Biologia de microlepidópteros (Gracillariidae) associados a *Daphnopsis fasciculata* (Meisn.) Neveling (Thymelaeaceae) e *Psychotria suterella* Müll. Arg. (Rubiaceae) na Mata Atlântica.
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Aprovada em _____ de ______________ de _____.

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Agradeço inicialmente aos meus pais Genírio e Damaris pelo apoio em todos os momentos da minha formação escolar e acadêmica.

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RESUMO

Gracillariidae (Lepidoptera), embora ainda pouco estudada, é considerada a maior família de microlepidópteros minadores de folhas. Esses insetos, cujo tamanho não ultrapassa 10 mm de envergadura, chamam atenção pelas cores brilhantes e pelo desenvolvimento hipermetamórfico. Este trabalho teve o objetivo de descrever os estágios imaturos de duas novas espécies de gracilarídeos para a região Neotropical, associadas a duas hospedeiras distintas: *Daphnopsis fasciculata* (Meisn.) Neveling (Thymelaeaceae) e *Psychotria suterella* Müll. Arg. (Rubiaceae), cujas populações são encontradas no município de São Francisco de Paula, estado do Rio Grande do Sul, Brasil. As descrições e ilustrações tiveram como base a microscopia óptica e eletrônica de varredura. A anatomia das minas também foi explorada através de cortes histológicos. Análises filogenéticas com base em DNA mitocondrial (COI) incluindo espécies congeneres, também foram conduzidas, e revelaram uma grande magnitude de divergência genética entre as linhagens (de 23 a 30%). Embora pertencentes a subfamílias distintas (Phyllocnistinae e Oecophyllembiinae, respectivamente) ambas apresentam os primeiros instares do tipo *sap-feeding*, cuja larva é achatada e com aparelho bucal prognato, adaptado para dilacerar os tecidos foliares e possibilitar a ingestão de fluidos. A seguir na ontogênese, ambas apresentam também um instar do tipo *spinning*, cuja larva não se alimenta e possui as estruturas bucais atrofiadas, com exceção do espinerete usado para tecer o casulo pupal. A primeira espécie, encontrada em *D. fasciculata*, alimenta-se de tecido subepidérmico no primeiro instar, passando para o parênquima paliçádico nos demais instares. Já a segunda espécie, associada a *P. suterella*, alimenta-se de parênquima paliçádico durante todo o seu desenvolvimento. Estes gracilarídeos destacam-se por serem, respectivamente, o primeiro registro em Thymelaeaceae em nível mundial e o primeiro Oecophyllembiinae assinalado para o Brasil.
ABSTRACT

Although little studied yet, Gracillariidae (Lepidoptera) is the largest family of leaf-miner microlepidoptera. Their size does not exceed 10 mm (wingspan), but call the attention due to their brilliant color and by having hypermetamorphic development. The main goal of this study was to describe the immature stages of two new gracillariid species for the Neotropical region, associated with distinct host plants: *Daphnopsis fasciculata* (Meisn.) Neveling (Thymelaeaceae) and *Psychotria suterella* Müll. Arg. (Rubiaceae), from populations located in the São Francisco de Paula municipality, Rio Grande do Sul state, Brazil. Descriptions and illustrations were based on optical and scanning electron microscopies. The mine anatomy was explored by performing histologic sections. Phylogenetic analyses based on mitochondrial DNA (COI) including congeneric species were also conducted, and revealed a great magnitude of divergence among lineages (from 23 to 30% of genetic distance). Although belonging to different subfamilies (Phyllocnistinae and Oecophyllembiinae, respectively), both species present sap-feeding instars, in which the larvae is dorso-ventrally flattened and prognathous, adapted to lacerate leaf tissue and ingest fluids. Afterwards in ontogeny, both also present a spinning instar, a non-feeding morphotype bearing atrophied mouth parts, except the spinneret used to build the pupal cocoon. The first species, found on *D. fasciculata* feeds on subepidermic tissue during the first instar, changing to palisade parenchyma during other instars. The second species, associated with *P. suterella* feeds on palisade parenchyma throughout its development. These gracillariid species account respectively for the world record on Thymelaeaceae and the first Oecophyllembiinae to be found in Brazil.
CAPÍTULO I
INTRODUÇÃO GERAL

