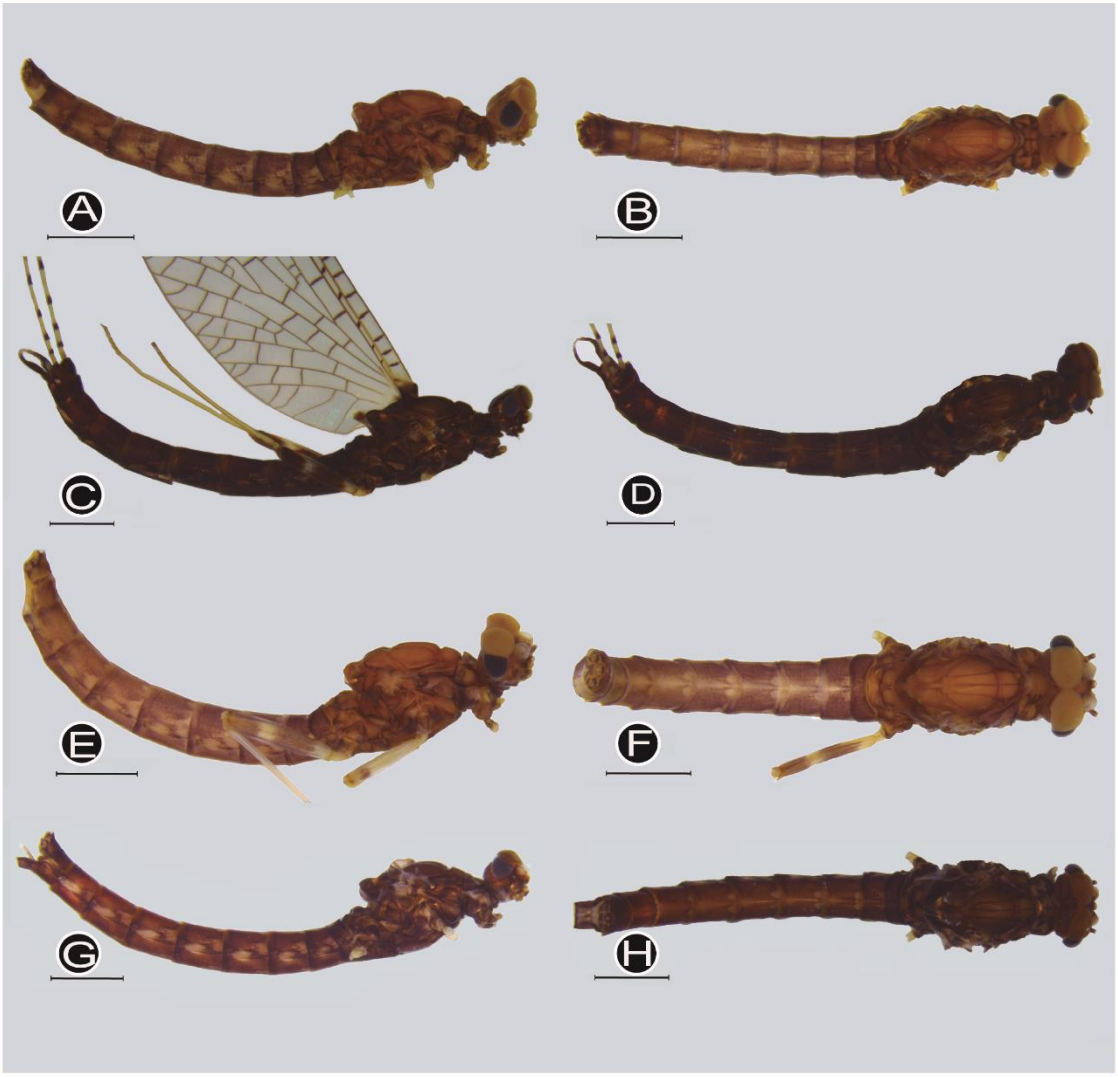

Figure 10. *Hagenulopsis diptera* Ulmer, 1920, male imagos in dorsal and lateral habitus sampled in Brazilian states. (A–B), Camacan (Bahia); (C–D), Itatiaia (Rio de Janeiro); (E–F), Campos do Jordão (São Paulo); (G–H), Grão Pará (Santa Catarina). Scale: A–H, 1 mm.

