UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO

CENTRO DE CIÊNCIAS HUMANAS E NATURAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

Evolução e Biogeografia de Atalophlebiinae (Ephemeroptera, Leptophlebiidae)

Felipe Donateli Gatti

Vitória, ES
Junho, 2021

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Coorientador: Prof. Frederico Falcão Salles

Tese submetida ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal do Espírito Santo, como requisito parcial para a obtenção do grau de Doutor em Ciências Biológicas.

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Felipe B. Gatti

SUMÁRIO

RESUMO

Atalophlebiinae (Ephemeroptera, Leptophlebiidae) é uma subfamília de efêmeras presente em áreas temperadas e montanhosas da América do Sul e Austrália. No primeiro capítulo deste trabalho, testamos a hipótese de que tanto a vicariância quanto a dispersão, relacionadas à separação da Gondwana, contribuíram para a origem, diversificação e moldaram a distribuição atual desse grupo. A hipótese foi testada usando filogenética bayesiana, datação molecular baseada em fósseis e estimativa de área ancestral para reconstruir a biogeografia das linhagens dentro do grupo. Os resultados sugerem que Atalophlebiinae se origina durante o período Cretáceo, a aproximadamente 108 milhões de anos atrás), após um evento vicariante, durante a segunda fase de separação do supercontinente Gondwana. Posteriormente, a linhagem se diversificou em um cenário referente ao corredor gondwânico formado pela América do Sul, Antártica e Austrália. Ao término da separação dos continentes que integravam o corredor, as especiações aconteceram dentro das atuais áreas de ocorrência. Assim, a diversidade e distribuição de Atalophlebiinae foram moldadas por complexos processos envolvendo vicariância, dispersão e especiação dentro do corredor Gondwânico. No segundo capítulo, considerando a complexa dinâmica da paisagem na América do Sul, investigamos a história evolutiva de *Massartella* Lestage, 1930 (Ephemeroptera, Leptophlebiidae, Atalophlebiinae). O gênero é atualmente composto por cinco espécies endêmicas do continente, adaptadas a ambientes frios e montanhosos em regiões de florestas tropicais. *Massartella* tem distribuição disjunta, ocorrendo nas montanhas ao longo da Mata Atlântica e na região venezuelana do Pantepui, mas está ausente entre essas áreas. Aqui usamos filogenética bayesiana e datação molecular baseada em fósseis para reconstruir a história evolutiva de *Massartella*. Os resultados recuperaram o monofiletismo do gênero, e das linhagens Pantepui e Mata Atlântica, e sugerem que o último ancestral comum desses clados viveu a aproximadamente 66 milhões de anos atrás. Os processos de diversificação começaram ao mesmo tempo nas duas linhagens, e as relações entre as espécies do Pantepui foram bem suportadas. Por outro lado, muitas relações entre as espécies nas montanhas da Mata Atlântica permanecem incertas. O monofiletismo recíproco das linhagens indica que não houve conexão subsequente a separação dos clados, ou a extinção dos dispersores. A biodiversidade nas montanhas tem a assinatura tanto de eventos geoclimáticos e processos ecológicos antigos quanto recentes, e as oscilações climáticas e ciclos de incursões marinhas na América do Sul podem ter sido responsáveis pelo isolamento das linhagens Pantepui e Mata Atlântica, bem como pelos processos de especiação nessas regiões.

Palavras-chave
área ancestral, dispersão, tempos de divergência, biogeografia histórica, Ephemeroptera, Leptophlebiidae, montanhas, Neotropical, América do Sul, vicariância.

ABSTRACT

Atalophlebiinae (Ephemeroptera, Leptophlebiidae) is a mayfly subfamily present in temperate and mountainous areas of South America and Australia. In the first chapter, we tested the hypothesis that both vicariance and dispersal related to the Gondwana breakup contributed to the origin, diversification and shaped the current distribution of this group. The hypothesis was tested using Bayesian phylogenetic trees, fossil based molecular dating and ancestral range estimation to reconstruct the biogeography of the lineages within this group. The results suggested an origin in the late Gondwana supercontinent for Atalophlebiinae after a vicariant event during the Cretaceous period (~108 mya). Subsequently, the lineage diversified into a scenario that refers to a Gondwanic corridor formed by South America, Antarctica and Australia. At the end of the separation of the continents that made up the Gondwanic corridor, speciation occurred within the current distribution areas. The diversity and current distribution of Atalophlebiinae were shaped by complex processes of vicariance, dispersal and speciation within the Gondwanic corridor during the second phase of the supercontinent breakup. In the second chapter, considering the complex landscape dynamic in the South America, we investigated the evolutionary history of *Massartella* Lestage, 1930 (Ephemeroptera, Leptophlebiidae, Atalophlebiinae). The genus is currently composed of five species endemic to this continent, adapted to cold environments and mountaintops in rainforests regions. *Massartella* has a disjunct distribution, occurring in mountains along the Atlantic Forest and in the Venezuelan Pantepui region, but being absent between these areas. Here we use Bayesian phylogenetic trees and fossil based molecular dating to reconstruct the evolutionary history of *Massartella*. Results recovered the genus, and the Pantepui and Atlantic Forest lineages as reciprocally monophyletic and suggest that the last common ancestor of these clades lived *ca*. 66 mya. The diversification processes started at the same time in both lineages, and the relationships among species of the Pantepui were recovered with high support. On the other hand, many relationships among taxonomic units in the Atlantic Forest mountains remained unclear. The reciprocal monophyly of clades indicates no subsequent connections between these areas, or the extinction of intermediates. Mountain biodiversity has the signature of both ancient and recent geoclimatic events and ecological processes, and climatic oscillations and cycles of marine incursions in the South America may have been responsible for isolating the Pantepui and Atlantic Forest lineages, as well as the speciation processes within these regions.

Keywords
ancestral area, dispersal, divergence times, historical biogeography, Ephemeroptera, Leptophlebiidae, mountains, Neotropical, South America, vicariance.

APRESENTAÇÃO

Atalophlebiinae (Ephemeroptera, Leptophlebiidae) é uma subfamília de efêmeras que incluía, até recentemente, grande parte dos gêneros da família Leptophlebiidae presentes no Hemisfério Sul (Kluge, 2009). Entretanto, após nova classificação (Monjardim et al., 2020), essa linhagem tornou-se um grupo menor, formada por gêneros adaptados aos ambientes frios e montanhosos da América do Sul e Austrália. Mesmo representando uma pequena fração da antiga classificação, Atalophlebiinae ainda é uma das linhagens mais diversas da família Leptophlebiidae (Monjardim et al., 2020).

Os gêneros que hoje compõem esta subfamília já foram agrupados, com base em caraceteres morfológicos, com táxons africanos e chamados de *cool-adapted* (adaptados ao frio, Pescador & Peters, 1980). Entretanto, análises moleculares sugeriram a existência de uma relação mais intíma entre as efêmeras com distribuição anfinótica, denominando o grupo de Paleoaustral (O'Donnell & Jockusch, 2008) e, posteriormente, Atalophlebiinae (Monjardim et al., 2020), sempre proximamente relacionado a um grupo de táxons de Madagascar. Assim, frente a esse padrão de distribuição e às relações filogenéticas, a hipótese da origem gondwânica do grupo foi sugerida por vários pesquisadores ao longo do tempo (Monjardim et al., 2020; Kluge, 2009; O'Donnell & Jockusch, 2008; Savage, 1987; Pescador & Peters, 1980).

Neste trabalho, usamos sequências de DNA de dois genes (*28S* e *COI*), registros fósseis, análises filogenéticas e biogeográficas para, no primeiro capítulo, datar e estimar a área de origem de Atalophlebiinae, bem como os eventos que podem ter contribuído para estabelecer o padrão de diversidade entre os gêneros que compõem a linhagem. No segundo capítulo, estabelecemos as relações filogenéticas e os tempos de divergência das espécies que compõem o gênero *Massartella* Lestage, 1930 (Atalophlebiinae). O gênero é endêmico da América do Sul e possui distribuição disjunta no continente, ocorrendo no Pantepui venezuelano e montanhas da Mata Atlântica (Derka et al., 2009; Domínguez et al. 2006; Derka, 2002; Pescador & Peters 1990).

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CAPÍTULO I

Gondwana breakup under the ephemeral look

Gondwana breakup under the ephemeral look*

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Abstract

Atalophlebiinae (Ephemeroptera, Leptophlebiidae) is a mayfly subfamily present in temperate and mountainous areas of South America and Australia. We tested the hypothesis that both vicariance and dispersal related to the second phase of Gondwana breakup—which began in the Early Cretaceous and resulted in the separation between Madagascar and India from Antarctica and Australia—contributed to the origin, diversification and shaped the current distribution of this group. The hypothesis was tested using Bayesian phylogenetic trees, fossil based molecular dating and ancestral range estimation to reconstruct the biogeography of the lineages within this group. The results suggested an origin in the late Gondwana supercontinent for Atalophlebiinae (85.76–136.63 mya) after a vicariant event during the Cretaceous period. Subsequently, the lineage diversified into a scenario that refers to a Gondwanic corridor formed by South America, Antarctica and Australia. At the end of the separation of the continents that made up the Gondwanic corridor, speciation occurred within the current distribution areas. The diversity and current distribution of Atalophlebiinae were shaped by complex processes of vicariance, dispersal and speciation within the Gondwanic corridor during the second phase of the supercontinent breakup. Mayflies have difficulty in crossing transoceanic barriers, which suggests that most living taxa are the result of more recent local ecological and historical processes.

Keywords

ancestral area, dispersal, divergence times, historical biogeography, vicariance

Introduction

Vicariance and dispersal associated with plate tectonics helped shape diversification and distribution patterns of biodiversity across the planet (Chamberland et al., 2017; Toussaint et al., 2017a; Jurado-Rivera et al., 2017). Gradual breakup of ancient supercontinents contributed to cladogenetic events, dividing lineages by vicariance (see Toussaint, Bloom, Short, 2017; Kim & Farrell, 2015; Sanmartín & Ronquist 2004) and dispersal through corridors that connected land fragments (see Reguero et al., 2014; Seton et al., 2012). Thus, vicariant events associated with the Gondwana breakup are commonly invoked to explain the disjunct distribution of ancient lineages across continents in the southern hemisphere (McCulloch, Wallis, Waters, 2016; Sanmartín & Ronquist 2004), while transoceanic dispersal would be responsible for this pattern of distribution in more recent lineages (Martín-Bravo & Daniel, 2016; Condamine, Sperling, Kergoat, 2013).

Gondwana breakup occurred gradually and can be divided into two phases. The first began in the Early Jurassic (~180 mya), resulting in the separation of West Gondwana (South America/Africa) from East Gondwana (Madagascar, India, Antarctica and Australia), ca. 140 mya (Mueller & Jokat, 2019; Thompson, Moulin, Aslanian, De Clarens, Guillocheau, 2019; Seton et al., 2012). However, it is important to note that southern South America was connected to the Antarctic Peninsula through the Weddellian Isthmus until the opening of Drake Passage at ca. 35 mya (Elsworth, Galbraith, Halverson, Yang, 2017). The second phase began in the Early Cretaceous (~135 mya), and resulted in the separation between Madagascar/India from Antarctica/Australia forming a corridor that connected fragments from South America, Antarctica and Australia at ca. 100 mya (Thompson et al., 2019; Gibbons, Whittaker, Müller, 2013; Seton et al., 2012; Seton et al., 2012). Concurrently to these events, rupture between Africa and South America and separation of Madagascar from India took place (Thompson et al., 2019; Gibbons et al., 2013; Seton et al., 2012).