Minas são canais ou galerias formados por larvas de insetos herbívoros dentro de tecidos das plantas, como parênquima ou epiderme, o qual fornece alimento e abrigo para as larvas (Hering, 1951). As minas podem ser construídas por representantes de quatro ordens de insetos: Coleoptera, Diptera, Hymenoptera e Lepidoptera (Sinclair & Hugues, 2010). Podem ser classificadas quanto à forma, por exemplo: minas serpenteantes, lineares, em forma de manchas ou blocos e digitadas; além disso, seu formato pode auxiliar na identificação tanto em nível genérico quanto específico (Moore, 1966). Alguns microlepidópteros podem passar todo o estágio larval dentro de uma única mina, em um grande número de angiospermas (De Prins & De Prins, 2018). Algumas dessas espécies estão associadas a hospedeiras de interesse econômico, tais como Citrus sp., Coffea sp., Malus sp. e Theobroma sp. (Sinclair & Hugues, 2010; De Prins & Kawahara, 2012).


O ciclo de vida é a característica mais marcante dessa família. Os ovos podem ser depositados na superfície adaxial ou abaxial da folha na planta hospedeira (Sinclair & Hugues, 2010). Após a eclosão, a larva entra em contato com a epiderme da planta, por onde penetra dando início a alimentação e a construção da mina (Davis, 1987). As larvas
apresentam hipermetamorfose, ou seja, transformações larvais morfológicas ao longo da ontogênese. Assim, podem ser divididas em três tipos morfológicos que podem variar de acordo com a espécie: 1) larva *sap-feeding* (primeiros ínstars), é caracterizada pela cabeça prognata com peças bucais adaptadas à dilaceração de tecidos e ingestão de líquidos; cabeça e corpo achatados com pernas e pseudopódios reduzidos ou, na maioria dos casos, ausentes; 2) larva *tissue feeding*, apresenta cabeça hipognata com peças bucais do tipo mastigadora; cabeça e corpo cilíndrico com pernas e pseudopódios geralmente presentes, sendo esse último, presente do terceiro ao quinto e no décimo segmento abdominal; 3) larva *spinning*, também conhecida como pré-pupa, caracterizada pelo aparelho bucal atrofiado apresentando somente o espinetere funcional, utilizado na construção do casulo. Esse último morfotipo é característico de *Phyllocnistis* Zeller, mas pode ser encontrado também em algumas espécies dos gêneros: *Marmara* Clemens, *Cameraria* Chapman, *Metrichocho* Busck e *Chrysaster* Kumata (Kumata, 1978). No entanto, Brito et al. (2013) descreveram o primeiro gracilarídeo desprovido de ínstars *sap-feeding*.

As espécies do grupo têm uma grande variação quanto a história de vida. Podem utilizar para a construção da mina, além de tecidos foliares, flores e frutos, podendo alterar sua forma de alimentação durante o seu desenvolvimento (Davis, 1987). Algumas espécies podem ser consideradas monófagas ou oligófagas, no entanto, representantes de Gracillariidae podem se alimentar de diversas famílias de plantas espalhadas nos diferentes continentes (De Prins & De Prins, 2018; Kawahara et al., 2017). As minas foliares também apresentam variações, com uso potencial como caracteres diagnósticos entre as espécies (Davis 1987). Algumas são estreitas e serpenteantes, podendo continuar assim até o final no estágio larval, apenas aumentando a largura das galerias. Outras começam serpenteantes formando manchas no decorrer do desenvolvimento, ou formam manchas desde o início do desenvolvimento, podendo cobrir parcialmente ou totalmente a superfície foliar. As fezes também podem auxiliar na identificação, já que algumas deixam rastros visíveis, muitas vezes com padrões distintos ao longo do desenvolvimento larval (Davis, 1987).

O objetivo deste trabalho foi descrever os estágios imaturos de duas novas espécies de gracilarídeos minadores de folhas associados a duas plantas hospedeiras, *Daphnopsis fasciculata* (Meisn.) Nevling (Thymelaeaceae) e *Psychotria suterella* Müll.Arg. (Rubiaceae), ocorrentes no município de São Francisco de Paula, estado do Rio Grande do Sul (RS), Brasil. Contempla-se a morfologia geral externa destes em nível de
microscopia óptica e de varredura, suas histórias de vida, incluindo a descrição anatômicas das minas, bem como inferências moleculares com base em DNA mitocondrial (COI) quanto as relações filogenéticas dentro da família. Os respectivos resultados integram dois artigos, um já publicado e outro que fará parte de uma publicação em colaboração, de maior escopo, incluindo a descrição de outras duas espécies.