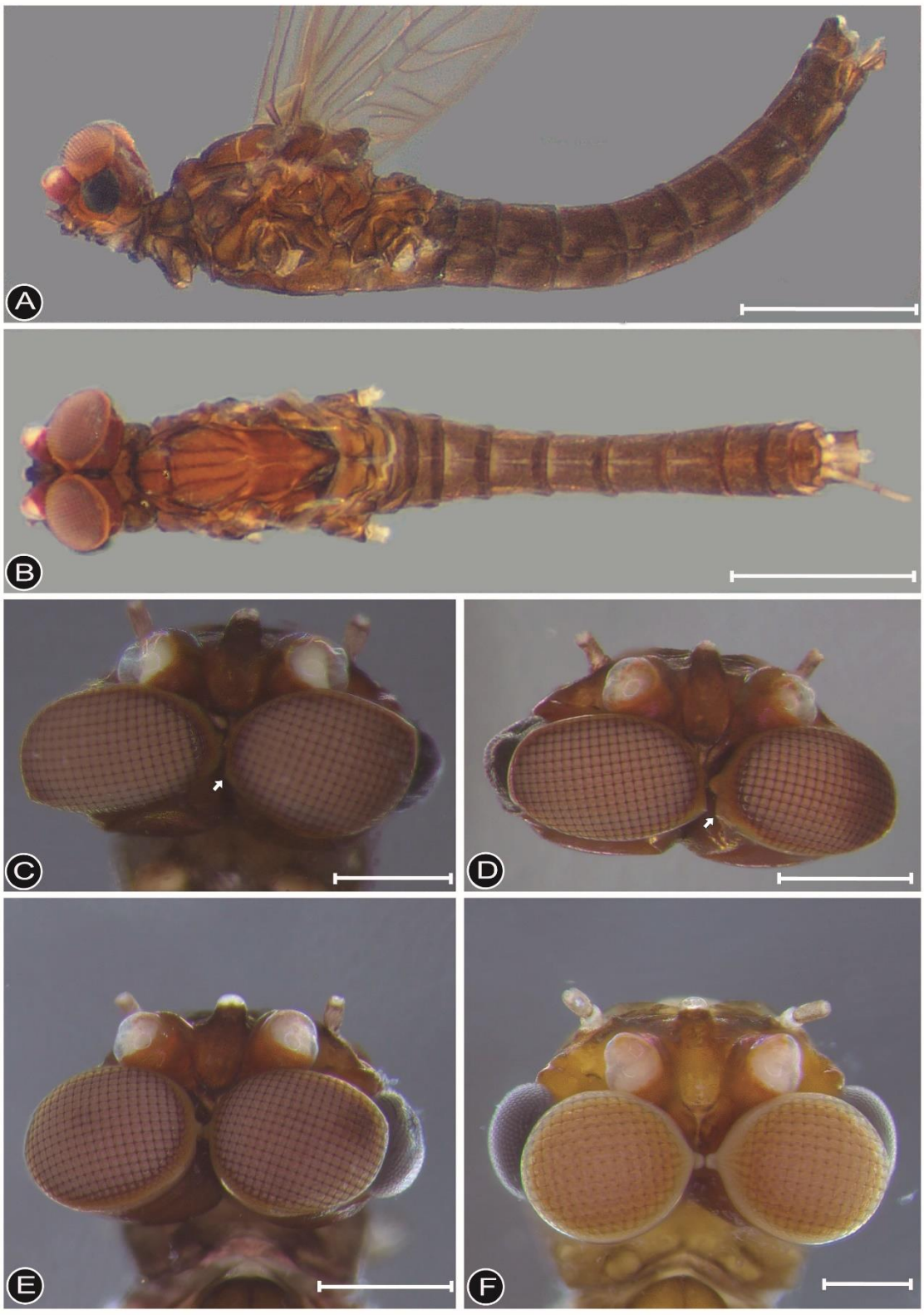

Figure 11. *Hagenulopsis minuta* Spieth, 1943. (A) Male imago in lateral habitus; (B) male imago in dorsal habitus; (C–F) upper portion of eyes, white arrows highlighting small protuberance on stalks. Scales: A–B, 1 mm; C–E, 0.3 mm; F, 0.2 mm.

CHAPTER 3

TITLE:

REDUCING THE HAECKELIAN DEFICIT IN *ASKOLA* (EPHEMEROPTERA: LEPTOPHLEBIIDAE): NYMPH ASSOCIATION OF *A. MUCUGE* CAMPOS, MARIANO & CALOR, 2019 USING MOLECULAR TOOLS ALSO REVEALS PUTATIVE CRYPTIC SPECIES IN THE GENUS

REDUCING THE HAECKELIAN DEFICIT IN *ASKOLA* (EPHEMEROPTERA: LEPTOPHLEBIIDAE): NYMPH ASSOCIATION OF *A. MUCUGE* CAMPOS, MARIANO & CALOR, 2019 USING MOLECULAR TOOLS ALSO REVEALS PUTATIVE CRYPTIC SPECIES IN THE GENUS

Running title: REDUCING THE HAECKELIAN DEFICIT IN *ASKOLA* (EPHEMEROPTERA: LEPTOPHLEBIIDAE): NYMPH ASSOCIATION USING MOLECULAR TOOLS ALSO REVEALS PUTATIVE CRYPTIC SPECIES IN THE GENUS

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Abstract

Once knowing the entire biodiversity is an almost impracticable task, continuous work is needed to fill knowledge gaps and overcome the shortfalls associated with the Earth's

biodiversity. In this scenario, Haeckelian shortfall refers to the scarcity of knowledge about the distinct developmental stages of the known species. This shortfall can be exemplified by *Askola*, a speciose mayfly genus within Hagenulinae, which comprises twelve valid species of which only two nymphs are currently described. Here, we aimed to reduce the Haeckelian shortfall associated with *Askola* proposing a molecular association of life stages based on COI sequences for *A. mucuge*. The nymph of this species can be differentiated from the two other known nymphs of the genus by the internal denticle medially located at the external incisor of the left mandible and by abdominal tergites without marks. In addition, our outcomes based on Automatic Barcode Gap Discovery also revealed cryptic species within *Askola froehlichi*, suggesting three independent lineages. Intraspecific threshold among these lineages was higher than 15%, thus we claim for more studies that should fill these gaps covering more individuals sampled, as well as the latitudinal distribution in order to understand the putative cryptic diversity within *A. froehlichi*.

Keywords: Leptophlebiidae, Hagenulinae, diversity, taxonomy, Neotropical, COI.

BACKGROUND

The study of biological diversity has great challenges, including filling the gaps in knowledge about the species. Despite the great effort made in recent years, there is a scarcity of knowledge about the species, including their identities (Linnean deficit), their geographic occurrence (Wallacean deficit), their abundances (Prestonian deficit), their phylogenetic relationships (Darwinian/Henningean deficit), their abiotic tolerances

(Hutchinsonian deficit), their interactions with other species (Eltonian deficit), its ecological traits and functions (Raunkiæran deficit), and their semaphoronts (Haeckelian deficit) (Hortal et al. 2015; Faria et al. 2020). Expanding sampling in poorly surveyed regions, cataloging information, organizing large databases, and using different available tools (e.g morphological analysis tools, molecular tools, stable isotopes, among others) have been helping us to reduce these gaps (see Hortal et al. 2015 and Faria et al. 2020 for further discussion on this issue).

Among the available tools, DNA barcode based on the mitochondrial gene cytochrome oxidase I (COI) has helped to overcome part of these deficits, mainly: 1) Linnean and Wallacean deficits as, together with morphology, facilitates the identification of species, enabling the finding of new species and the expansion of geographic records; 2) Darwinian/Henningean deficit as it, concatenated with other sources, has been used to propose phylogenetic hypotheses; 3) Haeckelian deficit as it allows the association of different semaphoronts. Indeed, the Barcode of Life initiative (Hebert et al., 2003a) has helped in accurate species identification and, due to its feasibility, it became widely used for taxonomy studies dealing with species delimitations and life stage associations. Studies on mayflies using COI have appeared since Ball and Hebert (2005) and henceforth, they became commonplace in mayfly studies (e.g. Williams et al. 2006; Alexander et al. 2009; Gattolliat & Monaghan 2010; Hojos et al. 2014; Salles et al. 2014; Múrria et al. 2015; Gattolliat et al. 2015; Angeli et al. 2016; Salles et al. 2016;; Molina et al. 2017; Gonçalves et al. 2017; Polato et al. 2018; Salles et al. 2019; León et al. 2020; Souto et al. 2021).