Atalophlebiinae (Ephemeroptera, Leptophlebiidae) are a cool-adapted mayfly subfamily with amphinotic distribution, present in temperate and mountainous areas of South America and Australia (Monjardim, Paresque, Salles, 2020; O'Donnell & Jockusch, 2008; Savage, 1987; Pescador & Peters, 1980). Previously, researchers also considered taxa from tropical areas of South America, Africa and Madagascar as part of this group, suggesting an ancient Gondwana origin of this subfamily (Kluge, 2009; Savage, 1987; Pescador & Peters, 1980). However, recent studies indicate a monophyletic lineage of only amphinotic taxa (Monjardim et al., 2020; O'Donnell & Jockusch, 2008). Ephemeroptera is one of the oldest insect lineages (Misof et al., 2014) and its amphinotic distribution pattern is recurrent in other taxa of the group, such as Ameletopsidae, Coloburiscidae, Nesameletidae and Oniscigastridae (see Sartori, Brittain, 2015; Edmunds 1972). Considering the role of plate tectonics in biogeography and our current knowledge of the cool-adapted mayflies, here we tested the hypothesis that Atalophlebiinae originated and diversified under the influence of both vicariant and dispersal events, during the second phase of Gondwana breakup, which would explain the disjunct distribution between South American and Australian taxa and their absence in tropical Africa, Madagascar and Indo-Malayan regions.

Materials and Methods

Taxon sampling and molecular dataset

We sequenced 76 specimens (Table S1) for two molecular markers (Table S2): the D2–D5 region of the *28S* ribosomal RNA gene (Gillespie, Cannone, Gutell, Cognato, 2004; Gillespie et al., 2005) and a partial region of the *Cytochrome c oxidase subunit 1* gene (Folmer, Black, Hoeh, Lutz, Vrijenhoek, 1994). We also used 59 sequences available on GenBank to complement our data set (Table S1). Nine outgroup genera were selected based on a recent phylogeny of the Ephemeroptera (Monjardim et al., 2020; Ogden et al., 2019; O'Donnell & Jockusch, 2008) and the availability of fossils for calibration. Our data matrix comprised 19 genera, representing approximately 55% of the generic composition of Atalophlebiinae. All specimens sequenced in this research are stored in the Museu de Entomologia of the Universidade Federal de Viçosa, under the care of the authors (FFS) or in the Coleção Zoológica Norte Capixaba, Universidade Federal do Espírito Santo, Brazil.

Phylogenetic analyses

Sequences were aligned in Geneious 9.0 (www.geneious.com) and nucleotide substitution models for each marker were selected using the Corrected Akaike Information Criterion (AICc) in jModelTest2 (Darriba, Taboada, Doallo, Posada, 2012) on CIPRES (Miller, Pfeiffer, Schwartz, 2010). Saturation level of sequences was verified by Xia's test (Xia, Xie, Salemi, Chen, Wang, 2003) in DAMBE 7 (Xia, 2018) and the third codon position of *COI* was consequently excluded from the analyses. The concatenated molecular data matrix comprised 1,622 base pairs (1,196 bp from *28S* and 426 bp from *COI*) for 97 operational taxonomic units. Models selected for each partition were GTR+G to *28S* and TrN+G to *COI* (Table S3).

Phylogenetic tree was inferred using Bayesian inference in MrBayes 3.2.7a (Ronquist et al., 2012) on CIPRES (Miller et al., 2010). Eight Markov chain Monte Carlo (MCMC) iterations were run simultaneously for 1.58 million generations with sampling trees every 1,000 generations and 25% of burn-in, until the convergence diagnostic reached the stop value (standard deviation of split frequencies < 0.01). Support of nodes was provided by posterior probabilities (PP) as directly estimated from the majority rule consensus topology. Considering recent discussions about statistical significance (see Amrhein, Greenland, McShane, 2019; Hurlbert, Levine, Utts, 2019; Pike, 2019; Wasserstein, Schirm, Lazar, 2019), the logic, background knowledge and experimental design were considered alongside PP to reach a conclusion and decide on its certainty. Therefore, nodes with PP value higher than 0.85 were considered well-supported.

Divergence times

We used the relaxed uncorrelated lognormal molecular clock with a tree prior using the birth–death incomplete sampling algorithm (Stadler, 2009). Substitution models for each partition (*28S* and *COI*) were selected according to AICc (Table S3) and monophyly was forced based on BI results. Clock points calibration was based on seven date priors based on fossils (n=5), geological event and probabilities of dispersal (n=1, adapted from Landis, 2017) and secondary data derived from previous analyses (n=1).

1) Root was calibrated to represent the minimum and maximum (242–290 mya) ages of fossil species *Protereisma permianum* Sellards 1907 (Protereismatidae), believed to be one of the stem groups of Ephemeroptera (Sroka, Staniczek, Bechly, 2015; Godunko, Staniczek, Bechly, 2011; Grimaldi & Engel, 2005), and the mayfly *Triassonurus doliiformis* Sinitshenkova & Papier, 2005 (Siphlonuridae), the lineage with the oldest origin in our dataset (Lognormal distribution, offset= 242.0, Mean= 8.5, Standard deviation= 1.0, mean in real space). 2) Oligoneuriidae initial diversification was calibrated based on *Incogemina nubile* Storari & Rodrigues & Saraiva & Salles 2020. (Oligoneuriidae) (Lognormal, offset= 112.6, M= 30.0, S= 1.0, mean in real space). 3)

Leptophlebiidae initial diversification was calibrated based on the age of ~175 mya given by Grimaldi & Engel (2005) for origin of the family (Normal distribution, Mean= 175.0, Sigma= 25.0). 4) Leptophlebiinae initial diversification was calibrated based on the fossil *Aureophlebia sinitshenkovae* Peters & Peters 2000 (Leptophlebiinae) (Lognormal, offset= 89.3, M= 20.0, S= 1.0, mean in real space). 5) *Paraleptophlebia* initial diversification was calibrated based on the fossil *Paraleptophlebia prisca* (Pictet & Hagen, 1856) (Lognormal, offset= 33.9, M= 13.0, S= 1.0, mean in real space). 6) Calibration of the most recent common ancestor of Atalophlebiinae from the lineage that originated *Radima* Akers, Peters & Peters, 2003 (Lognormal, offset= 85.0, M= 21.0, S= 1.0, mean in real space) considered the final period of separation between Madagascar/India and Antarctica/Australia (~100 mya, Thompson et al. 2019; White, Gibson, Lister, 2013; Gibbons et al. 2013; Seton et al., 2012) and low probability of dispersal between these areas after separation (see Suplementary data of Landis, 2017; Sanmartín & Ronquist 2004). 7) Divergence between *Atalophlebia* Eaton, 1881 and *Atalomicria* Harker, 1954 was calibrated with the fossil *Atalophlebia culleni* Etheridge & Olliff 1890 (Lognormal, offset= 2.6, M= 20.0, S= 1.0, mean in real space). Fossil information can be accessed in the Fossilworks Paleobiology Database (http://fossilworks.org).

The input file was constructed in BEAUti v2.5.2 and run in BEAST2 v2.5.2 (Bouckaert et al., 2019) for 100 million generations and trees sampled every 10,000 generations. The convergences of runs and the effective sample size (ESS > 1,000) of parameters were examined in Tracer v1.7.1 (Rambaut, Drummond, Xie, Baele, Suchard, 2018). A tree with maximum clade credibility topology, using a burn-in of 25%, was constructed with TreeAnnotator v2.5.2, and analyses were run on CIPRES (Miller et al., 2010).

Ancestral area reconstruction

As suggested by Ree & Sanmartín (2018), the model for reconstruction of ancestral areas was chosen to consider the structure and assumptions of models, and not a statistical method that assumes probabilistic equivalence between different models (e.g., AIC). We selected Dispersal-Extinction-Cladogenesis (DEC; Ree, Moore, Webb, Donoghue, 2005), which is a model that allows the incorporation of fossil and geological information, and to co-estimate phylogeny as a stochastic process in continuous time and incorporates both vicariance and dispersal (Ree & Sanmartín, 2018; Ronquist & Sanmartín, 2011; Ree & Smith, 2008).

Genera occurrences were defined by presence or absence in South America (A), Madagascar (B), Australia (C) and New Zealand (D). Gondwana breakup is well documented (see Mueller & Jokat, 2019; Elsworth et al., 2017; Bache et al., 2014; White et al., 2013; Seton et al., 2012) and the probability of dispersal was assigned according to the availability of connections between areas across four time slices: (*t1*) 108 to 85 mya; (*t2*) 85 to 50 mya; (*t3*) 50 to 35 mya; and (*t4*) 35 to 0 mya. As suggested by Landis (2017), the probability of dispersal (constrained to sum to 1) was attributed to short-distances dispersal (*s*= 0.7); medium-distances dispersal (*m*= 0.2); and long-distances dispersal (*l*= 0.1). Thus, probability of medium- are implied to exist in short-, and long-distance dispersal are implied to exist between all area-pairs. Therefore, short-distance has value 1 (*s*+*m*+*l*) and medium-distance has value 0.3 (*m*+*l*) (Table S4; adapted from Landis, 2017).

Time slices and dispersal probabilities considered five geological events: the separation between Madagascar/India and Antarctica associated with low probability of dispersal after separation (~85 mya, Suplementary data of Landis, 2017; White et al., 2013; Gibbons et al., 2013; Sanmartín & Ronquist 2004); Tasman Sea opening (~50 mya, Bache et al., 2014; White et al., 2013); the opening of Drake Passage and Tasman Gateway with consequent Antarctica glaciation (~35 mya, Elsworth et al., 2017; Scher et al., 2015). The analyses were run using the 'BioGeoBEARS' package (Matzke, 2014) on R (R Core Team, 2020) under the RASP interface (Yu, Harris, Blair, He, 2015). The resulting phylogeny from BEAST2 was used as a guide tree (consensus tree).

Results

Bayesian inference recovered Atalophlebiinae as monophyletic with high support (Figure 1, Figure S1) and revealed new phylogenetic relationships among genera in internal clades. *Garinjuga* Campbell & Suter, 1988 appears as sister to all other Atalophlebiinae genera, including *Massartella* Lestage, 1930 which, in turn, is sister to the remaining Atalophlebiinae. Two other clades contain genera from South America and Australia (Figure 1).

Estimated age for the origin of Atalophlebiinae was 107.83 mya [median age, 95% highest posterior density interval (95% HPD): 85.76–136.63, Figure 1, Table 1, node 1], with initial diversification at 94.52 mya (68.53–123.44, Figure 1, Table 1, node 2). Thus, both the origin and initial lineage divergence occurred in the Cretaceous, during the second phase of Gondwana breakup. The result suggests that most living genera originated within their current distribution area.

Inference of ancestral areas (Figure 2, Table 1) suggested that Atalophlebiinae originated in the Gondwana supercontinent after a vicariant event (node 1). Subsequently, this lineage diversified into a scenario that refers to the Gondwanic corridor formed by South America, Antarctica and Australia (node 2). Then vicariance again separated the lineage leading to *Massartella* from the remaining Atalophlebiinae at *ca.* 85 mya (node 3). Other Australian (*Jappa* Harker, 1954 and the clade *Atalomicria* + *Atalophlebia*) and South American (*Penaphlebia* Peters & Edmunds 1972 and the clade *Massartellopsis* Demoulin, 1955 + *Meridialaris* Peters & Edmunds, 1972) sister lineages resulted from vicariant events at *ca*. 57 mya (nodes 5, 9).

