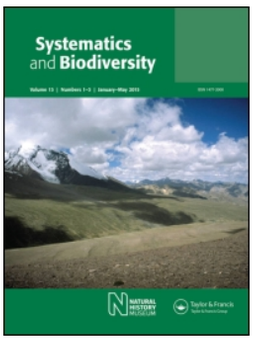

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Research Article

Cryptic diversity in the mayfly *Leptohyphodes inanis* (Pictet) (Ephemeroptera: Leptohyphidae) across water basins in Southeastern Brazil

PAULA MALAQUIAS SOUTO[1,2], LUIZ FELIPE LIMA DA SILVEIRA[3], DANIELA MAEDA TAKIYA[4] & FREDERICO FALCÃO SALLES[5]

[1]Programa de Pós-graduação em Biologia Animal (PPGBAN), Universidade Federal do Espírito Santo, Vitória, ES 29075-910, Brazil
[2]Linking Landscape, Environment, Agriculture and Food (LEAF), Tapada da Ajuda, 1349-017 Lisboa, Portugal
[3]Department of Biology, Western Carolina University, Cullowhee, NC 28723, USA
[4]Laboratório de Entomologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-901, Brazil
[5]Museu de Entomologia, Departamento de Entomologia, Universidade Federal de Viçosa, Minas Gerais 36570-900, Brazil

Leptohyphodes inanis (Pictet) is an enigmatic species with a rare trait among leptohyphid males – large and divided compound eyes. In addition, the color of its upper portion is variable across – but not within – populations. However, the geographic variation of this trait and its relation to gene flow across populations remain unknown. Here, we analyzed individuals across Southeastern Brazil (19° to 24°S and 40° to 48° W) to (i) assess genetic (COI) and eye color variation, and (ii) evaluate if *L. inanis* is a single species, by combining Bayesian phylogenetic analyses (including two other leptohyphid genera – *Tricorythopsis* and *Tricorythodes*) and species delimitation methods: ABGD and mPTP. To further investigate within-species variation in phenotypic traits, we evaluated quantitative and qualitative morphological traits of 1,252 individuals. We found that genetic variation in *L. inanis* is largely unrelated to eye color, and that pairwise genetic divergences in COI mtDNA are remarkably higher (up to 30.7%) than previously found in other mayfly lineages. *L. inanis* was recovered as monophyletic, although results suggest it includes three to seven cryptic species, each one related to mountain ranges across Southeastern Brazil. Furthermore, we found no genetic variation among individuals of the same drainage basin, suggesting that populations might be largely isolated from one another. Because morphological traits traditionally used in Ephemeroptera taxonomy were ineffective in distinguishing the cryptic species, we propose *L. inanis* to be a species complex.

Key words: Canastra mountain range, DNA Barcode, Mantiqueira system, phylogenetics, species delimitation

Introduction

Most descriptions and species delimitations of insect taxa are based upon morphological characters alone, which sometimes are inaccurate and render species identification a difficult or impossible task when dealing with cryptic species (i.e., two or more distinct species morphologically indistinguishable and classified as a single species; Pfenninger & Schwenk, 2007). The study of cryptic species represents a challenge to taxonomists but is nevertheless crucial towards a comprehensive understanding of biodiversity patterns, and yields several implications for evolutionary theory, biogeography and conservation planning (Bickford et al., 2007).

Different studies on genetic variation, often associating morphological and geographic data, revealed the existence of cryptic species in several groups of animals and plants worldwide, in diverse types of habitats (e.g., Dawson & Jacobs, 2001; Feulner et al., 2006; Gómez et al., 2002; Grundt et al., 2006; Hebert et al., 2004; de Rezende Dias et al., 2018; Vrijenhoek et al., 1994). The advent of molecular techniques and analyses has brought about increased awareness of the importance of DNA data in insect taxonomy, which has proven very useful in the discovery of cryptic species (e.g., Bickford et al., 2007; Cardoni et al., 2015; Cook et al., 2008; Fujita et al., 2012; Hebert et al., 2003; Hendrich et al., 2015;

Correspondence to: Paula Malaquias Souto. E-mail: pmsoutobio@gmail.com

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Figs. 1 and 2. *L. inanis*, nymph (a) from Santa Teresa (Espírito Santo) and male subimago (b) from Peixe Tolo river (Serra do Intendente State Park, Minas Gerais). Photos by Frederico Salles.

Macher et al., 2016; Ossa-López et al., 2017; Petit & Excoffier, 2009; Silveira et al., 2016).

Insects are one of the most well represented groups in the cryptic species literature, being the description and recognition of them of great implication for human health (e.g., *Anopheles* malaria-transmitting mosquitoes), pest management (different species have variable pesticide resistance), and studies of coevolution and species interaction (Bickford et al., 2007). More recently, several studies using molecular tools have drawn attention to the existence of cryptic species complexes in Ephemeroptera (e.g., Gill et al., 2016; Macher et al., 2016; Ossa-López et al., 2017; Pereira-da-Conceicoa et al., 2012; Rutschmann et al., 2014; Williams et al., 2006), especially using information from the mtDNA cytochrome C oxidase subunit I (COI) gene. However, the within-species genetic variation remains largely unexplored in South American mayflies.

Leptohyphodes Ulmer, 1920 (Ephemerelloidea: Leptohyphidae) was established for eight male imagos from Brazil, described as "*Potamanthus* ? *inanis*" by Pictet (1843) (Ulmer, 1920). Ulmer (1921) placed in this genus another enigmatic taxon, *Tricorythus australis* Banks (1913), transferred later to *Tricorythodes* by Traver (1958) and, more recently, to *Macunahyphes* Dias, Salles & Molineri (2005). The most unique character of *Leptohyphodes* is a rare trait among leptohyphid males: the large and divided compound eyes. This feature is shared only with *Amanahyphes* Salles & Molineri, 2006 and *Leptohyphes populus* Allen (1973), a species known only from a male nymph from Amazonas State (Brazil). Thus, *Leptohyphodes* is a monotypic genus endemic to Southeastern Brazil. Its type species, *Leptohyphodes inanis* (Pictet, 1843) (Figs. 1 and 2), known from all the stages, also has an even rarer feature in Ephemeroptera: variation in the colour of the upper portion of the males' eyes.

Although type-specimens of *L. inanis* have black eyes, we have found that different populations show variable coloration of the upper portion of compound eyes, in shades of red or black, followed by no other obvious morphological distinction. In this context, the objective of the present work was to investigate whether eye coloration variation could be used to diagnose mtDNA lineages of *L. inanis*. Our findings suggest that *L. inanis* is a species complex, with each cryptic species mostly restricted to different mountain ranges in Southeastern Brazil. Although eye coloration couldn't distinguish cryptic species alone, as different cryptic species may have the same eye colour, it is a stable character within each of the delimited species. Due to the newly discovered cross-population variation in male eye colour, we also propose an expansion of the generic diagnosis.

Material and methods

Sampling, morphology, and taxonomy

Material examined is deposited at: (DZRJ) – Coleção Entomológica Prof. José Alfredo Pinheiro Dutra, Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; (CZNC) – Coleção Zoológica Norte Capixaba, Universidade Federal do Espírito Santo, São Mateus, Brazil; and (MZUSP) – Museu de Zoologia, Universidade de São Paulo, São Paulo, Brazil. Syntypes of *L. inanis*, deposited at Naturhistorisches Museum Wien, Vienna, Austria (NMW), were observed by photographs (courtesy of Dr. Ernst Bauernfeind, NHM).

Material studied was collected in Southeastern Brazil, in the states of Espírito Santo, Minas Gerais, Rio de Janeiro, and São Paulo, mostly in the domain of the tropical Atlantic Rainforest, but some from Brazilian Cerrado as well. The sampled area comprises three mountain ranges: Serra do Mar, Serra da Mantiqueira, and Serra do Espinhaço. Our study area spanned about 1000 km across the Brazilian Southeast, and ranged from 19° to 24° S, and 40° to 48° W. Maps were made using the program QGIS 3.105-A Coruña (QGIS 2018), and vectors subsequently edited in the Adobe Illustrator 2019 software. Shapefile of Brazilian hydrographic basins was downloaded through the website of the Brazilian Government's National Water Agency (www.ana.gov.br). Other shapefiles were downloaded from the Brazilian government's Brazilian Institute of Geography and Statistics website (www.ibge.gov.br).

The material is preserved in 96% ethanol; wings and genitalia were slide-mounted in Euparal®. Photographs and measurements were made with the Leica Application Suite CV3 Automontage Software and later edited using the Adobe Photoshop CC 2018 software. Terminology follows Molineri (2002, 2006).

Morphological characters commonly used in Leptohyphidae taxonomy were observed in nymphs and imagos. Generally, diagnostic characteristics of nymphs are: absence or presence of maxillary palp – when present, its number of segments; absence or presence of apical setae on the maxillary palp; fore femoral width/height ratio; chaetotaxy on the dorsum of femora; number and arrangement of denticles on tarsal claws; and colour pattern. Imago characteristics more commonly used are: wing venation; morphology of the male genitalia, and colour pattern. In addition, total body lengths were compared among populations to see if there is a separation of populations by size. Only fully mature nymphs and imagos were included in the measurements, yielding a total of 13 female nymphs, 11 male nymphs, 10 female imago, and 14 male imagos. Despite the vast material available (Appendix 1), only a small portion of it is in good condition of preservation and at the same development stage for comparison. The material that was not suitable for measurements or DNA was used as data for distribution and evaluation of eye colour and colour pattern of the individuals. The eye colours considered in this work were black and red, with the pale yellow colour described by Molineri (2005) (originally referred as "cream" by the author) being considered within the red spectrum.