In Neotropical leptophlebiids, the COI sequences have been employed in studies on species delimitation (*e.g*. Salles et al. 2016, *Hermanella* group), life stage association (*e.g*. Molina et al. 2017), and, after concatenated with the sequences of other genes, in

phylogenetic and biogeographic studies (*e.g.* Salles et al. 2019; Monjardim et al. 2020; Gatti et al. 2021). Although efforts to barcode species have been made in Neotropical leptophlebiids, few of them have been addressed to overcome our ignorance on life stages. However, as the barcode is a very effective tool to make the association between different semaphoronts, it is expected that there will be a decrease in the Haeckelian deficit (Faria et al. 2020) for the Neotropical Leptophlebiidae fauna in the next years.

Currently, the size of the Haeckelian deficit in Neoptropical Leptophlebiidae can be exemplified by the genus *Askola* Peters, which comprises twelve described species and only two of them have described nymphs, *A. froehlichi* Peters, 1969 and *A. maculatus* Campos, Mariano & Calor, 2019. In addition, just the former species has barcode sequences available (Monjardim et al. 2019). In this study, our aim was to describe the nymph stage of *Askola mucuge* associated by DNA barcode and provide new COI sequences for species of the genus.

MATERIAL AND METHODS

Sample, morphology, and taxonomy

The specimens were sampled in streams across Caatinga and Atlantic Forest ecoregions in Brazil (Fig. 1). Nymphs were sampled using aquatic net (D-net) and the winged specimens were sampled using light attraction (Calor & Mariano 2012). Thereafter, all material was preserved in ethanol 90–100%. Nymph mouthparts, legs and gills, male genitalia, and other structures were dissected and mounted on a slide using Euparal®. The wings were dry mounted on a slide. Terminology and measurements for descriptions followed Hubbard (1995) and morphological identifications followed original paper descriptions (*e.g.* Peters 1969; Campos et al. 2019) and additional

references (*e.g*. Domínguez et al. 2009). Images were taken from a Leica M205A stereomicroscope with subsequent improvements made on the software Adobe PhotoshopCS6®. The map was built using the software Qgis 3.6.3.

DNA sequences and genetic analyses

The legs from nymphs and imagos were dissected and used for the total DNA extraction, which was done using the DNeasy® Blood and Tissue Kit (Qiagen), according to the manufacturer's protocol. The specimens received an identification number (Table 01) to address the sequences with the specimen. The barcode region of the COI gene (Hebert et al. 2003a; 2003b) was amplified through polymerase chain reaction (PCR) using the primers HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO-1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (Folmer et al. 1994). The PCR program consisted of a first denaturation step at 94ºC (2 minutes), followed by 40 cycles of denaturation at 94ºC (1 minute), annealing at 50ºC (1 minute), and extension at 72ºC (2 minutes), and a final extension step at 72ºC (5 minutes). The purification was performed using a manufacturer's protocol of the Wizard® DNA Purification Kit (Promega). The bidirectional sequencing of the products was carried out by CREBIO (Centro de Recursos Biológicos e Biologia Genômica) at UNESP (São Paulo State University), Jaboticabal, São Paulo State, Brazil.

An alignment comprising 22 sequences was built (503bp), of which seven sequences were produced in this study, and the others were taken from BOLDSYSTEM or provided directly by other authors (Table 01). The chromatograms were edited manually to obtain consensus sequences and then, they were aligned using ClustalW (Thompson et al. 1994) in MEGA Software (Kumar et al. 2016). To evaluate species delimitation based on the barcode region of the COI gene, we used the Automatic Barcode

Gap Discovery (ABGD, using primary and recursive partitions) (Puillandre et al. 2012; Zhang et al. 2013). The analysis was carried out using the online server (ABGD, https://bioinfo.mnhn.fr/abi/public/abgd/). The ABGD analysis was performed using standard parameters with a relative gap width of 1.0. The Bayesian analysis (2 independent runs of 4 Monte Carlo-Markov Chains for 2000000 generations, 25% generation burn-in) was run using MrBayes 3.2.2 (Ronquist et al. 2012). In order to choose the best evolutionary models for the sequences, the Partitionfinder 2.1.1 (Lanfear et al. 2017) was used. The GTR+G, HKY+I, and GTR+I+G models were chosen for the first, second, and third positions of the codons. All sequences used are available on GenBank. To evaluate the Barcoding Gap (Fig. 2), the intra and interspecific divergences were obtained through Kimura-2-parameters in Mega Software. Separately, Barcoding Gap graphs (Figure 2) were built for each species delimitation approach used: traditional morphological knowledge and ABGD.

RESULTS

Species delimitation

Species delimitation method (AGBD) revealed that the *Askola* molecular operational taxonomic units previously circumscribed by morphology were mostly consistent, despite some few mismatches between morphological and molecular variation (Fig. 3). In the case of *A. mucuge* life stage association, the ABGD indicated the nymph (Nymph-ED07) and adult male (Male-ED06) included in the analysis as belonging to the same species, with 3.2% (K2P) divergence between them.

Our results also revealed high intraspecific variations for *A. froehlichi*. The genetic distances within this species ranged from 0.1% (EP398a and EP3996b) to 21.9%

(MM071b and AFr4). In fact, the ABGD method suggested three cryptic lineages inside *A. froehlichi* as follow: (i) the former lineage (AFL1) (AFr4, AFr10, AFr11, and MM071a) varying from 0.4% to 7.9%; (ii) the second lineage (AFL2) (EP3996b, EP398a, and EP5510a) from 0.1% to 0.5 %; and (iii) the third (AFL3) formed by a singleton (MM071b).

Interspecific divergences, considering the three *A. froehlichi* lineages, were greater than 15% reaching 25.5% as the highest value observed between *A. froehlichi* (AFL1, AFR11) and *A. mucuge* (ED07). The divergence between *A. froehlichi* lineages was 17.6% to 19.0% between AFL2 and AFL3, and 18.8% to 21.9% between the AFL1 and the two other lineages (AFL1 + AFL2).