Referências


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Systematics, Morphology and Biogeography

Phyllocnistis hemera sp. nov. (Lepidoptera: Gracillariidae): a new species of leaf-miner associated with Daphnopsis fasciculata (Thymelaeaceae) in the Atlantic Forest

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ABSTRACT

During recent studies performed in the Atlantic Forest, a new species of Phyllocnistinae (Gracillariidae), Phyllocnistis hemera sp. nov., leaf miner of Daphnopsis fasciculata (Thymelaeaceae) was discovered. The adults are described and illustrated as well as the immature stages, with notes on natural history including a description of the leaf mine. Additionally, DNA barcode sequences were compared to other representatives of Phyllocnistinae to test for the specific status of Phyllocnistis hemera and to infer phylogenetic relationships. This is the fifth species described for the genus Phyllocnistis in the Atlantic Forest and the first record of a gracillarid mining Thymelaeaceae leaves.

Keywords:
Microlepidoptera
New species
Morphology
DNA barcoding
Neotropical region

Introduction

The Gracillariidae (Lepidoptera) is one of the most diverse families of leaf mining microlepidoptera. Approximately 2000 species have been described in 106 genera, with cosmopolitan distribution but not found in the Antarctic region (Davis, 1987; De Prins and De Prins, 2017). Kawahara et al. (2017) carried out a phylogenetic analysis and proposed a new classification for the group, dividing Gracillariidae into eight subfamilies. The Phyllocnistinae, one of the subfamilies retained in the new classification, has been the recent focus of taxonomic studies of the Neotropical region (Kawahara et al., 2009; Davis and Wagner, 2011; Brito et al., 2012; De Prins et al., 2016; Brito et al., 2017a,b).

Phyllocnistinae is a monotypic subfamily represented by species of Phyllocnistis Zeller, 1848. This genus has currently 108 species described worldwide, but only 27 for the Neotropics (De Prins and De Prins, 2017; Brito et al., 2017a). Representatives of this genus can be distinguished from the other gracillarids by a set of typical fasciae and strigulae on the forewings, and by a set of tergal spines on the abdominal segments on the pupae. The larvae usually present three sap-feeding instars followed by a spinning instar (Davis, 1987). The sap-feeding instar feeds on cell fluid which is released by the laceration of leaf tissue; the spinning instar, which does not feed, is responsible for the construction of a silk cocoon within which pupation occurs. Only two species, P. citrella Stanton and P. tethys Moreira & Vargas, have information regarding the type of tissue used as food by the sap-feeding larvae. Those of P. citrella are known to feed on epidermal cells, while larvae of P. tethys feed upon the spongy tissue (Achor et al., 1997; Brito et al., 2012).

Hostplants are known only for a third of the species of Phyllocnistis. They belong to 34 families of angiosperms, 13 of which occur in the Neotropics (De Prins and De Prins, 2017). Recently, during collections performed in the Atlantic Forest, in southern Brazil, a gracillariid representative associated with Thymelaeaceae was found for the first time. The comparison at both morphological and molecular levels confirmed that it is a new Phyllocnistis species. Here we provide illustrations and description of the corresponding adult and immature stages, and highlight important characteristics regarding its life history and feeding habits in association with the characterization of the leaf mine. DNA barcode (COI) was obtained from some specimens in order to establish the specific status and its phylogenetic relationships with representatives of the Phyllocnistinae.

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0085-5626/© 2017 Sociedade Brasileira de Entomologia, Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Material and methods

The specimens were reared in small plastic vials under controlled abiotic conditions (14h light/10h dark; 25 ± 2 °C) at the Laboratório de Morfologia e Comportamento de Insetos, Departamento de Zoologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul state (RS), Brazil, during May 2016, January, June and July 2017. They came from field-collected leaf mines associated with the host plant Daphnopsis fasciculata (Meisn.) Neveling (Thymelaeaceae), at the Centro de Pesquisa e Conservação da Natureza (CPCN Pró-Mata/PUCRS 29’28’36”S, 50’10’01”W), São Francisco de Paula, RS.

Morphological analysis

Adults were pinned and dried. Immature stages were fixed with Dietrich’s fluid and preserved in 75% ethanol. At least 3 specimens of each stage were used to describe the immatures. For adult morph morphology the specimens were cleared in 10% potassium (Paiva et al., 2006). Photographs were taken under a Leica DM 2500-LED light microscope with a Leica DFC 7000T camera.