DNA sequences and genetic analyses

Genomic DNA was extracted from imagos and/or nymphs of the following members of Leptohyphidae: *L. inanis*, *Tricorythodes eduardoi* (Almeida & Mariano, 2015), *Tricorythopsis chiriguano* Molineri, 2001, *Tricorythopsis gibbus* (Allen, 1967), and *Tricorythopsis spongicola* Lima, Salles & Pinheiro, 2011 (Supplementary material Table S1). We used the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following a modified protocol of the manufacturer.

Partial sequences of the mitochondrial cytochrome oxidase I (COI) gene were amplified by polymerase chain reactions (PCR) using the primers LCO-1490 or C1-J-1718 in combination with HCO-2198 (Folmer et al., 1994; Simon et al., 1994). We used a two-part PCR program with five initial cycles with an annealing temperature of 45°C followed by 35 cycles with annealing temperature of 49°C. Amplicons were purified and sequenced by Macrogen (Seoul, Korea).

Resulting electropherograms from both DNA strands were analysed using Geneious 8.1.7 (http://www.geneious.com, Kearse et al., 2012), adjusted manually to generate a consensus sequence for each specimen. Sequences were checked with Basic Local Alignment Search Tool (BLAST; Altschul et al., 1997) against the GenBank nucleotide database to ensure that the amplified product was correct and not contaminated. Individual sequences were aligned using ClustalW (Thompson et al., 1994) implemented in Geneious and translated into amino acids to ensure nonamplification of numts. The final alignment included sequences with 443 bp. The sequences were registered on the Genbank and BOLD platforms (Clark et al., 2016; Ratnasingham & Hebert, 2007).

Pairwise divergences between COI sequences of specimens of *Leptohyphodes* and related species were calculated by Kimura-2 parameter (K2P, Kimura, 1980) implemented in MEGAX (Kumar et al., 2018; Stecher et al., 2020). The K2P model accounts for different transition (purine-purine and pyrimidine-pyrimidine exchanges) and transversion (purine-pyrimidine interchanges) rates and has been used extensively in DNA barcoding studies. Moreover, this model is widely used for studies of cryptic species and intra- and interspecific variation in Ephemeroptera (Ball et al., 2005; Webb et al., 2007; Alexander et al., 2009; Zhou et al., 2009; Hwang et al., 2013; Ossa-López et al., 2017) and we used it in order to be able to compare our results with other K2P divergences cited in the literature.

Phylogenetic and phylogeographic analyses. PartitionFinder 2 (Lanfear et al., 2016) was used to detect the most appropriate model scheme for the gene COI based on the Bayesian information criterion (BIC). COI was initially partitioned with respect to codon

positions. The three-partition scheme was selected and appropriate models for each were SYM + I for 1st codon, F81 + I for 2nd codon, and GTR + G for 3rd codon positions.

Bayesian inference (BI) analysis was performed using MrBayes version 3.2.2 (Ronquist et al., 2012) at the CIPRES portal (Miller et al., 2010). Four independent Metropolis-Coupled Markov chain Monte Carlo (MCMCMC) analyses each with four chains were run for 20,000,000 generations, sampling trees every 2,000 generations. The initial 25% of sampled trees were discarded as burn-in. Convergence among independent analyses was assessed by monitoring the values of standard deviation of split frequencies (<0.05) in MrBayes and parameter sampling was assessed with Tracer version 1.7.1 (Rambaut et al., 2018) by the effective sample size (ESS) criterion (>200). A 50% majority-rule consensus post-burn-in tree was constructed and values of posterior probabilities (pp) were calculated.

Maximum likelihood (ML) topological estimation was conducted in the program IQ-TREE (Nguyen et al., 2015) as implemented in W-IQ-TREE (http://iqtree.cibiv.univie.ac.at/, Trifinopoulos et al., 2016). We performed 1,000 ultrafast boot-strap replicates (UFBoot) (Hoang et al., 2018) and 1,000 Shimodaira-Hasegawa approximate likelihood ratio test replicates (SH-aLRT) (Anisimova et al., 2011) to investigate nodal support across topologies.

A Bayesian posterior probability (pp) ≥0.95, SH-aLRT ≥80, and UFBoot ≥95 were recognized as indicating strong support for a given node (Erixon et al., 2003; Minh et al., 2013, 2017). Final trees, including both BI and ML approaches, were previewed at Figtree version 1.4.4 (Rambaut, 2018) and posteriorly edited in Adobe Illustrator CC 2019.

Parsimony haplotype networks were constructed for the same COI dataset (except of outgroups) using the Median-joining method (Bandelt et al., 1999) implemented in PopART version 1.7 (Population Analysis with Reticulate Trees, Leigh & Bryant, 2015), with epsilon set to 0. Haplotype networks are an intuitive method for visualizing relationships between individual genotypes at the intraspecific level, as well as, to infer the biogeographical history of populations (Leigh & Bryant, 2015). The final figure was edited in Adobe Illustrator CC 2019.

Species delimitation analyses. For species delimitation, we excluded identical sequences to avoid false positives (Ahrens et al., 2016). Identical sequences were identified and excluded using the perl script uniqHaplo.pl (available at: http://raven.wrrb.uaf.edu/~ntakebay/teaching/programming/perl-scripts/perl-scripts.html). Only five sequences were excluded (vouchers ENT1724, ENT1739, ENT2543, ENT2559, and ENT2569). We compared the outcomes of the threshold-based ABGD (Automatic Barcode Gap Discovery), and the phylogenetically-aware multi-rate Poisson tree process (PTP, Kapli et al., 2017).

ABGD suggests species boundaries by seeking barcoding gaps (i.e., a gap between the distribution of intra- and interspecific genetic divergences) in a matrix of pairwise distances based on two key settings: prior intraspecific divergences (P), and relative gap width (X) (Puillandre et al., 2012). This method sorts the dataset into hypothetical species based on a fixed overarching divergence threshold, and then recursively computes lineage-specific thresholds. We ran the ABGD on the graphic web version (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html), and compared the outcomes of an array of settings to account for parameter sensitiveness, including a X value of 1 and 1.5 (method default), and two P ranges – a more restrict (Pmin: 0.001 – Pmax: 0.1; method default) and a broader one (Pmin: 0.0001 – Pmax: 0.2). Since the ranges of P and X values examined recovered the same outcomes, we regarded only the default parameters in our results and discussion. The ABGD results were exposed according to the number of species recovered by the analysis (e.g. ABGD4 means that the analysis recovered 4 species).

The PTP method (Zhang et al., 2013) and its multi-rate implementation (Kapli et al., 2017) differ from ABGD as it infers putative species based on trees. Because mPTP fits multiple distributions to each delimited species to account for differences in sampling intensity and/or population history, it usually leads to results that are robust to model violations (Kapli et al., 2017; Blair & Bryson Jr., 2017). We ran the mPTP analysis on the web server (https://mcmc-mptp.h-its.org/mcmc/) using default settings, using the resulting ML tree obtained in IQ-TREE as input. The input tree used was obtained through the methods described above (see *Phylogenetic and phylogeographic analyses*).

Results

Morphology

Mean (and standard deviation) body length measurements (mm) of nymphs and imagos (females and males) from different *L. inanis* populations can be found in Supplementary material Table S2. All material examined agree with the species redescription by Molineri (2005), with no variation in the diagnostic characters, except by body length which reached a greater variation according

Figs. 3–8. *L. inanis*, male nymph habitus, showing morphological geographical diversity. In the Parque Nacional de Campos do Jordão, São Paulo State, it is possible to find nymphs with two eye colours, with red (3) and pale yellow ("cream") eyes (4) as described by Molineri (2006). Both colours were considered within the spectrum of red. 5 Parque Nacional do Itatiaia, Minas Gerais State, 6 Parque Nacional da Serra da Bocaina, São Paulo State, 7 Parque Estadual da Serra do Intendente, Minas Gerais State, 8 Reserva Biológica Augusto Ruschi, Espírito Santo State.

to material examined here [5.5–10.3 in nymphs, 5.6–9.4 in adults in our study; 8.3–8.5 in nymphs, 5.5–7.6 in adults in Molineri (2005)], and the colour of the upper portion of male compound eyes.

Adults and nymphs of *L. inanis* show variation in the colour pattern (Figs. 3–16), body length, and amount of bristles throughout the body; but these features overlap among populations. Black eyes were only found in four populations in our sample of 1,252 specimens of *L. inanis*, all collected far away from Rio de Janeiro, the presumed type locality (see Remarks in Taxonomy section): three from the Serra do Espinhaço (Serra do Capanema, Serra da Canastra, and Serra do Intendente in Minas Gerais State), in or near the Brazilian Cerrado biome; and one from Reserva Biológica Augusto Ruschi (Santa Teresa, Espírito Santo), part of Serra da Mantiqueira, in the Brazilian Atlantic Rainforest. The latter, unfortunately, could not be added to the molecular dataset due to DNA deterioration of the sample. All other populations have red or pale yellow eyes, but the latter was found in specimens from Serra da Mantiqueira and Serra do Mar, occurring together with

Figs. 9–16. *L. inanis*, male habitus, showing the morphological geographical diversity. 9 subimago from Parque Nacional do Itatiaia, Minas Gerais State, 10 imago from Parque Nacional da Serra da Bocaina, São Paulo State, 11 imago from Parque Nacional da Serra dos Órgãos, Rio de Janeiro State, 12 imago from Parque Estadual do Campos do Jordão, São Paulo State, 13 imago from Itabirito, Minas Gerais State, 14 imago from Parque Estadual da Serra do Intendente, Minas Gerais State, 15 imago from Parque Nacional da Serra da Canastra, Minas Gerais State, 16 subimago from Reserva Biológica Augusto Ruschi, Espírito Santo State.