Taxonomy

Askola mucuge Campos, Mariano & Calor, 2019

(Figs. 4–5)

Mature nymph (Figs. 4D, 5A–G)

Body length: 6.01 mm (n = 2)

General coloration (Fig. 4D): Yellowish-brown.

Head: frons orangish-brown and vertex reddish-brown. Upper portion of compound eye brown, lower portion black. Ocelli white surrounded black, lateral ocelli twice than mid ocellus. Antennae. Scape dark brown and pedicel washed dark brown, flagellum pale brown. Clypeus orangish-brown with distal margin truncated. Labrum almost as wide as clypeus; two equal size denticles on anteromedial emargination (Fig. 4A). Mandible (Figs. 4B) yellowish-brown, dark brown on molar and incisors; outer margin with 6–8 filiform long setae located submedially. Maxilla (Figs. 4D–E) translucent yellowish-

brown; maxillary palp with segment II ¼ longer than segment I and twice the length of segment III. Hypopharynx (Fig. 4C) translucent yellow. Labium (Fig. 4F) translucent yellowish-brown, covered by short and long setae; labial palp, dorsal surface of segment III with spine-like setae. Thorax: pronotum yellowish-brown with diffuse black marks. Meso and metathorax brown. Legs yellowish-brown, washed with dark brown on tibiae and tarsi; femora I–III orangish-yellow with dark brown mark on apex. Abdomen: terga yellowish-brown remaining brown toward posterior segments; terga washed with black on lateral margins; gills, lamella gray with black trachea (Fig. 4G). Sterna yellowish brown with lateral margins of terga I–VIII black. Caudal filaments orange-brown.

Nymph association: mitochondrial DNA COI.

Analyzed material: **BRAZIL**, Bahia State, Palmeiras, Caeté-Açú, Córrego do Batista, 12º 37′ 23″S, 41º 29′ 06.4″W, Light atraction, 09.vi.2019, Oliveira I., Miranda M and Calor R., 2 mature nymphs, 2 imagos ♂ and 1 imago ♀ (MHNBA); same data as for preceding, except for Mucugê, Projeto Sempre-viva, Córrego do Boiadeiro, 12º 52′ 44.9″S, 41º 19′ 36.3″W, el. 974m, Malaise trap, 10.i.2015, Dias E and Campos R, 3 imagos ♂ (*Paratype*s, MHNBA).

Remarks

The nymphs of *Askola mucuge* differ from the *A. maculatus* by an average size of 6.01 mm while *A. maculatus* present an average size of 4.20 mm (Campos et al. 2019). Regarding the body coloration, *A. mucuge* presents an abdominal coloration yellowish-brown washed with black on lateral margins resembling more *A. froehlichi* than those nymphs of *A. maculatus* that presents abdominal segments dark brown. In its turn, nymphs of *A. mucuge* can be separated from both species by a medial denticle at the external incisor of the left mandible (Fig. 4B).

DISCUSSION

Here, it was possible to associate the imago and the nymph of *A. mucuge*, with less than 3.5% of intraspecific variation. Comparing our results with other conspecific distances within neotropical leptophlebiids sampled at the same localities such as those described in Salles et al. (2016) to *Hydromastodon sallesi* Polegatto & Batista 2007 (2.8–3.1% K2P) seems like we have enough evidence to recognize the life stage association to *Askola mucuge* as suggested by ABGD (Figure 2A–B). Studies such as Ball et al. (2005) also find intraspecific threshold varying up to 3.4% (K2P) to *Maccaffertium vicarium* (Heptageniidae), conversely, it is usually found higher intraspecific distances among mayflies species (see, Morinière et al. 2017; Gonçalves et al 2017; Souto et al 2021).

Askola froehlichi presents a wide intraspecific molecular variation (0.1–21.9%). These values are high even for Mayflies, which can present values higher than those observed for other groups (see, Morinière et al. 2017). This is a widespread species recorded in Atlantic Forest throughout Brazilian coast from Santa Catarina to Bahia states, where, in the last state, it reaches the arid region of Caatinga (Campos et al. (2019). In this study, we could analyze some available sequences from different Brazilian states such as Bahia (EP3398a and EP3398b), Espírito Santo (AFR4), Minas Gerais (EP5510a, EP398a, and EP3996b), and São Paulo (AFR10 and AFR11). Our results based on ABGD suggest that *A. froehlichi*, as currently known, is formed by a complex of cryptic species, comprising at least three independent lineages (Figure 3). Despite our outcomes being limited by covering just part of the species distribution, they reinforce the morphological evidence (e.g. variation in nymph morphology; variation in adult coloration) raised by Da-Silva (2002) and Campos et al. (2019), which suggests cryptic species under the name

A. froehlichi. In face of this case, we claim for more studies, which should fill these gaps covering more individuals sampled, as well as the latitudinal distribution in order to understand the putative crypt diversity within *A. froehlichi*.

CONCLUSION

The present study contributes to reducing the Haeckelian gap in *Askola*, with the description of the nymph of *Askola mucuge*. In addition, this study helps us to have a first overview of intraspecific molecular variation of the most widely distributed species of *Askola*. In fact, despite the great diversity, few Neotropical leptophlebiids have barcode sequences available. Therefore, it is desirable that the DNA barcode database for this family be expanded, enabling a better understanding of intra- and inter-specific variations and the congruence between species delimitation based on morphological and molecular data. Additionally, a large database could facilitate the association between semaphoronts and the development of a more integrative taxonomy. Currently, several research groups are dedicated to the study of Ephemeroptera diversity in the Neotropical region, hence we expect that these efforts facilitate the construction of a library representative of DNA barcode for the order in the region, such as those that have been made for other regions (Webb et al. 2012; Morinière et al. 2017).

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Conflict of interest

The authors declare no competing interests.