Discussion

The topology of Atalophlebiinae herein proposed contains four main lineages: *Garinjuga* from Australia, *Massartella* from South America, and two others with genera from South America and Australia region (Figures 1, 2 and S1). *Garinjuga* samples and new sequences addition (Table S1) helped to establish the relationship among main lineages of the group, which in previous research were unclear (see Monjardim et al., 2020). *Jappa* and *Austrophlebioides* Campbell & Suter, 1988 belong to the clade supported by node 8 (Figures 1 and 2) diverging from the results of Monjardim et al. (2020). This study did not recover any previously proposed clades within Atalophlebiinae based on morphological data (see Finlay & Bae, 2008; Christidis, 2006; Pescador & Peters, 1980). Considering that they occur in similar environments throughout their distribution, and therefore experience similar ecological filters, many species may have evolved similar characteristics independently (see Bower & Winemiller, 2019). This indicates that some morphological similarities shared between Atalophlebiinae genera may be the result of evolutionary convergence.

The results suggest that the most recent common ancestor between Atalophlebiinae and the lineage that originated the Malagasy group (here represented by *Radima*) lived in Gondwana in the Cretaceous period (Figures 1 and 2, Table 1, node 1). These lineages diverged allopatrically during the second phase of the supercontinent breakup (Thompson et al., 2019; Gibbons et al., 2013; Seton et al., 2012) and the vicariant event that promoted speciation is probably related to the separation between Madagascar/India from Antarctica/Australia. The breakup process between these areas began at *ca*. 135 mya, with the migration of the Indian Plate, culminating in the opening of the Indian Ocean at *ca*. 100 mya (Thompson et al., 2019; Gibbons et al., 2013; Seton et al., 2012).

The initial divergence in Atalophlebiinae occurs in a scenario that refers to the Gondwanian corridor formed by South America, Antarctica and Australia (Thompson et al., 2019; Reguero et al., 2014; Seton et al., 2012) during the Upper Cretaceous (Figures 1 and 2, Table 1, node 2). The ancestor of the *Garinjuga* lineage was probably limited to the geographic area where the divergence occurred (area C), which can be explained, for example, by a peripatric speciation, while the remaining lineage (node 3) inherits the entire ancestral range (scenario 3 in Ree et al., 2005). Subsequently, a vicariant event isolated an ancestral population (node 3) in South America that gave rise to the *Massartella* lineage. Changes in sea level, associated with temperature increase on the planet, culminated in several cycles of marine transgressions in Patagonia during the Upper Cretaceous and Paleocene (Haq, 2014; Parras & Griffin, 2013; Le Roux, 2012a; Malumián & Náñez, 2011), which could have prevented dispersal events between South America and the Antarctic Peninsula. In addition, the temperature and precipitation calculated for these periods indicates a climate warm and humid subtropical temperate in this region (Varela et al., 2018; Le Roux, 2012b). This fact may have induced mayfly populations to seek colder habitats in mountains, leaving the lowlands, which could also have promoted allopatric speciation. Events of the same nature could have also been responsible for isolating the lineages that originated *Atalophlebia* + *Atalomicria* and *Jappa* in Australia from *Massartellopsis* + *Meridialaris* and *Penaphlebia* in South America (node 5 and 9). Thus, marine transgressions and/or climate changes were probably strong enough to isolate Atalophlebiinae populations, as this group is adapted to cold streams and rivers and is extremely intolerant of saltwater (Dos Santos et al., 2018).

Massartella descended from the oldest split which produced one lineage presently restricted to South America. Nowadays it has a wide and disjunct distribution on the tabletops of the Pantepui region and in the mountains of the Atlantic Forest (Domínguez, Molineri, Pescador, Hubbard, Nieto, 2006; Pescador & Peters, 1990). *Massartellopsis* and *Meridialaris* speciated during the uplift of the Andes (Figures 1 and 2, Table 1, node

6), a region where they are currently found, but where *Massartella* does not occur (Hoorn et al. 2010; Derka Svitok, Schlögl, 2009; Domínguez et al., 2006).

New Zealand emerges as an important factor in the diversification of Atalophlebiinae, after events of unclear nature (Figures 1 and 2, Table 1, node 4 and 8), possibly related to extinction events (or with sample gap), during the initial period of its separation from Australia (~85 mya) until the opening of the Tasman Sea (~50 mya, see Bache et al., 2014). An ancestral population isolated in this region (node 10) was the origin of the lineage of *Neozephlebia* Penniket, 1961, while another lineage increased its range by dispersal to Australia (node 11). Subsequently, peripherical speciation formed the lineage (node 19) in New Zealand region, while others (node 12) inherited the entire ancestral range (scenario 3 in Ree et al., 2005). Later, that lineage (node 12) was divided by a vicariant event, probably related to landscape changes during the opening of the Tasman Sea and Tasmanian Gateway at *ca.* 50–35 mya (see Scher et al., 2015; Bache et al., 2014). After the separation of the Gondwanic corridor (~35 mya, Elsworth et al., 2017; Scher et al., 2015; Seton et al., 2012), speciation occurred within the current distribution areas, indicating that Atalophlebiinae was unable to disperse across transoceanic barriers and suggests that most living taxa are the result of more recent local historical and ecological processes.

The biogeographic history of Atalophlebiinae is congruent with events that occurred during the second phase of the gradual process of Gondwana breakup in the Cretaceous and Paleogene. Our results provided evidence that vicariance and dispersal both played roles in the history of diversification prior to the completion of the second phase of Gondwana breakup. This pattern, together with natural extinction processes and its low dispersal capacity across transoceanic barriers, may explain its absence in other regions that form the circum-Antarctic pattern, such as tropical Africa, Madagascar and India.

Figures and table

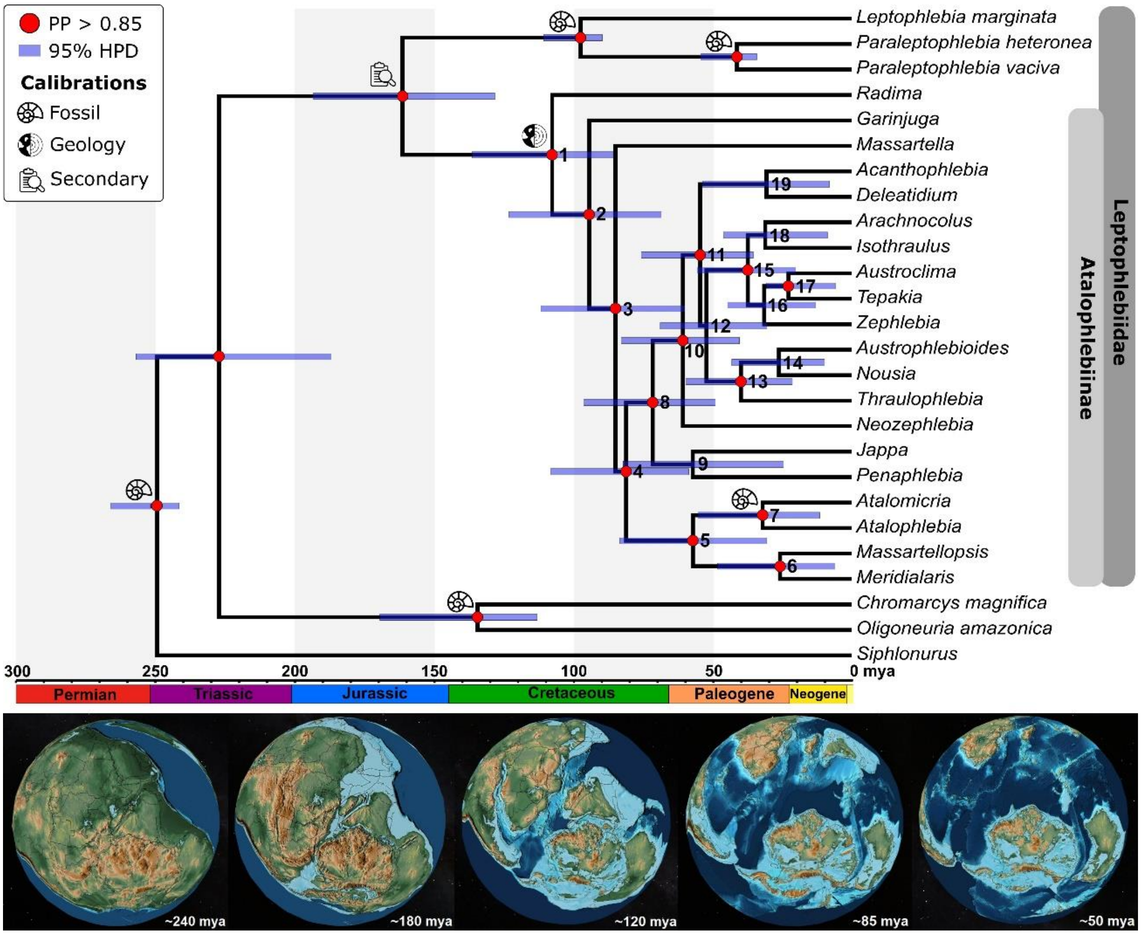

Figure 1. Time-calibrated [millions of years ago (mya)] phylogeny for Atalophlebiinae. Posterior probabilities (PP) referring to Bayesian inference tree (Figure S1). HPD: highest posterior density. Nodes used to calibrate tree were identified with specific symbols according to type of calibration (top-left box). Node numbers refer to Figure 2 and Table 1. Palaeogeographical maps reconstructed by Scotese's PALEOMAP Project available in Global Geology (Scotese & Dreher, 2012).

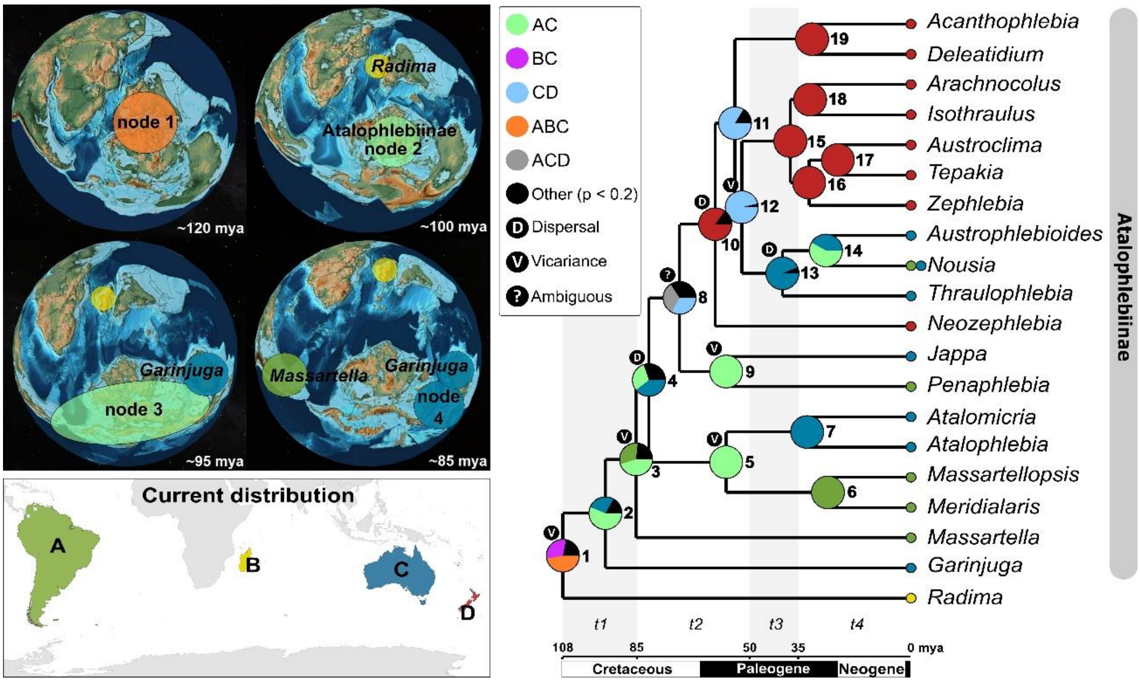

Figure 2. Reconstruction of ancestral area for Atalophlebiinae using Dispersal-Extinction-Cladogenesis (DEC) model. Palaeogeographical maps show probable scenarios of origin and initial diversification of the group. Nodes were colored according to the areas with highest relative probability inferred by DEC. Node numbers refer to Figure 1 and Table 1. The lower-left map represents the three areas (A, C, D) and Madagascar (for *Radima* outgroup) of current genera distribution implemented combined with time slices (*t1*, *t2*, *t3* and *t4*) in the matrix of dispersal probabilities (Table S4). Palaeogeographical maps reconstructed by Scotese's PALEOMAP Project available in Global Geology (Scotese & Dreher, 2012).