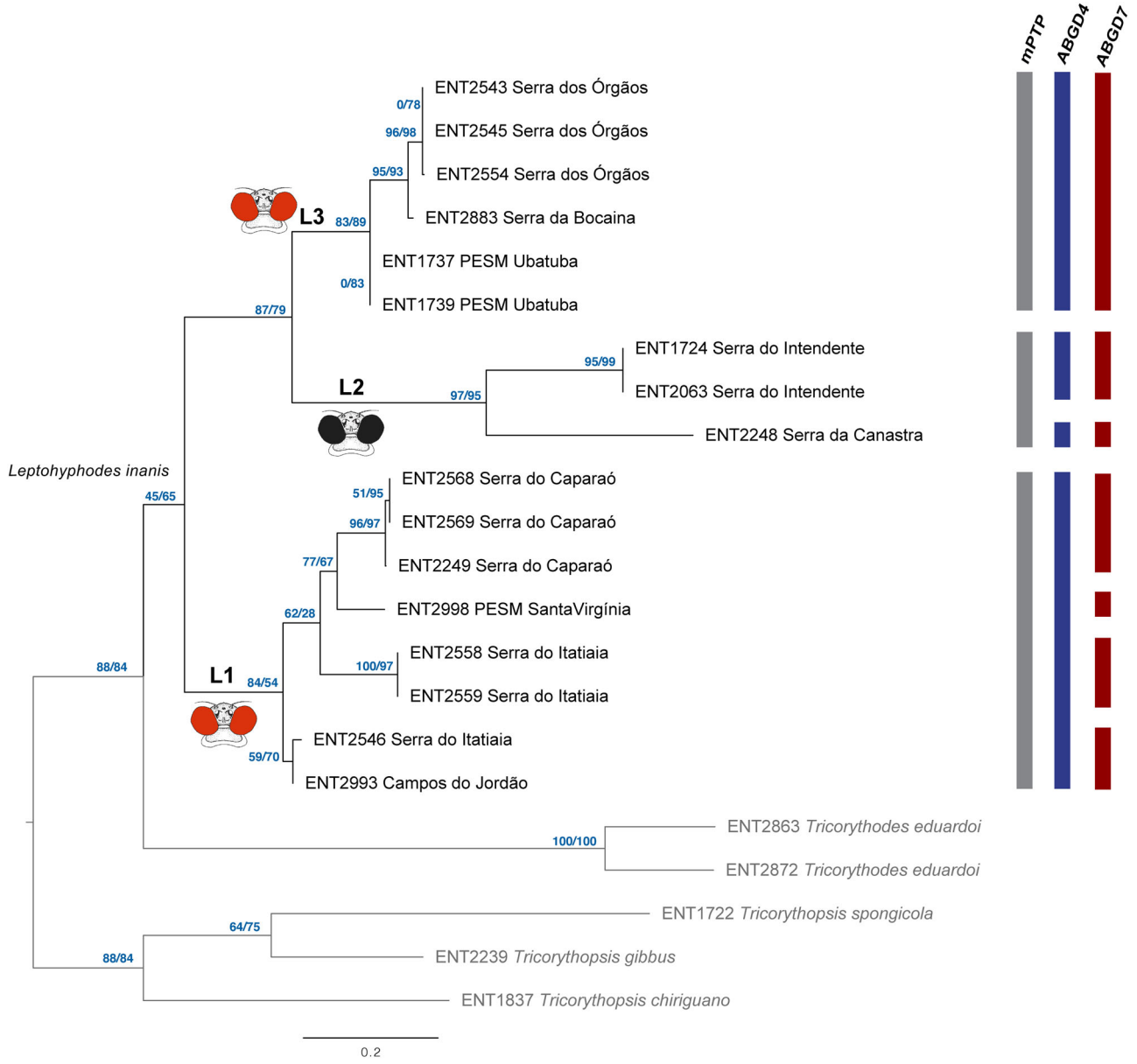


Fig. 17. Maximum likelihood tree of COI sequences (443 bp) of *L. inanis* (Log-likelihood = −2597.853). Shimodaira-Hasegawa approximate likelihood ratio test replicates (SH-aLRT) support (%) / ultrafast bootstrap support (UFBoot) are given above branches. Lineage 1 (L1) comprises sequences of specimens from Serra da Mantiqueira (except one sequence from Serra do Mar), all with red eyes. Lineage 2 (L2) comprises sequences of specimens from Serra do Espinhaço, all with black eyes. Lineage 3 (L3) comprises sequences of specimens from Serra do Mar, all with red eyes. Bars indicate species delimitations based on the distance-based (ABGD) and tree-based (mPTP) models. Figure of male imago head modified from Molineri (2005).

specimens having red eyes. For example, specimens of Campos do Jordão population vary regarding body general colour and the colour of the upper portion of male compound eyes: most specimens ($n = 31$) feature shades of orange, including red (Fig. 3), and few specimens ($n = 10$) have shades of grey, and the upper portion of male compound eyes pale yellow (Fig. 4). Both variations were treated herein as with colour in the red spectrum, because they largely overlapped.

In conclusion, variations found in colour pattern, body length, and amount of bristles throughout the body overlapped, being ineffective in differentiating populations.

Phylogeny and genetic diversity

BI and ML topologies were identical (Fig. 17 and Supplementary material Fig. S1), with all

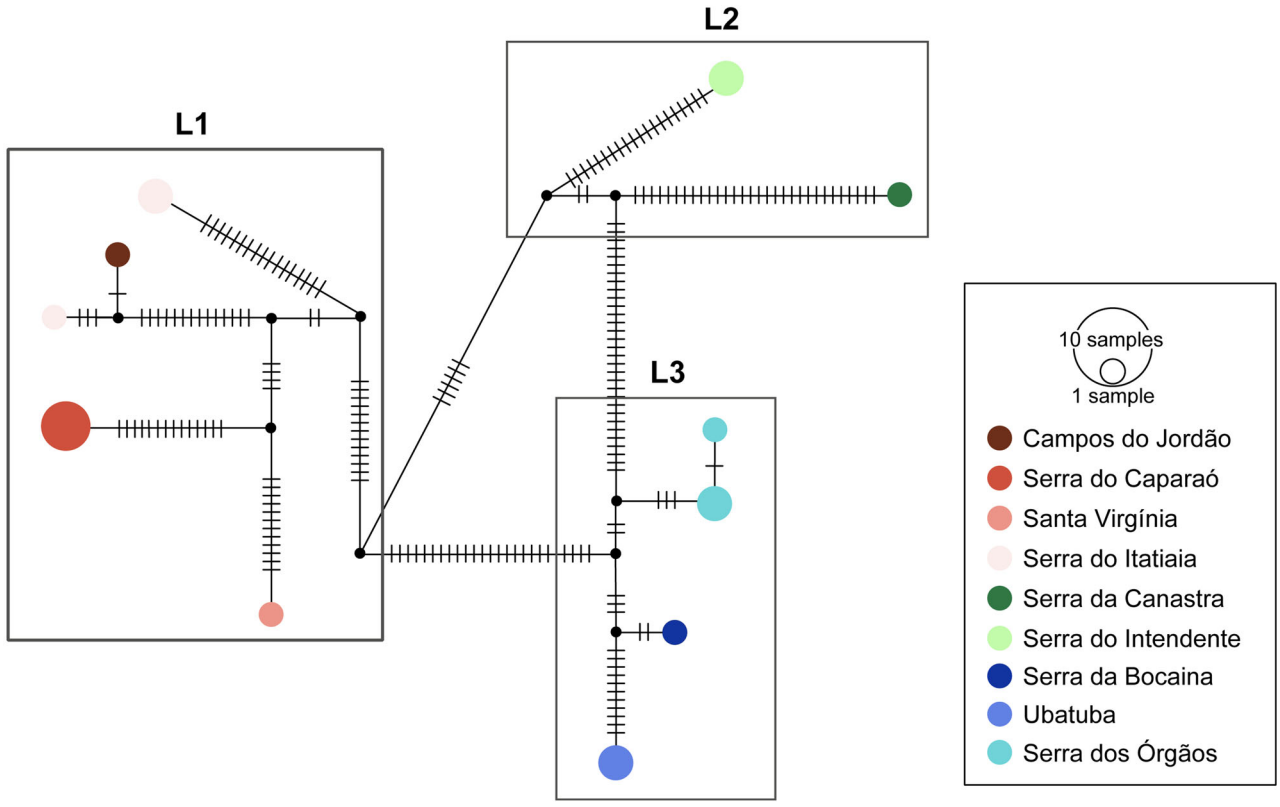


Fig. 18. Haplotype network of COI sequences of *L. inanis*. Colours indicate collecting locality of haplotypes according to legend. Size of circles related to the frequency of haplotypes and dashes on branches represent the number of mutations between haplotypes.

Leptohyphodes sequences grouped together with low support (pp = 0.76, SH-aLRT = 45, UFBoot = 65). Three lineages were recovered: Lineage 1, comprising sequences of specimens from the Serra da Mantiqueira (except one sequence from Serra do Mar; see below), all (including the sequence from Serra do Mar) with eyes coloured within the spectrum of red (pp = 0.92, SH-aLRT = 84, UFBoot = 54); Lineage 2, comprising sequences of specimens from the Serra do Espinhaço, all with black eyes (pp = 1.0, SH-aLRT = 97, UFBoot = 95); and Lineage 3, comprising sequences of specimens from the Serra do Mar, all with eyes coloured within the spectrum of red (pp = 0.97, SH-aLRT = 83, UFBoot = 89). Pairwise K2P divergences among all 17 *Leptohyphodes* sequences ranged from 0 to 30.7% (Supplementary material Table S3; see Appendix 2). Considering individuals belonging to the three above-mentioned lineages, interlineage K2P divergences ranged 22.7–24.3%, while estimates of average evolutionary divergence within each lineage were 10% for Lineage 1; 12% for Lineage 2; and 4% for Lineage 3, displaying a clear barcoding gap between lineages. Divergences of 0% were found only when comparing sequences from the same population (e.g., sequences from Caparaó and Itatiaia) and identical sequences (Supplementary material Table S3).