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Figures

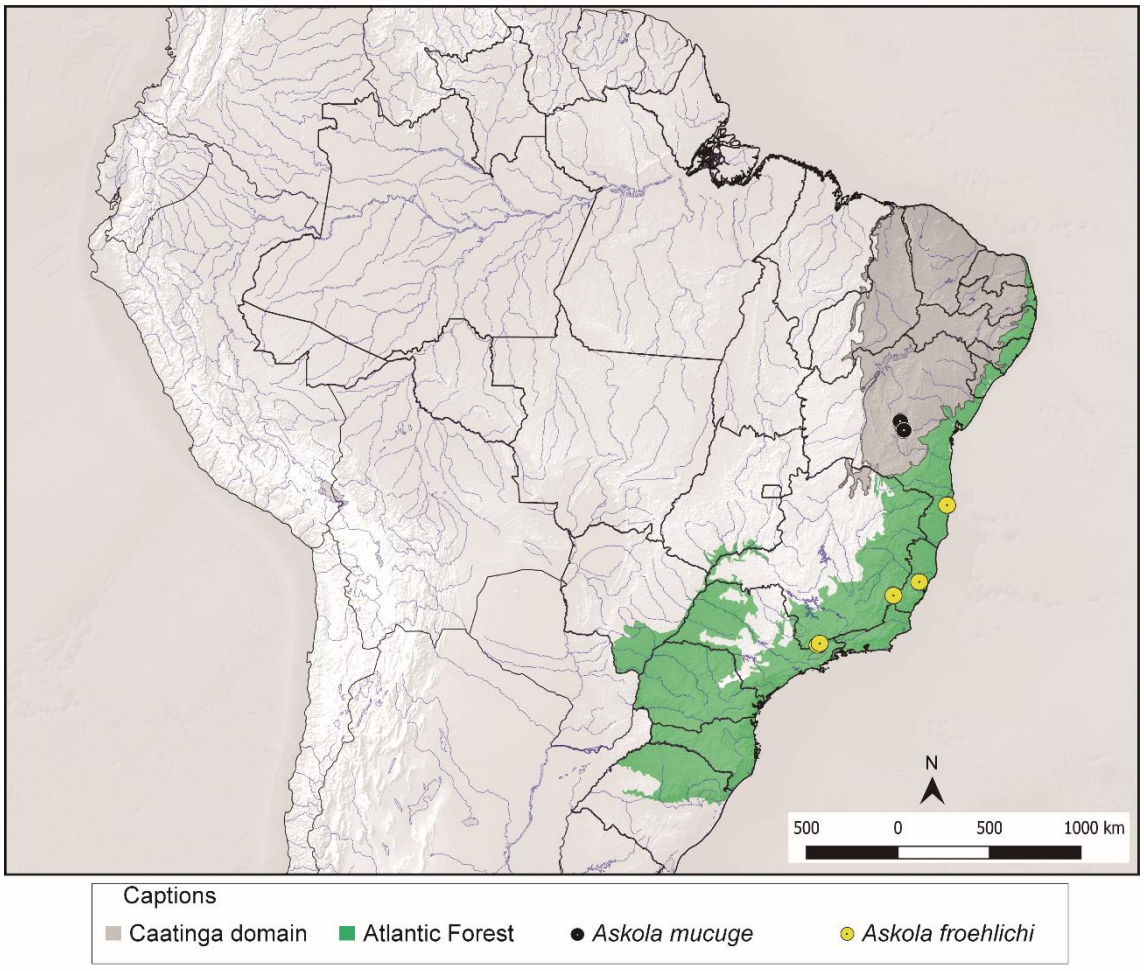

Figure 1. Distributional map, South America, in part. Sampling sites.

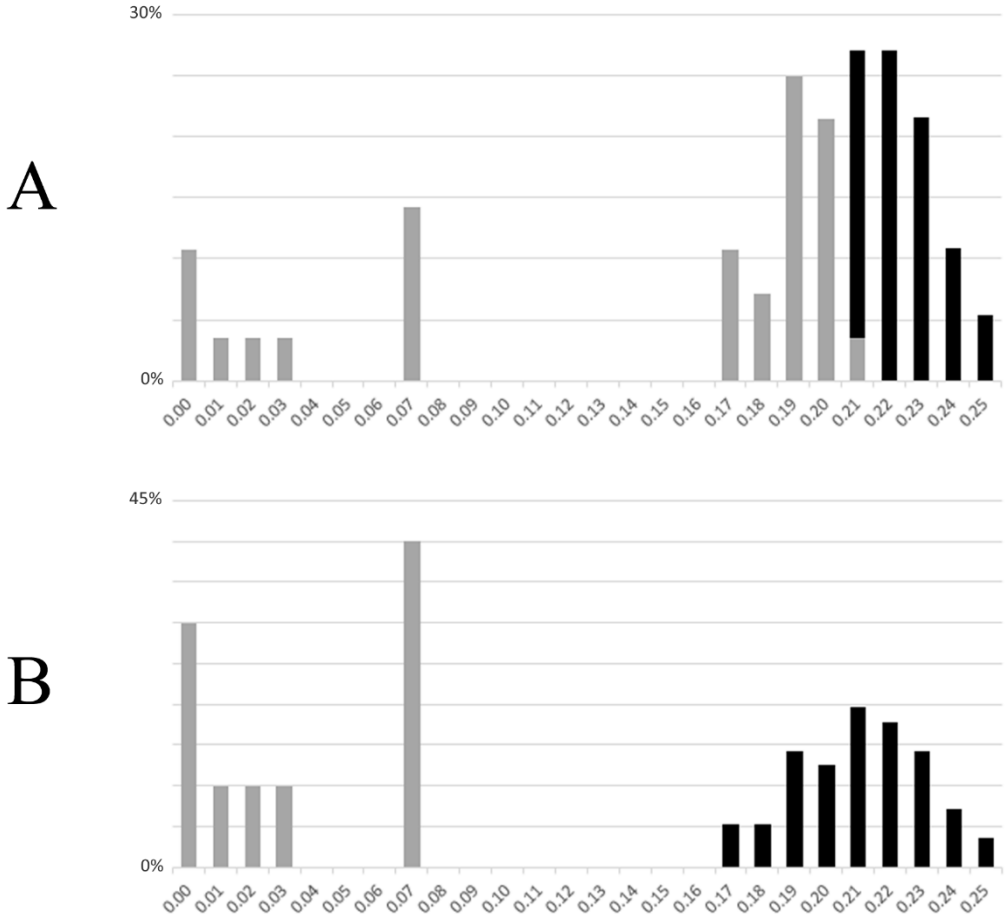

Figure 2. Intra and interspecific divergences comparisons obtained by Kimura-2-parameter (K2P) analyzed by methods: A, Morph; B, ABGD. Gray and black bars represent intra and interspecific divergences, respectively.