Table 1. Combined results of divergence time and ancestral area evolution of Atalophlebiinae based on BEAST2 time tree and Dispersal-Extinction-Cladogenesis (DEC). Node numbers refer to Figures 1 and 2; HPD: highest posterior density; s.w.a.: speciation within area.

Clade	**Divergence time (mya)**		**DEC**		
	Median	**95% HPD**	**Ancestral area**	**Probabilities**	**Event**
Root	249.95	242.09–266.57	-	-	-
Oligoneuriidae + Leptophlebiidae	227.69	187.32–257.53	-	-	-
Oligoneuriidae (Chromarcys + Oligoneuria)	134.62	113.24–169.86	-	-	-
Leptophlebiidae	161.68	128.42–193.84	-	-	-
Leptophlebiinae (*Leptophlebia* + *Paraleptophlebia*)	97.58	89.77–110.9	-	-	-
Paraleptophlebia	41.36	34.19–54.5	-	-	-
Node 1	107.83	85.76–136.63	ABC	0.47	vicariance
Node 2	94.52	68.53–123.44	AC	0.56	s. w. a
Node 3	85.05	60.11–111.87	AC	0.45	vicariance
Node 4	81.21	58.67–108.4	C	0.39	dispersal
Node 5	57.18	30.64–83.5	AC	1	vicariance
Node 6	25.83	6.09–48.3	A	1	s. w. a
Node 7	32.1	11.53–55.45	C	1	s. w. a
Node 8	71.66	49.07–96.49	CD	0.33	ambiguous
Node 9	57.24	24.60–82.25	AC	1	vicariance
Node 10	60.79	40.42–83.02	D	0.84	dispersal
Node 11	54.6	35.45–75.74	CD	0.83	s. w. a
Node 12	52.3	30.55–69.01	CD	0.96	vicariance
Node 13	39.9	21.37–59.72	C	0.94	dispersal
Node 14	26.26	9.83–43.26	AC	0.58	s. w. a
Node 15	37.44	20.24–55.71	D	1	s. w. a
Node 16	31.55	13.05–44.72	D	1	s. w. a
Node 17	22.8	5.95–30.78	D	1	s. w. a
Node 18	31.17	8.74–46.21	D	1	s. w. a
Node 19	30.7	8.05–53.74	D	1	s. w. a

Supporting information

Table S1. List of Atalophlebiinae and outgroups used in this research, including GenBank accession numbers and specimen voucher numbers.

Taxon	GenBank Accession Code		Voucher	Location
	28S	*COI*		
Leptophlebiidae				
Atalophlebiinae				
Acanthophlebia cruentata	EU874479.1	KX038486.1	-	New Zealand
Arachnocolus phillipsi	EU874482.1	KX038355.1	-	New Zealand
Atalomicria sp.	EU874483.1	JN289985.1	-	Australia
Atalophlebia sp.	EU874484.1	KP697596.1	-	Australia
Austroclima sp.	EU874485.1	KX037957.1	-	New Zealand
Austrophlebioides sp.	AY749929.1	KP697628.1	-	Australia
Deleatidium cornutum	EU874488.1	KX038144.1	-	New Zealand
Garinjuga sp.	this research	JN290008.1	-	Australia
Garinjuga sp.	this research	JN289965.1	-	Australia
Garinjuga sp.	this research	JN289971.1	-	Australia
Garinjuga sp.	this research	JN290009.1	-	Australia
Isothraulus abditus	EU874489.1	MH127965.1	-	New Zealand
Jappa sp.	EU874490.1	KP697484.1	-	Australia
Massartella alegrettae	this research	this research	DZUP515217	Brazil
Massartella alegrettae	this research	this research	DZUP515218	Brazil
Massartella brieni	this research	this research	LabEnt3228a	Brazil
Massartella devani	this research	this research	TD006a	Venezuela
Massartella devani	this research	this research	TD006b	Venezuela
Massartella hirsuta	this research	this research	TD007c	Venezuela
Massartella venezuelensis	this research	this research	TD002a	Venezuela
Massartella sp.	this research	this research	Ep4007	Brazil
Massartella sp.	this research	this research	Ep5260	Brazil
Massartella sp.	this research	this research	FG001a	Brazil
Massartella sp.	this research	this research	FG001g	Brazil
Massartella sp.	this research	this research	FG001h	Brazil
Massartella sp.	this research	this research	FG001j	Brazil
Massartella sp.	this research	this research	FG002d	Brazil
Massartella sp.	this research	this research	FG010e	Brazil
Massartella sp.	this research	this research	FG010f	Brazil
Massartella sp.	this research	this research	FG011e	Brazil
Massartella sp.	this research	this research	FG011f	Brazil
Massartella sp.	this research	this research	FG011g	Brazil
Massartella sp.	this research	this research	FG011h	Brazil
Massartella sp.	this research	this research	FG011i	Brazil

Massartella sp.	this research	this research	FG011j	Brazil
Massartella sp.	this research	this research	FG017a	Brazil
Massartella sp.	this research	this research	FG017b	Brazil
Massartella sp.	this research	this research	FG026a	Brazil
Massartella sp.	this research	this research	FG026b	Brazil
Massartella sp.	this research	this research	FG026c	Brazil
Massartella sp.	this research	this research	FG026d	Brazil
Massartella sp.	this research	this research	FG028a	Brazil
Massartella sp.	this research	this research	MM076	Brazil
Massartella sp.	this research	this research	TD005a	Venezuela
Massartella sp.	this research	this research	TD008a	Venezuela
Massartella sp.	this research	this research	TD008d	Venezuela
Massartella sp.	this research	this research	TD009	Venezuela
Massartella sp.	this research	this research	FG027	Brazil
Massartella sp.	this research	this research	FG036a	Brazil
Massartella sp.	this research	this research	FG036b	Brazil
Massartella sp.	this research	this research	FG036c	Brazil
Massartella sp.	this research	this research	MM006a	Brazil
Massartella sp.	this research	this research	MM006b	Brazil
Massartella sp.	this research	this research	MM040a	Brazil
Massartella sp.	this research	this research	MM040b	Brazil
Massartella sp.	this research	this research	FG024b	Brazil
Massartella sp.	this research	this research	FG029a	Brazil
Massartella sp.	this research	this research	FG029b	Brazil
Massartella sp.	this research	this research	FG038a	Brazil
Massartella sp.	this research	this research	FG038b	Brazil
Massartella sp.	this research	this research	FG002e	Brazil
Massartella sp.	this research	this research	FG003a	Brazil
Massartella sp.	this research	this research	FG003b	Brazil
Massartella sp.	this research	this research	FG011k	Brazil
Massartella sp.	this research	this research	FG014a	Brazil
Massartella sp.	this research	this research	FG014d	Brazil
Massartella sp.	this research	this research	FG017d	Brazil
Massartella sp.	this research	this research	FG017e	Brazil
Massartella sp.	this research	this research	FG017f	Brazil
Massartella sp.	this research	this research	FG019	Brazil
Massartella sp.	this research	this research	FG037a	Brazil
Massartella sp.	this research	this research	FG037b	Brazil
Massartella sp.	this research	this research	FG037c	Brazil
Massartella sp.	this research	this research	FG039a	Brazil
Massartella sp.	this research	this research	FG039b	Brazil
Massartella sp.	this research	this research	Ep1797b	Brazil
Massartella sp.	this research	this research	Ep4803a	Brazil

Massartella sp.	this research	this research	Ep4803b	Brazil
Massartella sp.	this research	this research	Ep6792	Brazil
Massartella sp.	this research	this research	Ep6875b	Brazil
Massartella sp.	this research	this research	Ep6880b	Brazil
Massartella sp.	this research	this research	FG010a	Brazil
Massartella sp.	this research	this research	FG010b	Brazil
Massartella sp.	this research	this research	FG010c	Brazil
Massartella sp.	this research	this research	FG010d	Brazil
Massartellopsis irarrazavali	EU874492.1	-	-	Chile
Meridialaris diguillina	EU874493.1	-	-	Chile
Meridialaris laminata	EU874494.1	-	-	Chile
Neozephlebia scita	EU874495.1	KX038315.1	-	New Zealand
Nousia sp. 1	EU874498.1	MG976136.1	-	Australia
Nousia sp. 2	EU874497.1	MG976137.1	-	Australia
Penaphlebia barriai	EU874499.1	-	-	Chile
Penaphlebia chilensis	EU874500.1	-	-	Chile
Penaphlebia vinosa	EU874501.1	-	-	Chile
Tepakia sp.	EU874502.1	KX038039.1	-	New Zealand
Thraulophlebia sp.	EU874491.1	KC348534.1	-	Australia
Zephlebia sp.	EU874503.1	KX038630.1	-	New Zealand
Malagasy group				
Malagasy taxon 4	EU874474.1	-	-	Madagascar
Malagasy taxon 5	EU874475.1	-	-	Madagascar
Malagasy taxon 6	EU874476.1	-	-	Madagascar
Radima sp. 1	EU874473.1	-	-	Madagascar
Radima sp. 2	EU874477.1	-	-	Madagascar
Radima sp. 3	EU874478.1	-	-	Madagascar
Leptophlebiinae				
Leptophlebia marginata	EU874508.1	HQ563240.1	-	Norway
Leptophlebia vespertina	EU874509.1	-	-	Norway
Paraleptophlebia heteronea	EU874512.1	JQ663272.1	-	USA
Paraleptophlebia vaciva	EU874515.1	JQ663110.1	-	USA
Oligoneuriidae				
Chromarcys magnifica	MG516507.1	MG516460.1	-	Thailand
Oligoneuria amazonica	MG516508.1	KT201517.1	-	Brazil
Siphlonuridae				
Siphlonurus sp.	AY749958.1	KR141711.1	-	USA

Table S2. Details of the primers used in this research.

Gene	Primers label	Primers sequences (5' - 3')	Amplicon lengths
28S	D2-3549 F	AGTCGTGTTGCTTGATAGTGCAG	1219 bp
	D5-4749 R	GTTACACACTCCTTAGCGGA	
COI	LCO1409	GGTCAACAAATCATAAAGATATTGG	691 bp
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	

Table S3. Best substitution models inferred by jModelTest2 using Corrected Akaike Information Criterion (AICc).