Haplotype network

The COI haplotype network of *L. inanis* consists of 11 haplotypes, which can be clustered into three haplogroups similar to the three lineages in the phylogenetic analyses (Fig. 18). Two different haplotypes were sampled from populations from both Serra dos Órgãos and Itatiaia, and one of the Itatiaia haplotypes appears to be more similar to the Campos de Jordão haplotype, both localities in the Serra da Mantiqueira range.

Species delimitation

Results of both ABGD and mPTP agreed upon the existence of cryptic species within *L. inanis* as currently understood, but the former found four or seven species, whereas the latter found three species. The point of disagreement regarded populations from Serra da Canastra and Serra do Intendente, found to be the same species by mPTP but different species by ABGD (Fig. 17). Along with the genetic divergence described above, these analyses indicate that the genetic diversity observed within *L. inanis* relates to the geographic structure of the mountain ranges, and that the populations of the drainage basins are isolated across the Brazilian Southeast (Fig. 19).

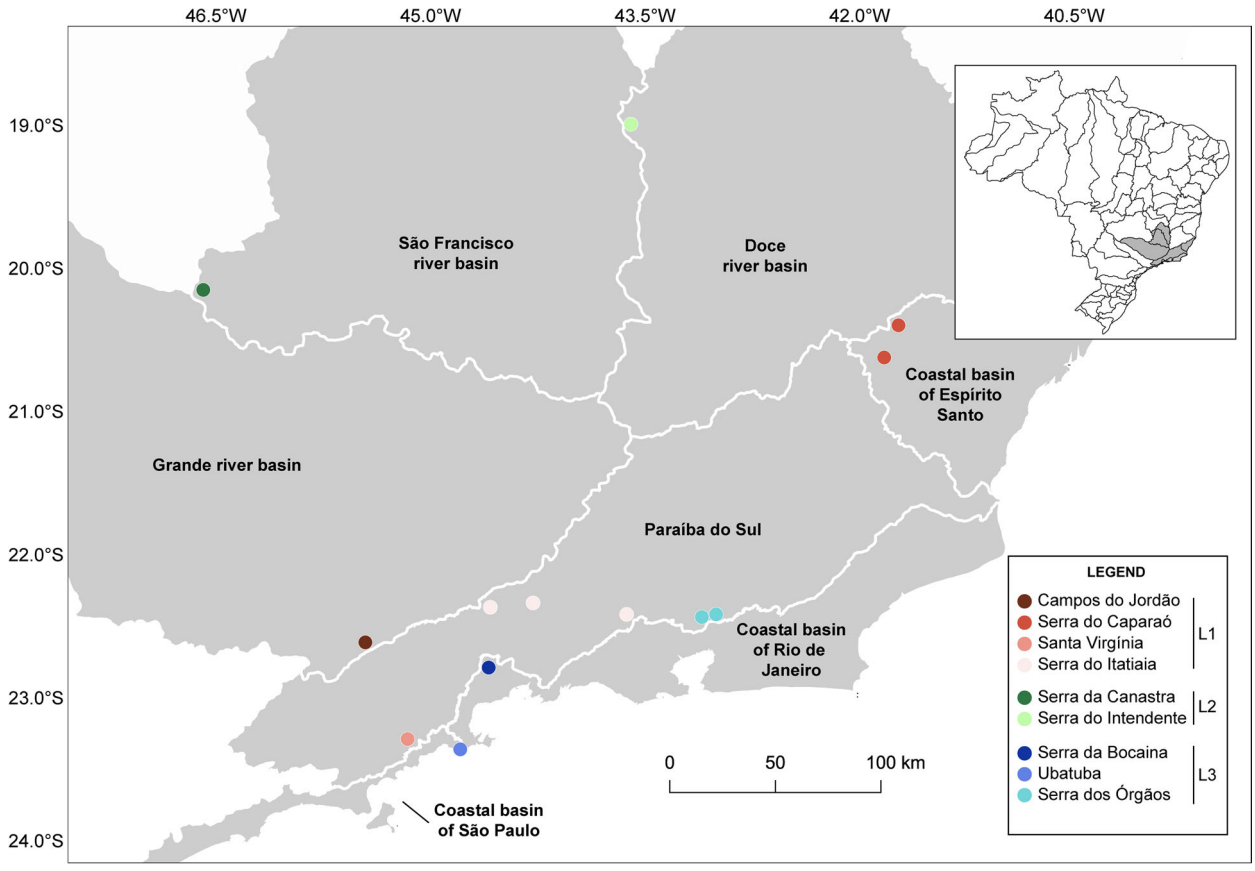


Fig. 19. Hydrographic map of Southeastern Brazil, showing the five hydrographic sub-basins where individuals of the molecular study were collected. Circles represent sample points of each individual used in phylogenetic and species delimitation analyzes.

Taxonomy

***L. inanis* (Pictet, 1843)**

Potamanthus? *inanis*. Pictet, 1843: 232 (orig. descr.); Eaton, 1886: 296 (male).

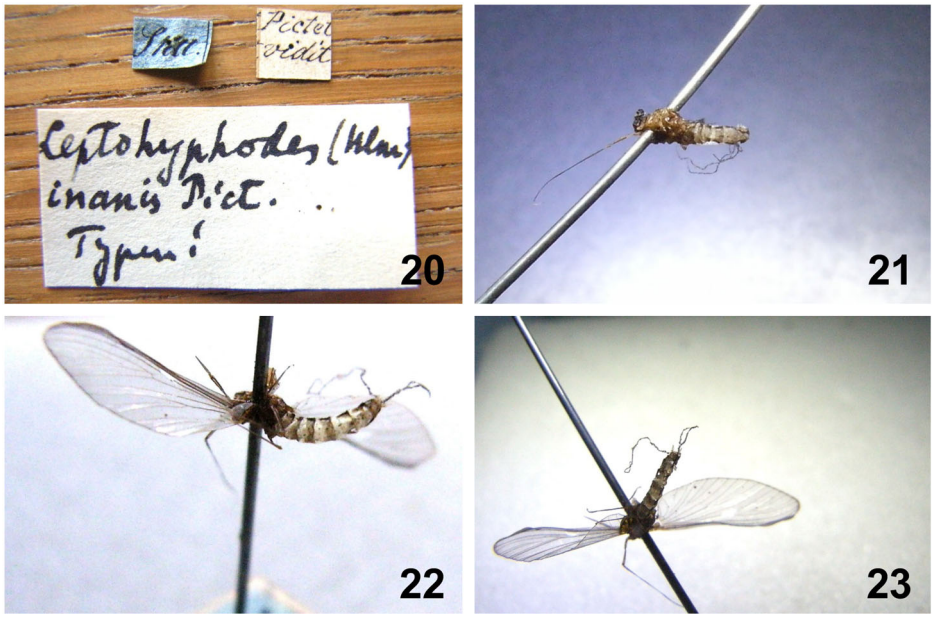
Potamanthus inanis. Walker, 1853: 544, 547; Eaton, 1871: 91 (male).

L. inanis. Ulmer, 1920: 51; Lestage, 1931a: 74; Lestage, 1931b: 60; Traver, 1958: 496 (male, female, nymph); Hubbard, 1982: 274; Molineri, 2005: 250 (redescription).

Leptohyphodes sp. Traver, 1944; Molineri, 2005: 250.

Measurements. Body length: nymph ♂ 5.5–9.0 mm (n = 11), nymph ♀ 6.4–10.3 mm (n = 13); imago ♂ 5.6–9.4 mm (n = 14), imago ♀ 6.0–7.5 mm (n = 10).

Diagnosis. According to Molineri et al. (2015), *Leptohyphodes* shares many characters with *Amanahyphes* Salles & Molineri, 2006, which suggest a close phylogenetic relationship between them (sister group relationship recovered in the unpublished phylogenetic analysis proposed by Baumgardner, 2008) and differentiate them from other Leptohyphidae genera. These features are: male eyes enlarged and divided in two portions, a rare feature in Leptohyphidae; elongate wings; two-segmented forceps arising from posterolateral projections of the styliger plate; nymphal legs long and slender with claws showing two sets of denticles (a marginal row basally and a double submarginal row subdistally); and operculate gills subtrapezoidal in shape, narrowest proximally, with a transverse weaker line near apex, and with inner margin nearly reaching midline of the body distally (Molineri, 2005, Salles & Molineri, 2006). However, the penes, eggs, and gills structures can easily differentiate the two genera. The penes of *Amanahyphes* show small spines subdistally on the lateral margin, which are absent in *Leptohyphodes*. Furthermore, eggs of *Leptohyphodes* have only one polar cap, while *Amanahyphes* have two – a blunt one formed by coiled threads, and a large conic structure composed of triangular plates on the other pole. Finally, gills of *Leptohyphodes* present a small, flap-like lamellae on the ventral side, which are absent in *Amanahyphes*. Here we propose an expansion of the generic diagnosis to include the colour variation found



Figs. 20–23. *L. inanis*, male imago, syntypes (NMW). 20 labels of an unphotographed individual, 21 dorsal view from one individual showing the head, 22 lateral view from a different individual showing the left wing and abdomen. 23 ventral view from the same individual in 22 showing the male genitalia. Photos by Ernst Bauernfeind.

in the male compound eye that can be black, red, or pale yellow.

Remarks. The monotypic *Leptohyphodes* Ulmer, 1920 (Ephemerelloidea: Leptohyphidae), endemic to Southeastern Brazil, was established for eight males imagos described as "*Potamanthus ? inanis*" by Pictet (1843) (Ulmer, 1920). Ulmer (1921) placed in this genus another enigmatic taxon, *T. australis* Banks (1913) known from male and female imagos, transferred later to *Tricorythodes* by Traver (1958), and more recently to *Macunahyphes* Dias, Salles, & Molineri (2005).