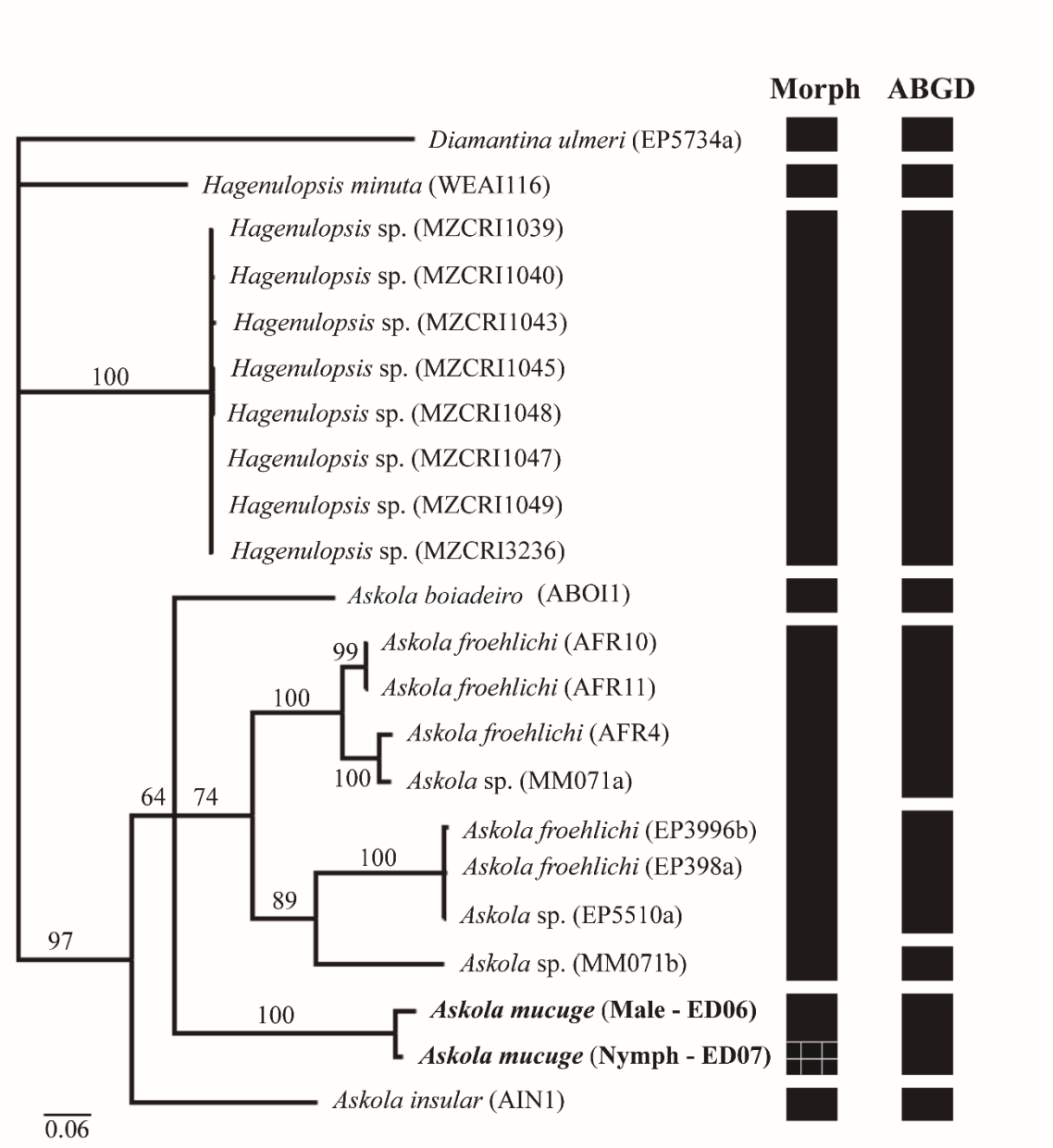

Figure 3. Bayesian inference tree obtained using mitochondrial cytochrome c oxidase subunit I (COI) under Morph and ABGD. Since the analyzed dataset delimitations under ABGD and ABGDr provided the same number of groups (n = 9), here are shown the results regarded to ABD. Crosshatched lines mean Morph method was not performed.

Figure 4. *Askola mucuge*, paratype and vouchers. A, male imago (paratype); B, male imago (voucher); C, female imago; D, nymph (voucher).