Genes	Evolution model	AICc	wt
28S	**GTR+G**	**12516.44047**	**0.727725**
	TIM2+G	12518.407	0.272233
	TIM1+G	12535.95078	4.22E-05
COI	**TrN+G**	**4160.520256**	**0.684445**
	TIM1+G	4164.340662	0.101332
	TIM3+G	4164.478022	0.094606

Table S4. Probability of dispersal according to the availability of connections among areas across four time slices. South America (A), Madagascar (B), Australia (C) and New Zealand (D).

Time Slice	Areas	A	B	C	D
0 - 35 mya (*t4*)	**A**	1	0.1	0.1	0.1
	B	0.1	1	0.1	0.1
	C	0.1	0.1	1	0.3
	D	0.1	0.1	0.3	1
35 - 50 mya (*t3*)	**A**	1	0.1	0.3	0.3
	B	0.1	1	0.1	0.1
	C	0.3	0.1	1	0.3
	D	0.3	0.1	0.3	1
50 - 85 mya (*t2*)	**A**	1	0.1	0.3	0.3
	B	0.1	1	0.1	0.1
	C	0.3	0.1	1	1
	D	0.3	0.1	1	1
85 - 108 mya (*t1*)	**A**	1	0.3	1	1
	B	0.3	1	0.3	0.3
	C	1	0.3	1	1
	D	1	0.3	1	1

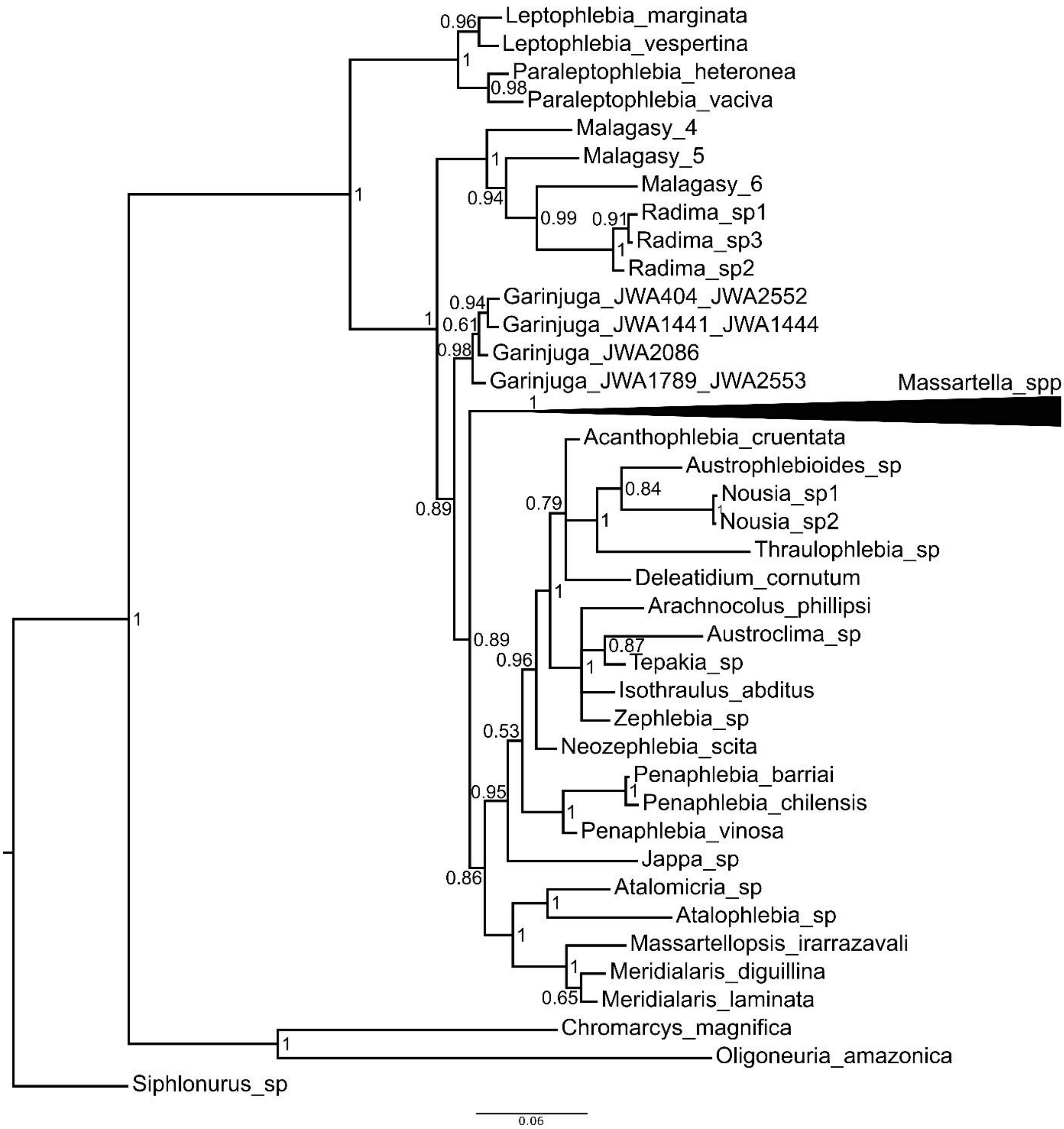

Figure S1. Phylogenetic relationships of Atalophlebiinae inferred by Bayesian analysis on a concatenated molecular dataset (*28S+COI*).

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CAPÍTULO II

Evolutionary history of *Massartella* (Ephemeroptera, Leptophlebiidae) suggests ancient connection between Pantepui and Atlantic Forest highlands

Evolutionary history of *Massartella* (Ephemeroptera, Leptophlebiidae) suggests ancient connection between Pantepui and Atlantic Forest highlands*

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Abstract

South America's complex geological and environmental dynamics contributed to the origin of Neotropical biodiversity and shaped the pattern of species distribution on the continent. *Massartella* Lestage, 1930 (Ephemeroptera, Leptophlebiidae, Atalophlebiinae) is a genus currently composed of five species endemic to South America, adapted to cold environments and mountaintops in rainforests regions. This genus has a disjunct distribution, occurring in mountains along the Atlantic Forest and in the Venezuelan Pantepui region, but being absent between these areas. Here we use Bayesian phylogenetic trees and fossil based molecular dating to reconstruct the evolutionary history of *Massartella*. Results recovered the genus, and the Pantepui and Atlantic Forest lineages as reciprocally monophyletic and suggest that the last common ancestor of these clades lived *ca*. 66 mya (median age, 95% HPD: 40.46–95.95 mya) during the Cretaceous–Paleogene. The diversification processes started at the same time in both lineages, and the relationships among species of the Pantepui were recovered with high support. On the other hand, many relationships among taxonomic units in the Atlantic Forest mountains remained unclear. The reciprocal monophyly of clades indicates no subsequent connections between these areas, or the extinction of intermediates. Mountain biodiversity has the signature of both ancient and recent geoclimatic events and ecological processes, and climatic oscillations and cycles of marine incursions in the South America may have been responsible for isolating the Pantepui and Atlantic Forest lineages, as well as the speciation processes within these regions.

Keywords

disjunct distributions, divergence times, Ephemeroptera, Leptophlebiidae, mountains, Neotropical, South America.

Introduction

Disjunct distributions result from geological events, environmental changes and/or dispersal. South America has gone through complex geological and landscape dynamics, including uplift of large mountain ranges, river course changes (Hoorn et al., 2010), cycles of marine incursions (Le Roux, 2012a; Bloom & Lovejoy, 2011, 2017), and habitat retractions and expansions (Ledo & Colli, 2017; Leite et al., 2016; Sobral-Souza et al., 2015), which contributed to the origin of Neotropical biodiversity and shaped the pattern of species distribution on the continent (Meseguer et al., 2020; Sánchez-Herrera et al., 2020; Hoorn et al., 2010). A recurring biogeographic pattern in South America is the close relationship among taxa from the Amazon and the Atlantic Forest regions, usually associated with recent reconnections between these forests, ancient geoclimatic events, and dispersal (Pirani et al., 2020; Cabanne et al., 2019; Capurucho et al., 2018; Fabre et al., 2017; Prates et al., 2017; Batalha-Filho et al., 2013; Costa, 2003).

Massartella Lestage, 1930 (Ephemeroptera, Leptophlebiidae, Atalophlebiinae) is a mayfly genus endemic to South America, adapted to cold environments and mountaintops, occupying streams and rivers in mountainous regions of rainforests (Derka et al., 2009; Domínguez et al. 2006; Derka, 2002; Pescador & Peters 1990; Savage 1987). There are five described species with disjunct distributions: *Massartella brieni* (Lestage, 1924) and *Massartella alegrettae* Ulmer, 1943 in mountains along the Atlantic Forest; and *Massartella venezuelensis* Pescador & Peters, 1990, *Massartella devani* Derka, 2002 and *Massartella hirsuta* Derka, Svitok & Schlögl, 2009 occurring in the Guiana highlands and uplands of Southern Venezuela (Pantepui), but they are absent between these areas (Derka et al., 2009; Domínguez et al., 2006). Research shows that this genus probably originated in the Patagonian Shield, as part of an ancient lineage of Atalophlebiinae in southern South America, and it is phylogenetically close to Patagonian, Andean and Australian taxa (see Gatti et al. 2021; Monjardim et al. 2020; Savage 1987).

Considering the geological evolution and environmental changes in South America, here we investigated the phylogenetic relationships of *Massartella* species, estimated divergence times from their closest relatives, and inferred biogeographic scenarios. This is the first phylogenetic analysis to incorporate molecular data from all described *Massartella* species, adequately covering the geographic range of this genus in South America.

Materials and Methods

Taxon sampling and molecular dataset

All five described *Massartella* species and eight undescribed species currently in the process of description (Salles et al. and Derka et al. in preparation), from throughout their distribution (Figure 1, Table S1), were sampled and sequenced for two molecular markers (Table S2): the D2–D5 region of the *28S* ribosomal RNA gene (Gillespie et al., 2004, 2005) and a partial region of the mitochondrial *Cytochrome c oxidase subunit 1* gene (*COI*, Folmer et al., 1994). We selected 12 outgroups based on a recent phylogeny of Ephemeroptera (Monjardim et al., 2020; Ogden et al., 2019; O'Donnell & Jockusch, 2008) and the availability of fossils for date calibration (Table S3). All specimens sequenced in this research are stored in the Museu de Entomologia of the Universidade Federal de Viçosa, under the care of the authors (FFS) or in the Coleção Zoológica Norte Capixaba, Universidade Federal do Espírito Santo, Brazil.

Phylogenetic analyses

Sequences were aligned in Geneious 9.0 (www.geneious.com) and nucleotide substitution models for each marker were selected using the Corrected Akaike Information Criterion (AICc) in jModelTest2 (Darriba et al., 2012) on CIPRES (Miller et al., 2010). Saturation level of sequences was verified by Xia's test (Xia et al., 2003) in DAMBE 7 (Xia, 2018) and the third codon position of *COI* was excluded from subsequent analyses. The concatenated molecular data matrix comprised 1,624 base pairs (1,198 bp from *28S* and 426 bp from *COI*) and 91 sequences (Alignment S1). Models selected for each partition were General Time Reversible, including heterogeneous rates across sites following a gamma distribution (GTR+G) for *28S*, and Tamura-Nei also including gamma (TrN+G) for *COI* (Table S4).