The original taxonomic description of *L. inanis* does not provide a specific type locality or mentions the eye colour of specimens in the type series, which could give hints on the identity of, and variation within the species. Although not stated in the original description, the eye colour of all syntypes is clearly black (Fig. 21 and Ernst Bauernfeind pers. comm.).

Molineri (2005) redescribed the species based on adults and nymphs from Campos do Jordão (São Paulo State, Brazil) deposited in the Museu de Zoologia (Universidade de São Paulo, São Paulo, Brazil), but didn't examine the syntypes deposited in the Naturhistorisches Museum Wien, Vienna, Austria. According to the original description of the genus, Ulmer (1921) examined eight specimens bearing Pictet's handwritten label, who originally described the species *L. inanis*. However, one specimen might have been lost since Ulmer's revision of the NMW collection (Ernst Bauernfeind pers. comm). On a visit to the Hamburg Zoological Museum (ZMH), FFS found an adult specimen of *L. inanis* with black eyes, with a "Pictet vidit." label. The ZMH has a large part of the material studied by Ulmer and this specimen may be what is lacking in the syntypes series. Although, the precise collecting locality of syntypes is not stated anywhere, it is assumed that it is Rio de Janeiro (Brazil), because Heinrich Wilhelm Schott collected in Rio de Janeiro between 1817 to 1821, staying practically all the time restricted to the direct vicinity of the city (Schott, 1822). However, as discussed above, black eyes were only found in population restricted areas far from Rio de Janeiro (see Morphology in Results section).

Comment. As *Leptohyphodes* is a monotypic genus, the generic diagnostic features of both nymphal and adult stages are maintained for *L. inanis*. However, we can distinguish the populations based on eye colour, but only in males. Males from Serra do Espinhaço (Minas Gerais) and Santa Teresa (Espírito Santo) have compound eyes black. The males from the remaining populations have compound eyes red, which may have lighter or more vivid colours.

Type material. Syntypes (Figs. 20–23): seven ♂ imagos (NMW), Brazil. One syntype, left wing missing,

bearing labels: 1) blue and rectangular handwritten "Shtt." for Heinrich Wilhelm Schott, who collected the specimens; 2) white rectangular handwritten "Pictet vidit"; 3) white rectangular in Ulmer's handwriting "Leptohyphodes (Ulm.) inanis Pict. Typus". Five syntypes, each one bearing two labels: 1) blue and rectangular handwritten "Shtt."; and 2) white rectangular handwritten "Pictet vidit". One syntype bearing the label "Pictet vidit"

Type locality. Brazil.

Distribution. Southeastern Brazil (Espírito Santo, Minas Gerais, Rio de Janeiro, and São Paulo) within the domains of the tropical Atlantic Rain Forest and Brazilian Cerrado, occurring between 46 and 1,550 m of altitude. The records in Serra da Canastra (MG) are new records for the species in the Cerrado biome.

Material examined. Appendix 1.

Discussion

Variation in male compound eye colour

In our analysis, we found phylogenetic signal in the eye colour: black eye species within *L. inanis* were found to be monophyletic, while red eyes seem to be a plesiomorphic state or were acquired independently at least twice in lineages 1 and 3. Moreover, we found that compound eye colour is not a good diagnostic character to separate the putative lineages or cryptic species, except for L2, whose two lineages have black eyes. However, eye colour is still an important trait in characterizing populations, because within each lineage eye colour is consistent. We stress that these results are contingent on the populations and the dataset included, which highlights the need for more comprehensive analyses about *L. inanis* diversity.

Genetic diversity within *L. inanis*

We found high genetic divergence within *L. inanis* (maximum intraspecific = 30.7%), much higher than normally observed among conspecific individuals of mayflies and insects in general. Divergences of 0% were found only when comparing sequences of the same population, which can be an artifact of a restricted geographic scale of sampling, as species sampled throughout a larger geographic range tend to display higher genetic divergences (Bergsten et al., 2012). As a comparison, the K2P divergence between the two sequences of *T. eduardoi* was 15.9% and those were sampled from individuals collected in very distant geographic localities, one from Roraima State (Northern Brazil) and the other from Espírito Santo State (Southeastern Brazil).

Previous studies in mayflies suggested that low values of genetic divergence are expected when conspecific sequences are compared, and that high values may indicate the existence of multiple species – a canonical pattern in animals (Ball et al., 2009; Webb et al., 2012). For example, Ball et al. (2009) found maximum intraspecific divergence of 3.4%, and Zhou et al. (2009) reported a 2% divergence criterion across species. However, high values of intra- and interspecific K2P divergences are not uncommon in mayflies. In a DNA barcoding survey of mayflies, Ball et al. (2005) found mean values of intra- and interspecific genetic diversity of 1% and 18%, respectively. Divergences similar to those found in *L. inanis* are also seen in other related taxa, such as 21.9 in *Caenis amica* Hagen, 1861; 21.0 in *Caenis diminuta* Walker, 1853; 20.4 in *Caenis punctata* McDunnough, 1931 and *Eurylophella macdunnoughi* Funk, 1994; 20.6 in *Ephemerella excrucians* Walsh, 1862; including the leptohyphid *Tricorythodes explicatus* (Eaton, 1892) with 19.8 (Webb et al., 2012). These values are especially high when compared to other insect groups, e.g., butterflies (0.25 and 6.8%, respectively; Hebert et al., 2003).

In the Neotropics, at least three species of *Campylocia* Needham & Murphy, 1924 show high maximum intraspecific barcode divergences, between 7.2% and 10.0% (GonÇalves et al., 2017), and were interpreted as cryptic species complexes. Similarly, Ossa-López et al. (2017) suggested that the Andean species *Andesiops peruvianus* (Ulmer, 1920) is a species complex with genetic distances up to 24.5% for the COI gene, supporting the existence of four putative species coexisting in the Chinchiná River Basin (Caldas Department, Colombia). Our study agrees with the above-mentioned ones in finding cryptic species in Neotropical taxa.

The higher values observed in Ephemeroptera is probably related to their biology of most species: individuals are poor dispersers due to the short life span of winged forms, and also stenotopic, with strict conditions of water quality for immature survival (long dispersal events, however, have also been considered for some species – Sartori & Brittain, 2015). As discussed for *Campylocia* and other burrowing mayflies, the high values of genetic distance found in *L. inanis*, as well as in other related taxa, may be a result of their low dispersal ability, which can result in the genetic isolation of lineages (GonÇalves et al., 2017).

Naturally, there is no magic threshold of genetic distance above which species status can be postulated (Buckley et al., 2001). Nevertheless, the level of sequence divergence between the three *L. inanis* lineages found in our analyses exceeds that found between other Ephemeroptera species that are well established on morphological criteria. Therefore, we conclude that the current understanding of *L. inanis* is actually a complex of cryptic species. In addition, the haplotype network showed a high haplotype diversity, with many mutational steps between them. Thus, we interpret the high genetic divergences found as the outcome of geographic isolation between populations, rather than reproductive isolation.

The *L. inanis* species-complex

Genetic species suggested by our results are indistinguishable based on diagnostic morphological characters commonly used in Ephemeroptera taxonomy, considering both nymphs and adults. Therefore, to avoid taxonomic problems, we decided to keep all putative species under the same scientific name.

Defining which of the species delimited would be the name-bearer of *L. inanis* is impossible because the type locality and the morphological state of the type material is unknown. In addition, defining species based only on locality and genetics may create more problems in the future when one tries to identify *Leptohyphodes* material from other areas not covered in the present study or dealing with labels with incomplete data (e.g. "Brasil"). Finally, occasional gene flow between these cryptic species cannot be ruled out with the methods we used.

Therefore, after our analyses we consider *L. inanis* to be a group of cryptic species with three to seven putative species in advanced stages of a speciation process, belonging to three monophyletic lineages.

Speciation within Leptohyphodes

Genetic diversity within *L. inanis* crosses two spatial scales – one coarser, related to mountain ranges, and another finer one, tied to the water basins in the region. Our phylogenetic analyses found three distinct evolutionary lineages with moderate to high clade support, each one mostly related to different mountain chains in Southeastern Brazil. These three lineages were recovered as distinct species by mPTP. Lineage 2 and 3 are endemic to the Serra do Espinhaço and Serra do Mar, respectively, whereas lineage 1 is mostly restricted to Serra da Mantiqueira, with a single branch from Santa Virgínia, in the Serra do Mar (see below). Given the exceptionally high level of genetic distance across the three lineages, and their geographic distribution, these lineages might have been separated by older vicariant speciation events. Future studies with a denser sampling within and across basins are needed to determine the boundaries of gene flow across the cryptic species of *Leptohyphodes*. Interestingly, even fairly close populations might be genetically very distant. For example, samples from Ubatuba (SP) were found genetically closer to other species in the oceanic side of the Serra do Mar range hundreds of kilometers apart rather than the geographically much closer population of Santa Virginia.

The finding of mountain-specific cryptic species of *Leptohyphodes* underlines the underestimation of biodiversity in this lineage, and we anticipate that new samplings in other mountain ranges and/or even water basins are likely to find new species. Therefore, future studies tackling the diversity of *Leptohyphodes* should thoroughly take into account water basin traits. Reproductive isolation between species from Santa Virginia and Ubatuba might be driven by spatial distance, as they are in different water basins – the former sits on the Paraíba do Sul water basin, akin to its close-relatives, and the latter is on the Litoral Paulista basin. In fact, the two basins drain to opposite directions: creeks at Santa Virginia tend to drain northwards, but southwards at Ubatuba.