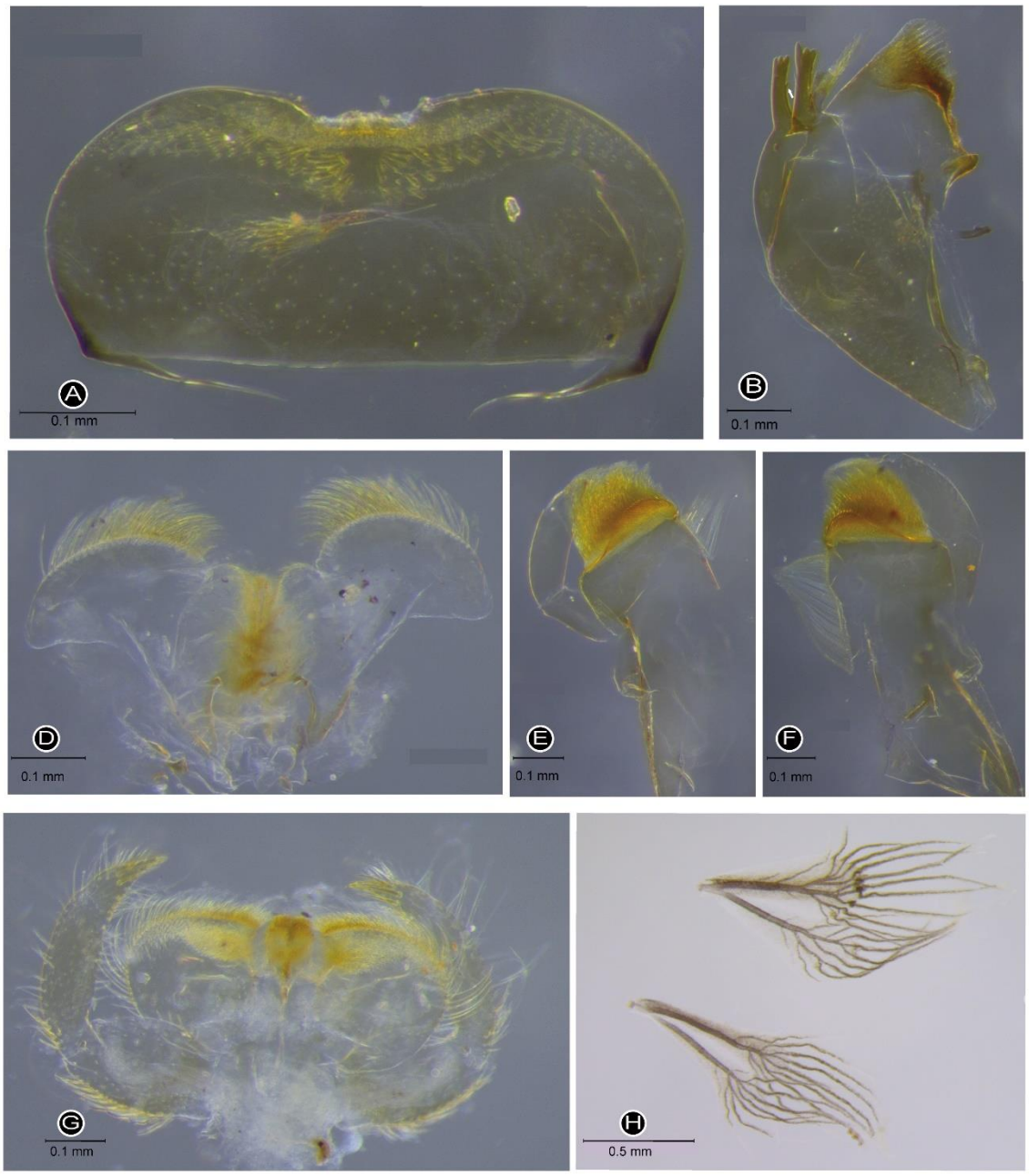

Figure 5. *Askola mucuge* Campos, Mariano & Calor, 2019, mouthparts. A, labrum in dorsal view; B, left mandible, white arrow indicating the medial denticle on external incisor; C, hypopharynx; D–E, maxilla; F, labium; G, gills.

Table 1. Analyzed specimens and vouchers information.

Voucher IDs	Species	coordinates	Reference
EP5734a	*Diamantina ulmeri*	12°36′0.2″S; 41°27′19.9″W	Salles et al. 2019
WEAI116-11	*Hagenulopsis minuta*	04°05′20″N; 52°40′37″W	Bold system
MZCRI1039-13	*Hagenulopsis* sp.	08°40′19″ N; 83° 30′ 40″W	Bold system
MZCRI1040-13	*Hagenulopsis* sp.	08°40′19″ N; 83° 30′ 40″W	Bold system
MZCRI1043-13	*Hagenulopsis* sp.	08°40′19″ N; 83° 30′ 40″W	Bold system
MZCRI1045-13	*Hagenulopsis* sp.	08°40′19″ N; 83° 30′ 40″W	Bold system
MZCRI1047-13	*Hagenulopsis* sp.	08°40′19″ N; 83° 30′ 40″W	Bold system
MZCRI1048-13	*Hagenulopsis* sp.	08°40′19″ N; 83° 30′ 40″W	Bold system
MZCRI1049-13	*Hagenulopsis* sp.	08°40′19″ N; 83° 30′ 40″W	Bold system
MZCRI3236-13	*Hagenulopsis* sp.	08°40′19″ N; 83° 30′ 40″W	Bold system
ABOI1	*Askola boiadeiro*	12°59′44.9″S; 41°19′36.3″W	This study
AFR4	*Askola froehlichi*	Brazil, Espírito Santo, Santa Teresa, Rebio Augusto Ruschi, 11.VIII.2017, Dias, Campos & Laurindo	This study
AFR10	*Askola froehlichi*	22º39′49″S, 45º26′32″W	This study
AFR11	*Askola froehlichi*	22º41′29″S, 45º27′58″W	This study
EP3998a	*Askola froehlichi*	20°28′19″S; 41°49′42″W	Salles et al. 2019
EP3996b	*Askola froehlichi*	20°28′19″S; 41°49′42″W	Salles et al. 2019
EP5510a	*Askola* sp.	20°28′57″S; 41°49′51″W	Salles et al. 2019
MM071a	*Askola* sp.	16°23′15.58″S; 39°10′11.06″W	Monjardim et al. 2020
MM071b	*Askola* sp.	16°23′15.58″S; 39°10′11.06″W	Monjardim et al. 2020
ED06	*Askola mucuge*	12º37′23″S; 41º29′06.4″W	This study
ED07	*Askola mucuge*	12º37′23″S; 41º29′06.4″W	This study

AIN1	*Askola insular*	27º17′35″S; 48º21′59″W	This study

Supplementary material.

Table 01. COI divergences obtained by Kimura-2-parameter (K2P).