The phylogenetic tree was inferred using Bayesian inference in MrBayes 3.2.7a (Ronquist et al., 2012) on CIPRES (Miller et al., 2010). Eight Markov chain Monte Carlo (MCMC) iterations were run simultaneously for 3.865 million generations with sampling trees every 1,000 generations and 25% of burn-in, until the convergence diagnostic reached the stop value [standard deviation of split frequencies < 0.01 and monitoring the effective sample size (ESS)]. Node support was provided by posterior probabilities (PP), as directly estimated from the majority rule consensus topology. Considering recent discussions about statistical significance (see Amrhein et al., 2019; Hurlbert et al., 2019; Pike, 2019; Wasserstein et al., 2019), the logic, background knowledge and experimental design were considered alongside PP and nodes with values higher than 0.85 were therefore considered well-supported.

Divergence times

We used the relaxed uncorrelated lognormal molecular clock with a tree prior to using the birth–death incomplete sampling algorithm (Stadler, 2009). Substitution models for each gene were selected according to AICc and monophyly was forced based on BI results. Clock point calibrations were based on seven date priors from fossils (n=5), geological event and probabilities of dispersal (n=1, adapted from Landis, 2017), and secondary data derived from previous analyses (n=1): 1) Root was calibrated to represent the minimum and maximum (242–290 mya) ages of fossil mayfly species *Protereisma permianum* Sellards 1907 (Protereismatidae), believed to be one of the stem groups of Ephemeroptera (Sroka et al., 2015; Godunko et al., 2011; Grimaldi & Engel, 2005), and the mayfly *Triassonurus doliiformis* Sinitshenkova & Papier, 2005 (Siphlonuridae), the lineage with the oldest origin in our dataset (Lognormal distribution, offset= 242.0, Mean= 8.5, Standard deviation= 1.0, mean in real space). 2) Oligoneuriidae initial divergence was calibrated based on *Incogemina nubila* Storari, Rodrigues, Saraiva & Salles 2020 (Oligoneuriidae) (Lognormal, offset= 112.6, M= 30.0, S= 1.0, mean in real space). 3) Leptophlebiidae initial divergence was calibrated based on the age of ~175 mya given by Grimaldi & Engel (2005) for origin of the family (Normal distribution, Mean= 175.0, Sigma= 25.0). 4) Leptophlebiinae initial divergence was calibrated based on the fossil *Aureophlebia sinitshenkovae* Peters & Peters 2000 (Leptophlebiinae) (Lognormal, offset= 89.3, M= 20.0, S= 1.0, mean in real space). 5) *Paraleptophlebia* initial divergence was calibrated based on the fossil *Paraleptophlebia prisca* (Pictet & Hagen, 1856) (Lognormal, offset= 33.9, M= 13.0, S= 1.0, mean in real space). 6) Calibration of the most recent common ancestor of Atalophlebiinae from the lineage that originated *Radima* Akers, Peters & Peters, 2003 (Lognormal, offset= 85.0, M= 21.0, S= 1.0, mean in real space) considered the final period of separation between Madagascar/India and Antarctica/Australia (~100 mya, Thompson et al. 2019; White et al., 2013; Gibbons et al. 2013; Seton et al., 2012) and low probability of dispersal between these areas after separation (see Suplementary data of Landis, 2017; Sanmartín & Ronquist 2004). 7) Divergence between *Atalophlebia* Eaton, 1881 and *Atalomicria* Harker, 1954 was calibrated with the fossil *Atalophlebia culleni* Etheridge & Olliff 1890 (Lognormal, offset= 2.6, M= 20.0, S= 1.0, mean in real space). Fossil information is available at the Fossilworks Paleobiology Database (http://fossilworks.org).

The input file was constructed in BEAUti v2.5.2 and ran in BEAST2 v2.5.2 (Bouckaert et al., 2019) for 100 million generations, and trees were sampled every 10,000 generations. The convergence of runs and the effective sample size (ESS > 1,000) of parameters were examined in Tracer v1.7.1 (Rambaut et al., 2018). A tree topology with maximum clade credibility, using a burn-in of 25%, was constructed with TreeAnnotator v2.5.2 and these analyses were run on CIPRES (Miller et al., 2010).

Results

Bayesian inference recovered *Massartella*, and both the Pantepui and Atlantic Forest lineages, as monophyletic with high support (Figure 2, Table 1). The last common ancestor among all these lineages lived *ca.* 66.84 mya [median age, 95% highest posterior density interval (95% HPD): 40.46–95.95 mya, Fig. 3 and Table 1], *i.e.*, between the Middle Cretaceous and the Paleogene. The early diversification in the Pantepui (56.01 mya, 95% HPD: 29.7–81.66 mya) and Atlantic Forest lineages (48.85 mya, 95% HPD: 21.99–76.55 mya) may have occurred at about the same time during the Upper Cretaceous–Paleogene (Figure 3, Table 1).

The relationships among Pantepui species were recovered with high support, and they form two clades, one composed by *M. venezuelensis* and *M. hirsuta* + *M. cf. devani*, and another including *Massartella* sp. 3 and sp. 1 + sp. 2 (Figures 2 and 3). On the other hand, in the Atlantic Forest lineage, the relationships among species are unclear, even though they comprise cohesive groups, with the exception of *M. brieni*. The monophyly of *M. brieni* was rejected, and the results suggest that this taxon is polyphyletic and represent a complex of cryptic species (Figures 2 and 3).

Discussion

The topology of *Massartella* herein recovered suggests a connection between Pantepui and Atlantic Forest highlands in the Cretaceous–Paleogene periods (66.84 mya, 95% HPD: 40.46–95.95 mya, Figure 3 and Table 1). The reciprocal monophyly of Pantepui and Atlantic Forest clades indicates no subsequent connections between these areas, or the extinction of intermediates. Species occurring in the southern portion of the Atlantic Forest are part of the most ancient lineage in the region, indicating that the last common ancestor between the two main clades of *Massartella* probably lived in southwestern South America, before the intensification of Andean uplift (see Hoorn et al., 2010).

Changes in oceans climate and the monsoons breakdown, related to tectonic processes, induced the climatic oscillations and cycles of marine incursions in the Cretaceous–Paleogene, making the climate in southwestern South America warm–temperate and humid (Varela et al., 2018; Le Roux, 2012ab; Scotese & Dreher, 2012; Iglesias et al., 2011). Therefore, *Massartella* ancestor would have restricted possibilities for suitable niches on highlands in warmer periods, such as the Cretaceous–Paleogene, and they must have taken advantage of the cooler periods of climatic oscillations to disperse across the continent (see Varela et al., 2018; Le Roux, 2012b; Iglesias et al., 2011). Thus, it is probable that the ancestor of *Massartella* dispersed to the Atlantic Forest mountains to the northeast and along the ancient cratonic landscape of the Andean region and/or some areas of table mountains in western Amazonia to the Pantepui highlands, settling in these regions before the climate warmed up. Subsequently, the apex of the temperature increase and the marine incursions may have been responsible for isolating the Pantepui and Atlantic Forest lineages and extinguishing ancestral populations in the lowlands and mid altitudes (Figure 3). Similar patterns of distribution and phylogenetic relationships have already been described for other groups of animals, but in more recent time frames (see Prates et al. 2017; Leite et al. 2015).

After the activation of the Circum-Antarctic Circulation (~ 35 mya), causing global temperature to decline, and the intensified of the Andes uplift the warm–temperate climate that characterized Patagonia was replaced by the cool–temperate (Elsworth et al., 2017; Iglesias et al., 2011; Hoorn et al., 2010). Thus, cold temperate forests and arid biomes emerged in the region (Le Roux, 2012b; Iglesias et al., 2011). This may be the reason for the absence of *Massartella* in Patagonia today, since rainforests are common habitats in their current distribution areas and must be important for their persistence. *Massartella* may have also suffered from competitive exclusion by other cold-adapted Leptophlebiidae, such as *Massartellopsis* Demoulin 1955, *Meridialaris* Peters & Edmunds 1972 and *Penaphlebia* Peters & Edmunds 1972, which originated in Patagonia

Shield in this context of landscape changes (Monjardim et al. 2020; Savage 1987), and occur from there to the Andean and Bolivian mountains (Domínguez et al., 2006).

We cannot say, however, that the ancestor of *Massartella* certainly lived in southwestern South America. Methodological limitations prevent us from making precise inferences about the suitability of ecological niches in very ancient times, thus we cannot rule out the hypothesis of colonization of Pantepui through the Brazilian Shield (see Derka et al., 2009). Nonetheless, climatic oscillations and cycles of marine incursions occurred on a continental scale in South America, and if the ancestor lived in the Brazilian and Guiana shields and the Amazon basin, these geoclimatic events would have potential to promote speciation of lineages in those regions as well.

In the Venezuelan highlands, each *Massartella* species are restricted to the specific tepui or the surrounding areas in La Gran Sabana (Figure 1A). Obviously, this scenario may change with increasing collecting efforts in poorly sampled areas of this region. On the other hand, we found both restricted and widely distributed species among different mountain ranges in the Atlantic Forest (Figure 1B). In the tropics, and especially for aquatic insects, the low seasonal variation in temperature produces greater thermal stratification along tropical mountains, selecting narrow thermal tolerances and limiting dispersal along altitudinal gradients, which in turn increases speciation rates (Polato et al., 2018; Janzen, 1967). However, in periods of climatic oscillation, the decrease in temperature would facilitate cold-adapted species on mountaintops to migrate downhill and use the valleys to disperse to other areas. Subsequently, as temperature increases, species would be induced to seek refuge in the highlands, not necessarily at the same place where they came from. These processes could lead to the rearrangement of range distributions, introgressions, hybridization, extinctions and speciation. Thus, the sum of these ecological and climatic processes likely promoted population divergence, higher speciation rates, higher cryptic diversity and higher accumulation of species over time in tropical mountains (Meseguer et al., 2020; Rahbek et al., 2019; Antonelli et al., 2018; Hazzi et al., 2018; Polato et al., 2018). These factors explain the complex distribution pattern and the phylogenetic relationship among *Massartella* species, since both the Pantepui and the Atlantic Forest mountains biota experienced vertical and horizontal migration with the climatic oscillations (Thomé et al., 2020; Rull et al., 2019a; Rull & Vegas-Vilarrúbia, 2019; Françoso et al., 2016). Thus, even though *Masartella* is part of an ancient lineage, its current distribution has been also influenced by recent geoclimatic events (e.g., Quaternary climatic oscillations).

Models based on future scenarios predict smaller potential distributions of species in the South American highlands due to global warming (Thomé et al., 2020; Rull et al., 2019b, Rödder et al., 2010). Therefore, *Massartella* is part of a large portion of Neotropical biodiversity that will be strongly affected by the accelerated climate change the planet is subject to. This fact, associated with habitat fragmentation and loss, can lead *Massartella* species to extinction, many probably still unknown to science.

In summary, the initial diversification of *Massartella* is older than previously thought and changes in the South American landscapes caused by ancient cycles of marine incursions and climatic oscillations may have promoted the divergence of Pantepui and Atlantic Forest lineages, and speciation within these regions. Mountain biodiversity has the signature of both ancient and recent geoclimatic events and ecological processes. Understanding how these complex environmental dynamics affected the patterns of diversification and distribution of the species that inhabit these regions are the keys to promote the conservation of Neotropical biodiversity.