Another unexpected result was that samples from Serra dos Órgãos – nested in lineage 3 – were found phylogenetically closer to one population from Serra da Bocaina (Coastal basin of Rio de Janeiro), rather to other samples from Paraíba do Sul water basin, such as Serra do Itatiaia and Santa Virginia species. The origin of these unexpected results may be hidden in geological phenomena present in streams – for example the stream piracy (or river capture) phenomenon, which correspond to the natural diversion of waters from one river basin to another due to tectonic causes (Ribeiro, 2006), may explain faunal similarities through dispersal of species to the new basin (Pereira et al., 2012). Stream piracy has been used to explain the occurrence of many shared species between neighboring basins in Neotropical freshwater fish studies, (e.g. Ribeiro, 2006; Ribeiro et al., 2006; Serra et al., 2007; Pereira et al., 2012), and can occur through several forms (e.g., absorption of one river by another) until the captured river suddenly changes direction at the point of capture, a place known as the elbow of capture (Oliveira, 2010). Several river capture areas have been described in recent years, including some for the mountain ranges studied here (see Salgado et al., 2018; Oliveira, 2010 for review on the subject). However, this phenomenon can be rare and remains poorly studied in Brazil (Salgado et al., 2018),

and usually provides explanation for distributions of animals without an aerial dispersal stages, as found in aquatic insects

The reasons why flying insects such as mayflies get "evolutionary trapped" in, or speciate across water basins remain elusive. One possible explanation involves dispersal limitation and allopatric speciation. On one hand mayflies have two flying stages (i.e. subimago and imago), which could actively fly from one site to another, even if only through shorter distances (Brittain & Sartori, 2009), tending to disperse upstream. On the other hand, mayflies have a very short adult lifespan, with one to two hours to several days, in addition to being extremely fragile (Brittain & Sartori, 2009; Sartori & Brittain, 2015). In fact, some tropical species of Leptohyphidae are especially short lived, having the entire winged stages lasting less than one hour (Sartori & Brittain, 2015), therefore limiting dispersal. Nymphs, otherwise, tend to drift downstream (Brittain & Sartori, 2009; Sartori & Brittain, 2015), not upstream, which might contribute to biased dispersal patterns in mayflies. In addition, *L. inanis* has many environmental limitations, due to its high sensitivity to changes in the environment, which limits the dispersion capacity and may explain the presence of several cryptic species (Souto & Salles, pers. comm.). Despite these limitations, the existence of an aerial/terrestrial stage is usually related to a distribution that coincides with mountain ranges instead of catchment areas of rivers limited by watersheds, as found for aquatic organisms without stages of land dispersion (Mamos et al., 2014). Thus, the chances of a dispersal incident cannot be discarded.

Studies on phylogeography of mountain organisms have been growing in recent years, but the vast majority of studies in this area are focused on terrestrial species (e.g. Çiplak et al., 2010; Kutnjak et al., 2014; Schmitt & Hewitt, 2004; Schönswetter & Tribsch, 2005; Zając et al., 2020). Also, within aquatic organisms, most studies focus on vertebrates (Barker et al., 2011; Bernatchez & Wilson, 1998; Doadrio et al., 2002; Firkowski et al., 2016; Schreiber, 2002). However, recently several articles on cryptic species hidden in watersheds and mountain ranges have been published, but mostly for nearctic and palearctic fauna (eg. Bálint et al., 2008; Mamos et al., 2014, 2016; Sworobowicz et al., 2015; Wattier et al., 2020). Since mountainous areas are characterized by a high diversity of species (Rahbek et al., 2019), it is expected that we will find substantial cryptic diversity in these areas (Mamos et al., 2014). Our findings in the present study will serve as a basis for deeper studies about the evolution of *L. inanis* and plausible explanations (whether hidden in river basins or in mountain ranges) for the high genetic diversity found and unexpected relationships between different populations.

Conclusion

After examining genetic variation within *Leptohyphodes*, its phylogeography and species boundaries, we found three monophyletic lineages, which refer to three to seven species hidden under *L. inanis*, which we propose to be a complex of cryptic species. These cryptic species are mostly isolated across water basins in Southeastern Brazil, implying that vicariant speciation might have shaped the current distribution within *Leptohyphodes*.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Supplementary material

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ORCID

Paula Malaquias Souto http://orcid.org/0000-0001-6995-9146
Luiz Felipe Lima da Silveira http://orcid.org/0000-0002-0648-3993
Daniela Maeda Takiya http://orcid.org/0000-0002-6233-3615
Frederico Falcão Salles http://orcid.org/0000-0001-8331-5929

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Appendix 1. List of material examined of *L. inanis* during the study. The list is organized by Brazilian states

BRAZIL, MINAS GERAIS, Conceição do Mato Dentro, Parque Estadual Serra do Intendente, S19º0'14″, W43º36'45″, 07.ix.2012, Salles, Rocha & Braga *leg.*, 4 male imagos; same locality, 08.ix.2012, same collectors, 11 subimagos (DZRJ); same data, 6 imagos (DZRJ). Itabirito, Serra do Capanema, Vale do Catana, Cachoeira do Cascalho, S20°12'26", W43°38'34"W, 9.x.2010, Ferreira-Jr. *leg.*, 5 subimagos (DZRJ); same locality, 10.x.2010, Ferreira-Jr. *leg.*, 9 subimagos (DZRJ); same data, 3 subimagos (DZRJ); Cachoeira da Carranca, S20°12'28", W43°38'26", 10.x.2010, Gonçalves *leg.*, 24 subimagos (DZRJ); Cachoeira dos Cruzados, S20°12'17", W43°38'10", 10.x.2010, Clarkson & Dumas *leg.*, 8 subimagos (DZRJ); same locality, 9.x.2010, Clarkson & Dumas *leg.*, 4 subimagos (DZRJ); São João Batista da Canastra, Parque Nacional da Serra da Canastra, S20°9'12″, W46°39'40″, 1,231 m, 15.xi.2014, Nessimian, Oliveira, Rocha & Souto *leg.*, 11 subimagos (DZRJ); same locality, 02.x.2015, Nessimian, Dumas, Rocha & Souto *leg.*, 15 imagos (DZRJ); Cachoeira do Jota, Rio Araguari, S20°5'50″, W46°40'13″, 1,141 m, 16.xi.2014, 15 imagos (DZRJ); same locality, 02.x.2015, Nessimian, Dumas, Rocha & Souto *leg.*, 1 imago (DZRJ); São Roque de Minas, Rio São Francisco, Casca D'Anta (high part), S20°14'37″, W46°38'43″, 956 m, 16.xi.2014, 6 subimagos (DZRJ); Parque Nacional da Serra da Canastra, spring of São Francisco river, S20°14'37″, W46°26'47″, 1,364 m, 15.xi.2014, Nessimian, Oliveira, Rocha & Souto *leg.*, 4 subimagos (DZRJ); Cachoeira do Rolinho, Ribeirão da Mata, S20°10'34″, W46°33'35″, 1,100 m, 16.xi.2014, Nessimian, Oliveira, Rocha & Souto *leg.*, 6 subimagos (DZRJ); Alto Caparaó, Parque Nacional do Caparaó, Rio José Pedro, Cachoeira das Andorinhas, S20°22'29″, W41°51'28″, 06.x.2010, Ferreira-Jr & Clarkson *leg.*, 1 subimago (DZRJ); Espera Feliz, Parque Nacional do Caparaó, Pedra Menina, S20°37'30″, W41°49'27″, 14.x.2011, Massariol & Raimundi *leg.*, 5 nymphs (CZNC Ep-4194); same locality, 14.x.2010, 1 nymph (CZNC). Bocaina de Minas, Córrego do Morro Cavado, Cachoeira Santa Clara, S22°18'54″, W44°35'45″, 27.i.2012, 1 subimago (DZRJ); Itamonte, Parque Nacional do Itatiaia, 20.xi.2004, 1 subimago (DZRJ); same locality, 20.xi.2009, 8 nymphs (DZRJ); first-order stream, 21.ix.2007, Jardim, Santos, Dumas & Nessimian *leg.*, 1 nymp (DZRJ 3168); Rio Aiuruoca, S22°20'59″, W44°41'36″, 20.xi.2004, Nessimian & Ferreira-Jr *leg.*, 1 subimago (DZRJ); Itatiaia, 03.xi.2007, 12 nymphs (DZRJ 3169); Santa Clara, Rio Preto, Poção do Maromba, 16.xii.2006, Moreira, Braga, Alecrim & Vanini *leg.,* 2 nymphs (DZRJ 2652). **ESPÍRITO SANTO**, Pedra Roxaa, Parque Nacional da Serra do Caparaó, 01.vi.2011, 2 nymphs (CZNC); same data, 1 nymph (CZNC Ep-6433); same data, 1 nymph (CZNC Ep-6434); Ibitirama, Parque Nacional da Serra do Caparaó, Rio Pedra Roxa and tributary, S20°23'48″, W41°44'8″, 1,063 m, 20.iv.2008, Salles, Massariol, Lima, Boldrini & Brito *leg.*, 2 nymphs (CZNC Ep-218); same data, 1 nymph (CZNC Ep-249); river from Tecnotruta, "Sonho Meu" property, S20°28'9″, W41°43'22″, 959 m, Salles, Massariol, Lima, Boldrini & Brito *leg.*, 2 nymphs (CZNC Ep-222); same data, 1 nymph (CZNC Ep-226); Santa Marta, 31.v.2011, 1 nymph (CZNC); Santa Teresa, Nova Lombardia, Capitel de Santo Antônio, Córrego Escavado, S19°52'32″, W40°31'47″, 705 m, 19.i.2008, Salles, Massariol, Lima, Boldrini & Angeli *leg.*, 1 nymph (CZNC Ep-339); same locality, 26.x.2008, 1 nymph (CZNC Ep-976); Capitel de Santo Antônio, S19°52'31″, W40°31'49″, 768 m, 24–26.x.2008, Salles, Lima, Brito, Soares, Rúbio & Silva *leg.*, 1 nymph (CZNC Ep-950); Reserva Biológica Augusto Ruschi, S19°55'22″, W40°33'13″, 20.ii.2009, 1 nymph (CZNC); same data, 2 nymphs (CZNC Ep-1135); Córrego Bragacho, S19°52'3″, W40°33'34″, 28.iv–27.v.2017, Costa & Salles *leg.*, 2 subimagos (CZNC); same locality and collectors. 26.v–21.vi.2017, 1 subimago (CZNZ);); same locality and collectors, 26.vii–23.viii.2017, 2 subimagos (CZNC);); same locality and collectors, 24.viii–30.ix.2017, 1 subimago (CZNC); same locality and collectors, 21.x.2017–18.xi.2017, 1 subimago (CZNC); same locality and collectors, 17 nymphs (CZNC). **RIO DE JANEIRO**, Itatiaia, Parque Nacional do Itatiaia, Córrego Simon, S22°25'55″, W44°36'25″, 1,149 m, 14.iv.2007, Moreira *leg.*, 1 nymph (DZRJ 2712); Rio Campo Belo tributary, S22°26'44″, W44°36'27″, 900 m, 15.iv.2007, Dumas & Santos *leg.*, 3 nymphs (DZRJ 2714); Itatiaia, S22°35'59″, W44°35'58″, 17.iv.2007, Dumas, Santos, Fernandes & Nessimian *leg.,* 4 subimagos (DZRJ 3163); Rio Marimbondo, S22°21'42″, W44°35'14″, 14.x.2000, Huamantinco & Nessimian *leg.*, 14 nymphs (DZRJ 3170); Rio Campo Belo, trail to Cachoeira Véu da Noiva, S22°25'42″, W44°37'11″, 16.iv.2007, Dumas, Santos, Ferreira-Jr & Nessimian *leg.*, 3 subimagos (DZRJ 3171); Córrego Maromba, below Cachoeira Véu da Noiva, S22°25'39″, W44'37'10″, 10.i–02.ii.2015, Takiya, Santos & Monné *leg.*, 11 subimagos (DZRJ 3172); Cachoeira Véu da Noiva, S22°25'38″, W44°37'6″, 12.x.2013, Silva, Santos & Souza *leg.*, 1 subimago (DZRJ 3173); same locality, 16.iv.2007, Dumas, Santos, Ferreira-Jr & Nessimian