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
ED06																					
ED07	0.032																				
AIN1	0.240	0.239																			
ABOI1	0.220	0.205	0.223																		
AFR4	0.236	0.241	0.223	0.211																	
AFR10	0.243	0.255	0.210	0.224	0.079																
AFR11	0.241	0.254	0.213	0.217	0.078	0.004															
MM071a	0.225	0.238	0.194	0.188	0.029	0.079	0.074														
Ep3996b	0.227	0.232	0.214	0.216	0.198	0.200	0.198	0.176													
Ep3998a	0.232	0.237	0.214	0.221	0.203	0.200	0.198	0.181	0.010												
MM071b	0.235	0.229	0.215	0.237	0.219	0.206	0.206	0.200	0.176	0.176											
Ep5510a	0.224	0.228	0.218	0.215	0.195	0.193	0.191	0.188	0.005	0.005	0.190										
MZCRI1040-13	0.263	0.270	0.281	0.253	0.251	0.247	0.239	0.266	0.275	0.270	0.267	0.264									
MZCRI1039-13	0.263	0.270	0.281	0.246	0.243	0.247	0.236	0.263	0.273	0.268	0.264	0.264	0.003								
WEAI116-11	0.226	0.228	0.219	0.255	0.261	0.273	0.275	0.252	0.244	0.239	0.236	0.235	0.226	0.220							
MZCRI1043-13	0.260	0.270	0.282	0.246	0.237	0.252	0.241	0.261	0.273	0.268	0.267	0.264	0.008	0.008	0.221						
MZCRI1045-13	0.263	0.270	0.281	0.247	0.243	0.247	0.237	0.266	0.270	0.265	0.262	0.264	0.003	0.003	0.222	0.008					
MZCRI1047-13	0.263	0.270	0.281	0.250	0.246	0.247	0.239	0.263	0.273	0.268	0.264	0.264	0.005	0.002	0.222	0.007	0.002				
MZCRI1048-13	0.263	0.270	0.281	0.250	0.246	0.247	0.239	0.266	0.270	0.265	0.262	0.264	0.006	0.003	0.222	0.008	0.000	0.002			
MZCRI1049-13	0.260	0.267	0.284	0.253	0.249	0.249	0.242	0.266	0.270	0.265	0.267	0.261	0.003	0.003	0.217	0.008	0.003	0.002	0.003		
MZCRI3236-13	0.260	0.267	0.279	0.247	0.244	0.244	0.237	0.260	0.276	0.270	0.267	0.266	0.006	0.003	0.220	0.008	0.003	0.002	0.003	0.003	
Ep5734a	0.298	0.307	0.280	0.270	0.282	0.286	0.281	0.268	0.286	0.289	0.276	0.285	0.287	0.284	0.292	0.289	0.287	0.287	0.287	0.289	0.284

CONSIDERAÇÕES FINAIS

De forma pioneira nos estudos em Leptophlebiidae neotropicais, a presente tese integrou dados morfológicos (paleontológicos e neontológicos) a uma abordagem filogenética Bayesiana a fim de compreender as relações internas e evolução no tempo-espaço da subtribo Hagenulina. Nossos dados corroboram com hipóteses anteriores sobre a monofilia da subtribo (e.g. Monjardim et al 2020), a qual possui 49 espécies e 11 gêneros. A subtribo foi suportada por quatro sinapomorfias: abdômen com projeções posterolaterais nos segmentos V ou VI ao IX, asa anterior com intercalares radiais IR1-2 e IRP conectadas, setor medial (MA) da asa anterior com veia MA2 *sagged* e setor cubital da asa anterior com veia intermedial cubital (Cu) ICu1 conectada a veia cubital posterior (CuP).

As análises biogeográficas indicam que Hagenulina surgiu como linhagem independente na América do Sul a cerca de 100 milhões de anos atrás (Cretáceo). A subtribo iniciou a sua diversificação por volta de 61 milhões de anos atrás (Cretáceo-Paleogeno), tendo suas linhagens internas alcançado a região das Antilhas (América Central) através de múltiplas dispersões a partir da América do Sul. Além disso, nossos resultados filogenéticos reacendem a discussão sobre a perda da asa posterior nos Leptophlebiidae neotropicais, indicando pela primeira vez uma possível parafilia do gênero *Hagenulopsis* Ulmer em relação ao gênero *Askola* Peters, no entanto, a relação filogenética entre esses dois gêneros necessita ser melhor estudada.

No contexto dos projetos de pesquisa em biodiversidade de Ephemeroptera, nosso estudo aponta perspectivas futuras para os estudos filogenéticos, enfatizando a possibilidade de análises morfológicas modernas. Isso é altamente salutar, uma vez que existe material antigo disponível em várias coleções científicas, os quais permitem uma análise morfológica detalhada, mas não permite a análise molecular. Certamente, não

podemos abrir mão dessa importante fonte de informação. Por outro lado, também entendemos que é importante ampliar o esforço de coleta, fixando material de forma adequada para análises moleculares futuras, assim será possível desenvolver uma taxonomia mais integrativa e a proposição de filogenias baseadas em múltiplas evidências.

O esforço para documentar e entender a diversidade é contínuo. Por isso, a documentação e catalogação, revisão, descrição de novas espécies com semaforontes associados, bem como a associação dos semaforontes para espécies já conhecidas são aspectos fundamentais para reduzir os déficits associados a Biodiversidade. Por exemplo, considerando a fauna da região das Antilhas, onde a maioria dos registros para a subtribo são oriundos de trabalhos descritivos pontuais (e.g. Peters 1981, Traver 1938, Kluge 1994) ou em sua maioria concentrados em Cuba (e.g. Gonzalez-Lazo et al. 2008; Naranjo, Peters & Castillo, 2021; Salina & Torres-Cambas 2021), faz-se necessário ampliar os esforços para melhor conhecermos a diversidade Hagenulina nas demais ilhas que compõem as Antilhas (e.g. Sartori 2021).

Nosso estudo, ainda no caminho para reduzir os déficits associados a biodiversidade, mais especificamente ligado ao déficit Haeckeliano, reforça a importância da integração dos dados moleculares (e.g. COI) com as análises morfológicas. Essa integração, nos possibilitou, ao mesmo tempo, realizar a associação molecular de ninfa e adulto de *Askola mucuge*, bem como explorar a variação molecular para o gênero *Askola*, lançando luz sobre a possibilidade de espécies crípticas para o gênero.

O nosso estudo é o mais abrangente até o momento sobre Hagenulina. Apesar disso, lacunas ainda precisam ser preenchidas. Neste sentido, como perspectivas futuras para os estudos da subtribo Hagenulina, sugerimos os seguintes próximos passos: 1)

Ampliar os estudos faunísticos na Região das Antilhas; 2) acondicionar os novos espécimes coletados de forma adequada a fim de possibilitar a extração de dados moleculares; 3) promover a integração dos dados morfológicos e moleculares; e 4) ampliar a amostragem e testar as hipóteses de relacionamento entre *Askola* e *Hagenulopsis.* A partir do presente trabalho e a partir do preenchimento dessas lacunas, será possível ter uma visão mais completa da diversidade e evolução de Hagenulina.

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