Figures and table

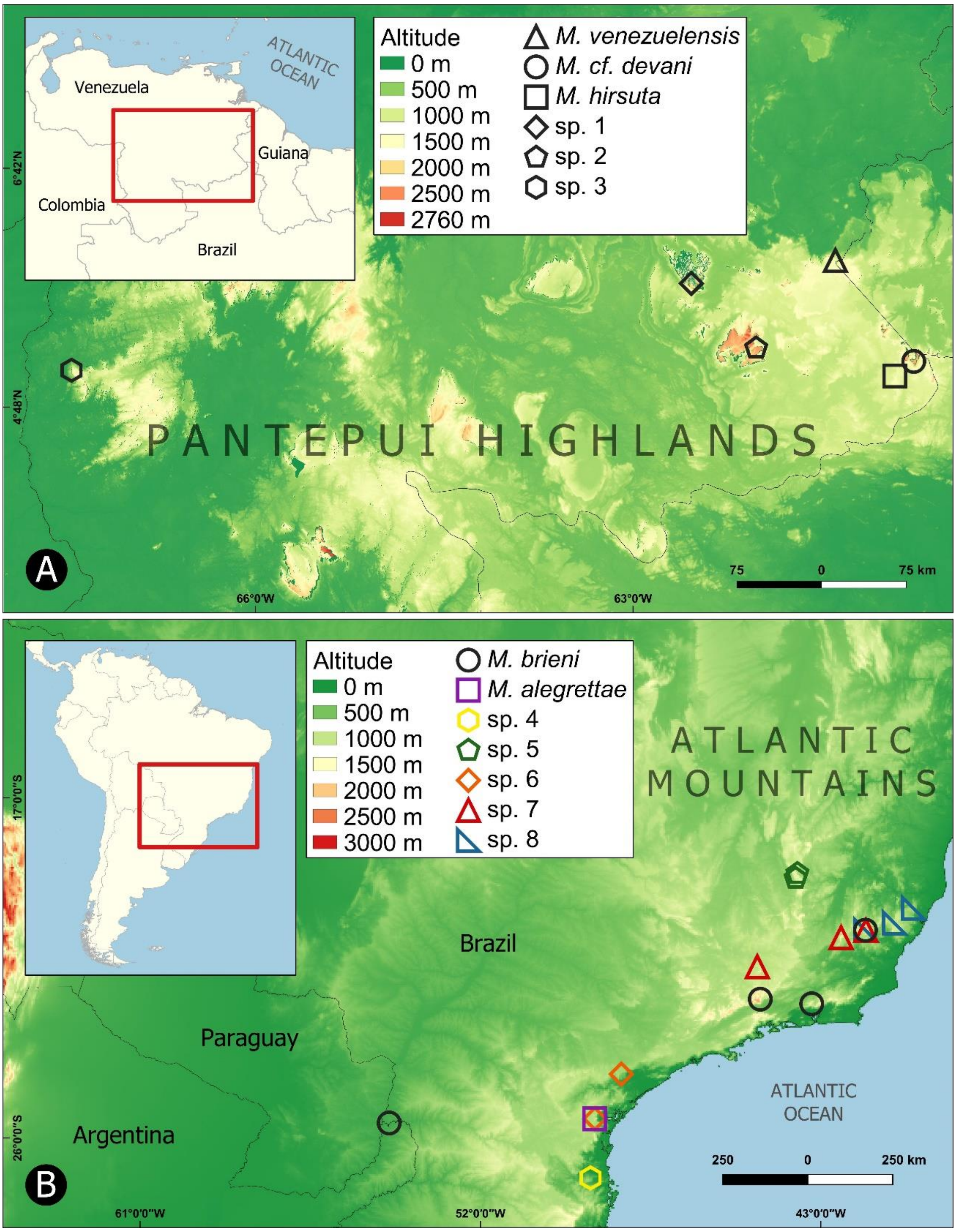

Figure. 1. Collecting localities. **A)** Pantepui highlands: ***M. venezuelensis*** (Salto del Danto), ***M. cf. devani*** (Mt. Roraima), ***M. hirsuta*** (Paraitepuy), **sp.1** (Auyán Tepui), **sp. 2** (Churí Tepui) and **sp. 3** (Cerro Cuao). **B)** Atlantic Forest mountains: ***M. brieni*** (PARNA Caparaó, PARNA Itatiaia, Petrópolis and Foz do Iguaçu), ***M. alegrettae*** (Mananciais da Serra), **sp. 4** (Serra do Itajaí), **sp. 5** (Serra do Cipó, Lapinha da Serra, Peixe Tolo canyon),

sp. 6 (Mananciais da Serra and PE Intervales), **sp. 7** (PARNA Caparaó, Serra do Brigadeiro and Carrancas) and **sp. 8** (REBIO Augusto Ruschi, Domingos Martins and PARNA Caparaó). For detailed coordinates, see Table S1.

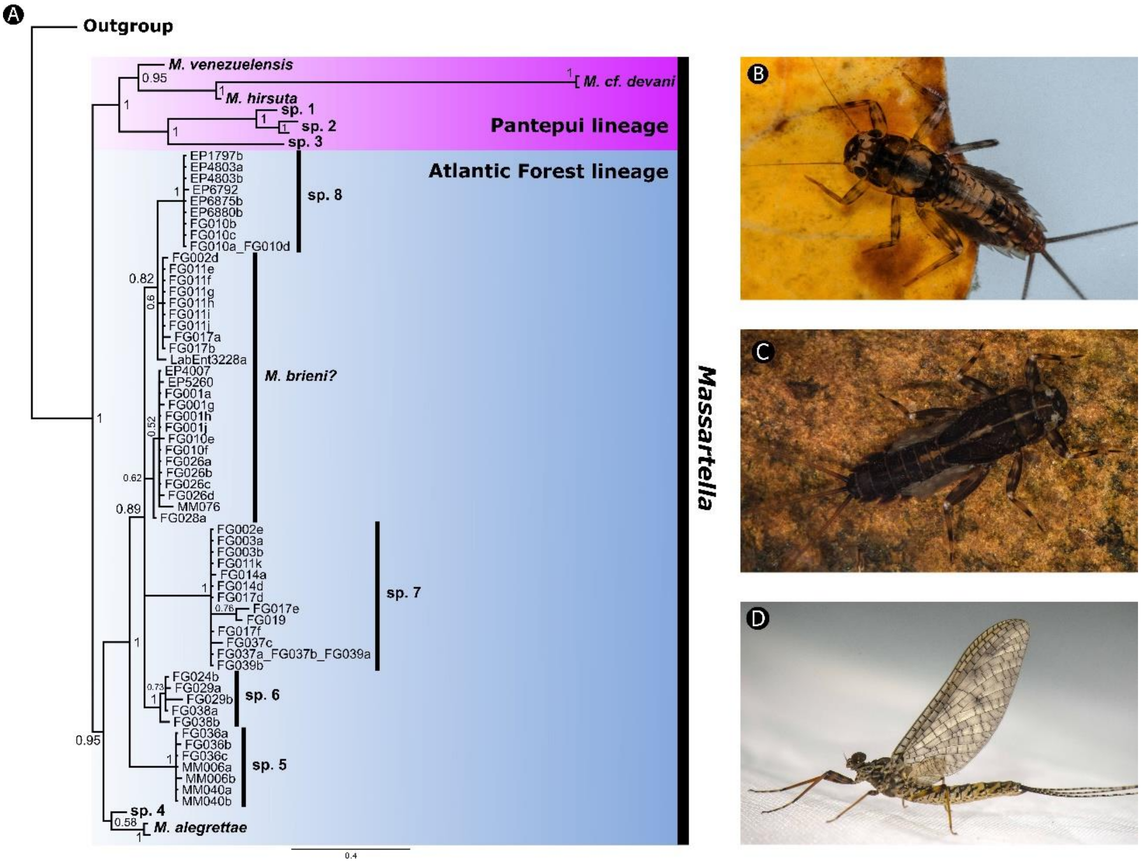

Figure 2. A) Phylogenetic relationships of *Massartella* inferred by Bayesian analyses on concatenated molecular dataset (*28S*+*COI*). *Massartella* spp. nymphs **(B–C)** and subimago **(D)**.

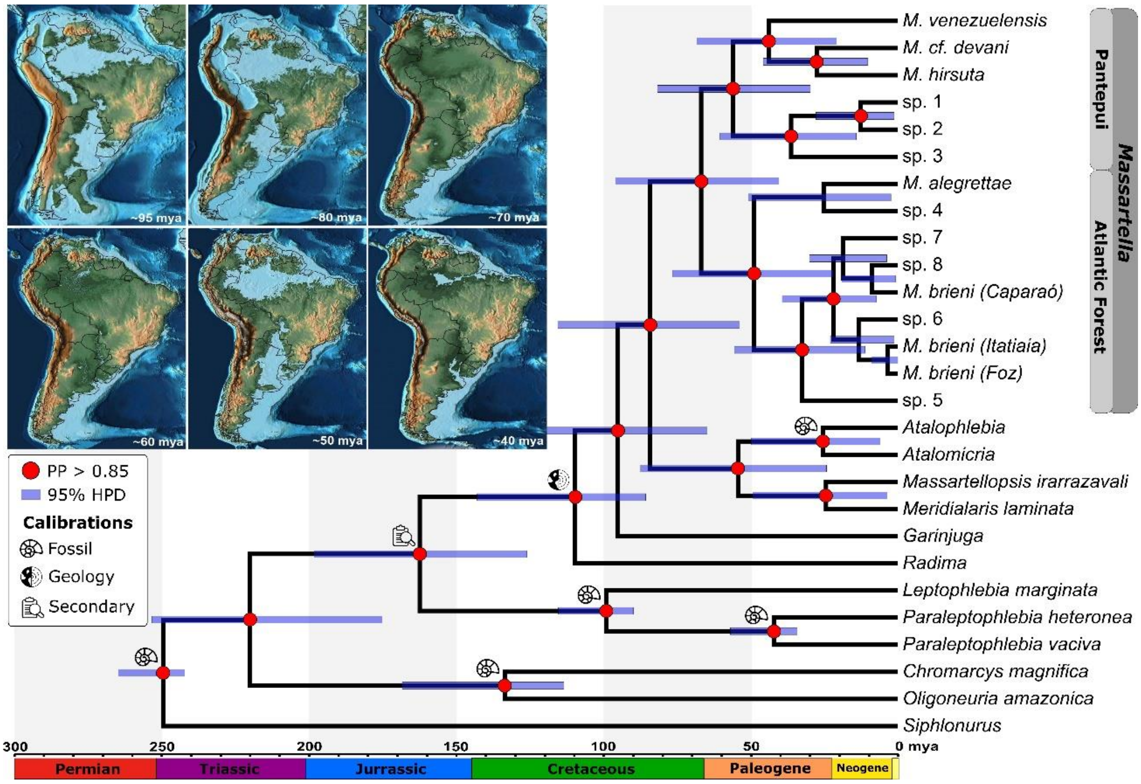

Fig. 3. Time-calibrated [millions of years ago (mya)] phylogeny for *Massartella*. Posterior probabilities (PP) referring to Bayesian inference tree (Figure 2). HPD: highest posterior density. Nodes used to calibrate tree were identified with specific symbols according to type of calibration (lower-left box). Palaeogeographical maps reconstructed by Scotese's PALEOMAP Project available in Global Geology (Scotese & Dreher, 2012).