leg., 1 subimago (DZRJ 3174); Rio Campo Belo, piscina do Maromba, S22°25'46″, W44°37'10″, 16.iv.2007, Dumas, Santos, Ferreira-Jr & Nessimian *leg.*, 8 subimagos (DZRJ 3175); Vale do Pavão, Rio Marimbondo, S22°21'43″, W44°35'15″, 28.i.2012, Sampaio, Oliveira & Gomes *leg.*, 22 subimagos (DZRJ 3176); Visconde de Mauá, Maromba, Rio Monjola, Cachoeira Véu da Noiva, S22°19'41″, W44°36'1″, 26.I.2012, Oliveira *leg.*, 1 subimago (DZRJ 3164); Rio Preto tributary, 15.x.2000, 4 nymphs (DZRJ 3166); Rio Preto, Cachoeira do Escorrega, S22°19'30″, W44°36'55″, 26.i.2012, Sampaio *leg.*, 1 subimago (DZRJ 3167); Resende, Serrinha do Alambari, Cachoreira dos Amores, S22°23'36″, W44°34'10″, 1,041 m, 10.ii.2016, Takiya & Santos *leg.*, 32 subimagos (DZRJ); same data, 1 nymph (DZRJ); Teresópolis, Parque Nacional da Serra dos Órgãos, Rio Beija-flor (pool), 27.x.2007, Azevedo, Dumas & Kaplan *leg.*, 1 nymph (DZRJ 2651); Rio Beija-flor, S22°26'50″, W43°0'20″, 19.vii.2000, 3 nymphs (DZRJ 2713); same locality, 11.xi.2011, Oliveira, Nessimian & Santos *leg.*, Rio Paquequer, S22°27'23″, W42°59'50″, 23–24.iii.2010, 1 subimago (DNA Voucher DZRJ ENT2547); same data, 1 subimago (DNA Voucher DZRJ ENT2557) same data, Passos & Nessimian *leg.*, 1 subimago (DNA Voucher DZRJ ENT2550); tributary of Rio Beija-flor, trail to Pedra do Sino, S22°26'54″, W43°0'27″, 1,332 m, 14.xi.2011, Oliveira *leg.,* 1 nymph (DNA Voucher DZRJ ENT2554); Petrópolis, Bonfim, Parque Nacional da Serra dos Órgãos, Rio Bonfim, S22°27'55″, W43°5'16″, 1114 m, 19.xii.2011, Oliveira, Dumas, Passos, Gomes & Nessimian *leg.*, 2 subimagos and 1 imago (DZRJ); same data, 1 subimago (DNA Voucher DZRJ ENT2545); Rio Bonfim, S22°27'51″, W43°5'21″, 19.xii.2011, Oliveira, Dumas, Passos, Gomes & Nessimian *leg.*,1 subimago (DZRJ); Guapimirim, Parque Nacional da Serra dos Órgãos, Rio Soberbo, Poço da Preguiça, S22°29'34″, W43°0'04″, 388 m, Silva, Nessimian, Dumas & Souto *leg.,* 1 subimago (DZRJ); Serra do Subaio, Rio Varginha, 20.vii.2000, 1 nympha (DZRJ 2688); Nova Friburgo, Rio das Flores, S22°24'36″, W42°29'41″, 971 m, 30.xi.2008, Sampaio *leg.*, 1 imago (DZRJ 1699); same data, Gonçalves *leg.*, 3 subimagos (DZRJ 1700); same river, S22°25'37″, W42°30'26″, 1,062 m, 30.xi.2008, Gonçalves *leg.*, 11 subimagos (DZRJ 1702); same locality, 01.xii.2008, Jardim *leg.*, 1 nymph (DZRJ 1701); same river, S22°25'07″, W42°29'55″, 993 m,30.xi.2008, Gonçalves *leg.*, 1 imago (DZRJ 1703); same data, Sampaio & Santos *leg.*, 10 subimagos (DZRJ 1704); Rio Macaé, S22°24'46″, W42°31'16″, 935 m, 14.ix.2008, Alecrim *leg.*, 11 subimagos (DZRJ 1705), same locality and collector, 12.ix.2008, 7 subimagos (DZRJ 1706); same locality and collector, 13.ix.2008, 2 subimagos (DZRJ 1707); same locality and collector, 12.ix.2008, 5 subimagos (DZRJ 1708); same locality and collector, 14.ix.2008, 3 subimagos (DZRJ 1709); same locality, 30.xi.2008, Gonçalves *leg.*, 4 subimagos (DZRJ 1710); same locality and collector, 15.ix.2008, 11 subimagos (DZRJ 1704); Córrego Verdun, S22°25'27″, W42°32'08″, 1,008 m, Santos *leg.* (DZRJ 1714); first order tributary of the Macaé river, S22°25'52″, W42°32'14″, 1,055 m, 29.xi.2008, Santos *leg.*, 20 imagos (DZRJ 1716); second order tributary of the Macaé river, S22°25'58″W42°32'24″, 1,103 m, 29.xi.2008, Santos *leg.*, 1 subimago (DZRJ 1717); second order tributary of the Macaé river, S22°25'34″W42°32'56″, 1,103 m, 29.xi.2008, Santos *leg.*, 6 subimagos (DZRJ 1718); Rio Macaé, S22°23'30″, W42°29'6″, 944 m, 30.xi.2008, Santos & Sampaio *leg.*, 1 subimago (DZRJ 1719); same data, 1 subimago (DZRJ 1720); second order tributary of the Macaé river, S22°23'39″, W42°30'8″, 956 m, 01.xii.2008, Sampaio & Santos *leg.*, 1 subimago (DZRJ 1721); Rio Macaé, Cascata da Fumaça, S22°21'56″, W42°15'13″, 368 m, 08.iii.2009, Gonçalves *leg.*, 1 subimago (DZRJ 1727); Lumiar, Rio Boa Vista, S22°19'1″, W42°17'23″, 910 m, 14.xi.2008, Gonçalves *leg.*, 3 subimagos (DZRJ 1722); same data, Nessimian & Sampaio *leg.*, 2 subimagos (DZRJ 1723); Lumiar, second order of the Córrego Santa Margarida, S22°20'35″, W42°18'0″, 844 m, 17.xi.2008, De-Souza *leg.*, 2 nymphs (DZRJ 1724); first order of the Córrego Santa Margarida, S22°20'10″, W42°17'34″, 970 m, 16.xi.2008, De-Souza *leg.*, 2 nymphs (DZRJ 1725); first order of theRio Toca da Onça, S22°23'24″, W42°20'5″, 716 m, 05.iii.2009, Gonçalves *leg.*, 9 subimagos (DZRJ 1726); Sana, Córrego da Ilha (second order tributary of Rio Boa sorte), S22°20'42″, W42°11'4″, 381 m, 19.ii.2009, Gonçalves *leg.*, 8 subimagos (DZRJ 1728); Sana, São Bento, Córrego do Colégio, S22°20'23″, W42°12'13″, 294 m, 19.ii.2009, Gonçalves *leg.*, 14 subimagos (DZRJ 1729); São Fidelis, Parque Estadual do Desengano, Morumbeca dos Marreiros, Ribeirão Macapá, S21°52'40″, W41°54'30″, 1,083 m, Dumas, Nessimian, Portela & Barbosa *leg.*, 13.iv.2016, 1 subimago (DZRJ DNA Voucher ENT3294); same river and collectors, S21°52'36″, W41°54'44″, 1,111 m, 1 subimago (DZRJ DNA Voucher ENT3295); Santa Maria Madalena, Parque Estadual do Desengano, Morumbeca dos Marreiros, tributary of Ribeirão Macapá, S21°52'39″, W41°54'55″, 1,110 m, Dumas, Nessimian, Portela & Barbosa *leg.*,1 subimago (DZRJ DNA Voucher ENT3296); Mangaratiba, BR101, Rio Muriqui, S22°54'56″, W43°56'09″, 18.ix.2007, Baptista, Mugnai & Oliveira *leg.*, 4 nymphs (DZRJ 1385); Rio

Claro Lídice, Rio Cotia, S22°50'8″, W44°12'32″, 02.x.2007, Nessimian, Baptista, Mugnai & Oliveira *leg.*, 1 nymph (DZRJ 1435); Angra dos Reis, Parque Nacional da Serra da Bocaina, Trilha do Ouro, Rio Santo Antônio, 03.x.2007, Nessimian, Baptista, Mugnai & Oliveira *leg.*, 1 nymph (DZRJ 1463); Córrego Maitaca, S22°54'58″, W44°37'47″, 442 m, 09.viii.2003, Oliveira *leg.*, 2 nymphs (DZRJ 4420); same locality and collector, 07.viii.2004, 1 nymph (DZRJ 1146); unnamed stream, S22°55'31″, W44°37'31″, 318 m, 09.viii.2003, 3 nymphs (DZRJ 428); same data, 2 nymphs (DZRJ 433); same locality and collector, 07.viii.2004, 24 nymphs (DZRJ 1159); same data, 21 nymphs (DZRJ 1168); Córrego do Forno, S22°55'34″, W44°37'25″, 318 m, 07.viii.2004, Oliveira *leg.*, 6 nymphs (DZRJ 1169); same data, 4 nymphs (DZRJ 1183); tributary of Rio Mambucaba, S22°54'41″, W44°37'52″, 586 m, 07.viii.2004, Oliveira *leg.*, 1 nymph (DZRJ 1212); Córrego Itapetininga, S22°54'44″, W44°33'12″, 586 m, 01.ix.2004, Oliveira *leg.*, 4 nymphs (DZRJ 1252); Córrego da Memória (frontier of the Rio de Janeiro and São Paulo states), S22°54'17″, W44°37'44″, 720 m,09.viii.2003, Oliveira *leg.*, 22 nymphs (DZRJ 412); same data, 7 nymphs (DZRJ 417); same locality and collector, 07.viii.2004, 11 nymphs (DZRJ 1124); same data, 122 nymphs (DZRJ 1130); same data, 5 nymphs (DZRJ 1205**). SÃO PAULO**, São José do Barreiro, Parque Nacional da Serra da Bocaina, Córrego das Posses, S22°46'7″, W44°36'36″, 1,270 m, 17.iii.2003, Oliveira *leg.*, 5 nymphs (DZRJ 9); same data, 1 nymph (DZRJ 21); same locality and collector, 07.viii.2003, 6 nymphs (DZRJ 382); same data, 4 nymphs (DZRJ 384); same locality and collector, 05.viii.2004, 1 nymph (DZRJ 962); same locality, 11.xii.2012, 3 subimagos (DZRJ); Ribeirão da Prata, S22°46'49″, W44°36'40″, 2 nymphs (DZRJ); same locality, 01.ix.2012, 3 nymphs (DZRJ); same locality, 19.xii.2010, 1 subimago (DZRJ); same data, 9 subimago (DZRJ); same locality, 18.xi.2012, 1 nymph (DZRJ); same locality, 07.viii.2003, Oliveira *leg.*, 122 nymphs (DZRJ 389); same data, 6 nymphs (DZRJ 465); same locality and collector, 5.viii.2004, 51 nymphs (DZRJ 985); Ribeirão do Boqueirão, S22°45'17″, W44°37'6″, 1,364 m, 23.ix.2006, Oliveira *leg.,* 1 nymph (DZRJ); same locality, 05.x.2007, Baptista, Mugnai, Nessimian & Oliveira *leg.*, 3 nymphs (DZRJ 1529); tributary of Rio Mambucaba, S22°43'47″, W44°37'5″, 1,550 m, 17.iii.2003, Oliveira *leg.*, 2 nymphs (DZRJ 37); same locality and collector, 06.viii.2003, 2 nymphs (DZRJ 364); same data, 3 nymphs (DZRJ 463); same locality and collector, 16 nymphs (DZRJ 921); tributary of Rio Mambucaba, S22°44'6″, W44°36'58″, 1,520 m, 7.viii.2003, Oliveira *leg.*, 12 nymphs (DZRJ 371); same data, 2 nymphs (DZRJ 372); same data, 9 nymphs (DZRJ 374); same data, 1 nymph (DZRJ 377); same data, 1 nymph (DZRJ 747); same locality, 21.iv.2006, 5 nymphs (DZRJ); Fazenda Barreirinha, tributary of Rio Mambucaba, S22°49'23″, W44°35'52″, 1,200 m, 7.viii.2003, Oliveira *leg.*, 2 nymphs (DZRJ 394); same locality and collector, 05.viii.2004, 1 nymph (DZRJ 1017); same data, 5 nymphs (DZRJ 1027); same data, 2 nymphs (DZRJ 1030); same data, 1 nymph (DZRJ 1035); same locality and collector, 5.viii.2004, 1 nymph (DZRJ 112); Rio Mambucaba, 05.x.2007, Baptista, Mugnai, Nessimian & Oliveira *leg.*, 4 nymphs (DZRJ 1546); Córrego Barra Branca, S22°51'10″, W44°36'7″, 1,040 m, 07.viii.2003, Oliveira *leg.*, 1 nymph (DZRJ 397); same data, 2 nymphs (DZRJ 464); same locality and collector, 5.viii.2004, 1 nymph (DZRJ 1046); same data, 4 nymphs (DZRJ 1049); Córrego do Moinho, S22°51'19″, W44°36'58″, 940 m, 08.viii.2003, Oliveira *leg.*, 15 nymph (DZRJ 402); same data, 1 nymph (DZRJ 468); same locality and collector, 06.viii.2004, 2 nymphs (DZRJ 1064); same data, 2 nymph (DZRJ 1069); same data, 1 nymph (DZRJ 1073); same data, 8 nymphs (DZRJ 1077); same data, 3 nymphs (DZRJ 1080); Córrego São Gonçalo, S22°52'29″, W44°36'6″, 920 m, Oliveira *leg.*, 1 nymph (DZRJ 474); same data, 5 nymphs (DZRJ 475); same data, 1 nymph (DZRJ 796); same locality and collector, 06.viii.2004, 26 nymphs (DZRJ 1094); same data, 37 nymphs (DZRJ 1103); Ubatuba, Parque Estadual da Serra do Mar, Km 2, BR101, 300 m from Cachoeira da Escada, S23°21'14″, W44°46'4″, 233 m, 9.ix.2011, Souto *leg.*, 4 subimagos (DZRJ); same locality and collector, 08.ix.2011, 10 subimagos (DZRJ); same data, 2 subimagos (DZRJ); same locality and collector, 04.vi.2011, Oliveira, Takiya, Nessimian & Souto *leg.*, 5 subimagos (DZRJ); Poço do Amor, Km 2, BR101, 146 m, 2 subimagos (DZRJ); Cunha, Parque Estadual da Serra do Mar, Núcleo Cunha-Indaiá, S23°16'34", W45°02'10", 1004 m, 24.vii.2012, Thiago Polizei & Lucas Costa *leg.*, 3 nymphs (DZRJ 3193); same data, 6 nymphs (DZRJ 3194); same data, 1 nymph (DZRJ 3195); São Luís do Paraitinga, Parque Estadual da Serra do Mar, Núcleo Santa Virgínia, S23°18'46", W45°07'12", 918 m, 28.vii.2012, Thiago Polizei & Lucas Costa *leg.*, 6 nymphs (DZRJ 3198); same data, 1 nymph (DZRJ 3200); same locality and collector, S23°20'36", W45°07'43", 823 m, 30.vii.2012, 1 nymph (DZRJ 3199); Caraguatatuba, Parque Estadual da Serra do Mar, S23°35'02", W45°24'58", 163 m, Thiago Polizei & Lucas Costa *leg.,* 04.viii.2012, 3 nymphs (DZRJ 3196); same data, 4 nymphs (DZRJ 3197); São Miguel Arcanjo, Serra de Paranapiacaba, Parque Estadual Carlos Botelho, S24°09'25″, W47°58'55″, 699 m,

16.vii.2013, Thiago Polizei & Lucas Costa *leg.,* 5 nymphs (DZRJ 3181); same data, 1 nymph (DZRJ 3184); same data, 3 nymphs (DZRJ 3186); same data, 3 nymphs (DZRJ 3187); same locality and collector, S24°04'32″, W47°58'43″, 717 m, 15.vii.2013, 8 nymphs (DZRJ 3182); same data, 1 nymph (DZRJ 3183); same data, 1 nymph (DZRJ 3185); Campos do Jordão, Parque Estadual do Campos do Jordão, S22°41'33", W45°27'54″, 1.555 m, 04.vii.2013, Thiago Polizei & Lucas Costa *leg.,* 6 nymphs (DZRJ 3188); same locality and collector, S22°39'50″, W45°27'06", 1.539 m, 05.vii.2013, 1 nymph (DZRJ 3189); same locality and collector, S22°41'39″, W45°27'54″, 1.537 m, 06.vii.2013, 1 nymph (DZRJ 3190); same locality and collector, S22°41'49″, W45°29'20″, 1.542 m, 06.vii.2013, 1 nymph (DZJR 3191); same locality, 14–16.xii.1987, 2 imagos (MZUSP); 16.xii.1987, 7 nymphs (MZUSP); Córrego Galharada, 25.ix.1997, 33 nymphs (MZUSP); same locality, 15.x.1998, 1 subimago (MZUSP); Ribeirão Grande, Parque Estadual de Intervales, S24°19'17", W48°23'29", 658 m, 06.vii.2012, 1 nymph (DZRJ 3192).