Table 1. Combined results of divergence time and posterior probabilities (PP) of *Massartella* and the Pantepui and Atlantic Forest lineages based on BEAST2 and MrBayes analyses. HPD: highest posterior density interval.

Clade	**Divergence time (mya)**		**PP**
	Median	**95% HPD**	
Massartella + Andean and Australian taxon	84.12	53.79–115.46	0.88
Massartella	66.84	40.46–95.95	1
Pantepui lineage	56.01	29.7–81.66	1
Atlantic Forest lineage	48.85	21.99–76.55	0.95

Supporting information

Table S1. Detailed location of the Massartella samples used in this research, including voucher numbers.

Voucher	**Species**	**Country**	**State**	**Locality**	**Coordinates**	
DZUP515217	*M. alegrettae*	Brazil	Paraná	Pico do Marumbi	-25.4933	-48.9783
DZUP515218	*M. alegrettae*	Brazil	Paraná	Pico do Marumbi	-25.4933	-48.9783
Ep4007	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.4722	-41.8291
Ep5260	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.6251	-41.8242
FG001a	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5028	-41.8189
FG001g	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5028	-41.8189
FG001h	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5028	-41.8189
FG001j	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5028	-41.8189
FG002d	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5028	-41.8189
FG010e	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG010f	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG011e	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG011f	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG011g	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG011h	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG011i	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG011j	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG017a	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8191
FG017b	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8191
FG026a	*M. brieni*	Brazil	Rio de Janeiro	PN Itatiaia	-22.4268	-44.6185
FG026b	*M. brieni*	Brazil	Rio de Janeiro	PN Itatiaia	-22.4268	-44.6185
FG026c	*M. brieni*	Brazil	Rio de Janeiro	PN Itatiaia	-22.4268	-44.6185
FG026d	*M. brieni*	Brazil	Rio de Janeiro	PN Itatiaia	-22.4268	-44.6185
FG028a	*M. brieni*	Brazil	Paraná	PN Iguaçu	-25.6036	-54.4145
LabEnt3228a	*M. brieni*	Brazil	Rio de Janeiro	Vale do Amor	-22.45	-43.2408
MM076	*M. brieni*	Brazil	Minas Gerais	PARNA Caparaó	-20.5028	-41.8189
TD006a	*M. cf. devani*	Venezuela	Bolívar	Mt. Roraima	5.1629	-60.7693
TD006b	*M. cf. devani*	Venezuela	Bolívar	Mt. Roraima	5.1629	-60.7693
TD007c	*M. hirsuta*	Venezuela	Bolívar	Paraitepuy	5.0468	-60.9191
TD002a	*M. venezuelensis*	Venezuela	Bolívar	Salto del Danto	5.9657	-61.3918
TD005a	sp. 1	Venezuela	Bolívar	Auyán Tepui	5.7837	-62.5368
TD008a	sp. 2	Venezuela	Bolívar	Churí Tepui	5.2693	-62.0216
TD008d	sp. 2	Venezuela	Bolívar	Churí Tepui	5.2693	-62.0216
TD009	sp. 3	Venezuela	Amazonas	Cerro Cuao	5.0927	-67.4617
FG027	sp. 4	Brazil	Santa Catarina	PN Serra do Itajaí	-27.057	-49.0877
FG036a	sp. 5	Brazil	Minas Gerais	Lapinha da Serra	-19.1085	-43.6672
FG036b	sp. 5	Brazil	Minas Gerais	Lapinha da Serra	-19.1085	-43.6672
FG036c	sp. 5	Brazil	Minas Gerais	Lapinha da Serra	-19.1085	-43.6672
MM006a	sp. 5	Brazil	Minas Gerais	PARNA Serra do Cipó	-19.2671	-43.5158

MM006b	sp. 5	Brazil	Minas Gerais	PARNA Serra do Cipó	-19.2671	-43.5158
MM040a	sp. 5	Brazil	Minas Gerais	Peixe Tolo canyon	-19.0033	-43.6193
MM040b	sp. 5	Brazil	Minas Gerais	Peixe Tolo canyon	-19.0033	-43.6193
FG024b	sp. 6	Brazil	São Paulo	PE Intervales	-24.3101	-48.2745
FG029a	sp. 6	Brazil	São Paulo	PE Intervales	-24.3101	-48.2745
FG029b	sp. 6	Brazil	São Paulo	PE Intervales	-24.3101	-48.2745
FG038a	sp. 6	Brazil	Paraná	Mananciais da Serra	-25.4898	-48.9748
FG038b	sp. 6	Brazil	Paraná	Mananciais da Serra	-25.4898	-48.9748
FG002e	sp. 7	Brazil	Minas Gerais	PARNA Caparaó	-20.5028	-41.8189
FG003a	sp. 7	Brazil	Espírito Santo	PARNA Caparaó	-20.3967	-41.7356
FG003b	sp. 7	Brazil	Espírito Santo	PARNA Caparaó	-20.3967	-41.7356
FG011k	sp. 7	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG014a	sp. 7	Brazil	Espírito Santo	PARNA Caparaó	-20.5112	-41.807
FG014d	sp. 7	Brazil	Espírito Santo	PARNA Caparaó	-20.5112	-41.807
FG017d	sp. 7	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8191
FG017e	sp. 7	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8191
FG017f	sp. 7	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8191
FG019	sp. 7	Brazil	Espírito Santo	PARNA Caparaó	-20.5112	-41.8070
FG037a	sp. 7	Brazil	Minas Gerais	Carrancas	-21.4697	-44.6799
FG037b	sp. 7	Brazil	Minas Gerais	Carrancas	-21.4697	-44.6799
FG037c	sp. 7	Brazil	Minas Gerais	Carrancas	-21.4697	-44.6799
FG039a	sp. 7	Brazil	Minas Gerais	Serra do Brigadeiro	-20.7019	-42.4636
FG039b	sp. 7	Brazil	Minas Gerais	Serra do Brigadeiro	-20.7019	-42.4636
Ep1797b	sp. 8	Brazil	Espírito Santo	Domingos Martins	-20.3154	-41.0556
Ep4803a	sp. 8	Brazil	Espírito Santo	REBIO Augusto Ruschi	-19.8539	-40.5606
Ep4803b	sp. 8	Brazil	Espírito Santo	REBIO Augusto Ruschi	-19.8539	-40.5606
Ep6792	sp. 8	Brazil	Espírito Santo	REBIO Augusto Ruschi	-19.925	-40.5561
Ep6875b	sp. 8	Brazil	Espírito Santo	REBIO Augusto Ruschi	-19.8765	-40.5282
Ep6880b	sp. 8	Brazil	Espírito Santo	Nova Lombardia	-19.8711	-40.5286
FG010a	sp. 8	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG010b	sp. 8	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG010c	sp. 8	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192
FG010d	sp. 8	Brazil	Minas Gerais	PARNA Caparaó	-20.5023	-41.8192

Table S2. Details of the primers used in this research.

Gene	Primers label	Primers sequences (5' - 3')	Amplicon lengths
28S	D2-3549 F	AGTCGTGTTGCTTGATAGTGCAG	1219 bp
	D5-4749 R	GTTACACACTCCTTAGCGGA	
COI	LCO1409	GGTCAACAAATCATAAAGATATTGG	691 bp
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	

Table S3. List of outgroups used in this research with GenBank accession numbers.

Taxon	GenBank Accession Code		Location
	28S	***COI***	
Leptophlebiidae			
Atalophlebiinae			
Atalomicria sp.	EU874483.1	JN289985.1	Australia
Atalophlebia sp.	EU874484.1	KP697596.1	Australia
Garinjuga sp.	this research	JN290008.1	Australia
Garinjuga sp.	this research	JN289965.1	Australia
Garinjuga sp.	this research	JN289971.1	Australia
Garinjuga sp.	this research	JN290009.1	Australia
Massartellopsis irarrazavali	EU874492.1	-	Chile
Meridialaris diguillina	EU874493.1	-	Chile
Meridialaris laminata	EU874494.1	-	Chile
Malagasy group			
Malagasy taxon 4	EU874474.1	-	Madagascar
Malagasy taxon 5	EU874475.1	-	Madagascar
Malagasy taxon 6	EU874476.1	-	Madagascar
Radima sp. 1	EU874473.1	-	Madagascar
Radima sp. 2	EU874477.1	-	Madagascar
Radima sp. 3	EU874478.1	-	Madagascar
Leptophlebiinae			
Leptophlebia marginata	EU874508.1	HQ563240.1	Norway
Leptophlebia vespertina	EU874509.1	-	Norway
Paraleptophlebia heteronea	EU874512.1	JQ663272.1	USA
Paraleptophlebia vaciva	EU874515.1	JQ663110.1	USA
Oligoneuriidae			
Chromarcys magnifica	MG516507.1	MG516460.1	Thailand
Oligoneuria amazonica	MG516508.1	KT201517.1	Brazil
Siphlonuridae			
Siphlonurus sp.	AY749958.1	KR141711.1	USA

Table S4. Best evolutions models inferred by jModelTest2 using Corrected Akaike Information Criterion (AICc).

Genes	Evolution model	AICc	wt
28S	**GTR+G**	**10696.36841**	**0.507053**
	TIM2+G	10696.42532	0.492827
	TIM1+G	10713.1043	1.18E-04
COI	**TrN+G**	**3683.176256**	**0.578302**
	TIM3+G	3686.284322	0.122249
	TIM1+G	3686.464762	0.111703

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CONCLUSÃO

Neste trabalho, avaliamos a história evolutiva e os eventos biogeográficos que influenciaram a distribuição de Atalophlebiinae. A adição de novas sequências de DNA às já disponíveis no GenBank ajudou a estabelecer as principais relações filogenéticas dos gêneros que compõem o grupo, que divergiu de estudos anteriores que usaram tanto dados morfológicos quanto moleculares. Assim, acreditamos que muitas características morfológicas compartilhadas entre esses gêneros, usadas para estabelecer linhagens no passado, podem ser resultado de convergência evolutiva. Além disso, sequências genéticas de gêneros patagônicos, australianos e neozelandeses, ainda não amostrados, podem rearranjar as relações do grupo e ajudar a preencher as lacunas da sua história evolutiva.

As estimativas dos tempos de divergência e as reconstruções de áreas ancestrais datam a origem de Atalophlebiinae para o Cretáceo, a aproximadamente 108 milhões de anos atrás, após um evento vicariante, provavelmente relacionado com a segunda fase da quebra da Gondwana, onde de Madagascar e Índia se separam da Antártida e Austrália. Após esse evento, o grupo se diversifica sob uma complexa dinâmica entre dispersão e vicariância no corredor gondwânico formado pela América do Sul, Antártida, Austrália e Nova Zelândia. Assim, a separação da Gondwana ajudou a moldar o atual padrão de distribuição e diversidade de Atalophlebiinae.

O sequenciamento e análises das amostras que cobrem amplamente a distribuição de *Massartella* ajudou a estabelecer o monofiletismo do gênero, bem como o de suas duas principais linhagens: Pantepui e Mata Atlântica. Além disso, contribuiu para identificar novas espécies, que estão em processo de descrição, nas duas regiões de ocorrência do grupo. As estimativas dos tempos de divergência datam que o último ancestral comum das linhagens Pantepui e Mata Atlântica teria vivido durante o período Cretáceo-Paleogeno, a aproximadamente 66 milhões de anos atrás. Assim, é provável que oscilações climáticas e/ou incursões marinhas, frequentes na América do Sul nesses períodos, tenham separado os clados e estabelecido o padrão disjunto de distribuição do gênero no continente.