For scanning electron microscopy, specimens were dehydrated in a Bal-tec CPD030 critical-point drier, mounted on metal stubs with double-sided tape and coated with gold in a Bal-tec CPD030 critical-point drier. Photographs were taken under a JEOL JSM5800 scanning electron microscope at the Centro de Microscopia Eletrônica (CME), UFRGS.

For histological sections, leaf fragments (0.5 cm²) with mines (n=6) were fixed in FAA (37% formaldehyde, acetic acid, 50% ethanol, 1:1:18, v/v) for 48 h, dehydrated in an n-butyl series, embedded in Paraplast and sectioned transversely (12 μm) in a rotary microtome (Jung Biocut). The sections were stained in eosin/H9262 and mounted in colorless varnish (Paiva et al., 2006). Photographs were taken under a Leica DM 2500-LED light microscope with a Leica DFC 580 camera.

Museum collections

The material examined was deposited in the following entomological collections:

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</table>

Molecular analysis

Genomic DNA was extracted from larval tissue of three specimens of P. hemera (LMCI 292-25C, D and E) using the Purelink genomic DNA extraction kit (Invitrogen) following manufacturer’s instructions. Polymerase chain reaction (PCR) was performed to amplify the DNA barcode region (sensu Hebert et al., 2003), i.e. a 660-base pair (bp) segment of the mitochondrial gene cytochrome c oxidase subunit I (COI), with the universal primers and conditions proposed by Folmer et al. (1994). Variants obtained matched the region sequenced in other Phyllocnistis deposited in the GenBank and BOLD Systems databases. PCR products were treated with exonuclease I and FastAP thermosensitive alkaline phosphatase (Thermo Scientific), sequenced using BigDye chemistry and analyzed in an ABI3730XL (Applied Biosystems). Sequences were automatically aligned using the algorithm Clustal X in MEGA v5 (Tamura et al., 2011) running in full mode. Data generated in this study were deposited in GenBank and BOLD (Table 1).

Phylogenetic trees were reconstructed to test the specific status of the new species and to infer its relationships within the genus. Representative taxa belonging to Phyllocnistis were incorporated, particularly Neotropical species already described (Table 1). The tree was rooted with species of Angelabella Vargas & Parra and Marmara Clemens and representatives of the subfamilies Oecophyllaeinae and Marmarinae, known to be closely related to Phyllocnistiane (Kawahara et al., 2017).

Phylogenetic reconstruction used distance (neighbor-joining [NJ]) and model-based methods (Maximum Likelihood [ML] and Bayesian inference [BI]). The substitution model GTR+G was used for ML and BI according to the Akaaike Information Criterion (AIC) performed in MEGA v5. The NJ and ML analyses were run in MEGA v5 using default parameters for tree inference. Monophyly confidence limits were assessed with the bootstrap method (Felsenstein, 1985) at 60% cutoff after 1000 bootstrap iterations. The BI analysis was implemented in BEAST v1.8.4 (Drummond et al., 2012), using an uncorrelated lognormal clock and a Yule prior on branching rates. Four independent runs of 10 million generations and a burn-in period of 10,000 (the first 1000 trees were discarded) were implemented; the remaining trees were summarized in TreeAnnotator v1.6.2 (Drummond and Rambaut, 2007) and used to infer a maximum a posteriori consensus tree. Bayesian posterior probabilities (BPP) were used as an estimate of branch support. Consensus trees were visualized and edited in FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/201/). The genetic distances between the same pairs of taxa used in the phylogenetic analysis (including outgroups) were analyzed using the Kimura 2-parameter (K2P) model, with 1000 bootstrap replications in MEGA v5.

Results

Phyllocnistis hemera Brito & Fochezato sp. nov. (Figs. 1–7)

Type material. MALE HOLOTYPE: São Francisco de Paula municipality, Rio Grande do Sul (RS), Brazil; preserved pinned and dried,

**PARATYPES:** Same locality, 28–30.VI.2017, G.R.P. Moreira and J. Foechezato coll., two males (LMCI 319-46 and one female (LMCI 306-43) donated to MCTP (60251, 60252, respectively); 01–02.VII.2017, G.R.P. Moreira and J. Foechezato coll., one female (LMCI 320-43), donated to MCTP (60253).

Additional specimens examined from the same locality and host plant, all preserved pinned and dried: 22–24.VI.2016, G.R.P. Moreira, R. Brito and J. Foechezato colls., four males (LMCI 306-26, 32, 36 and 40) with genitalia on slides (GRPM 50-144 to 147, respectively); three females (LMCI 306-34, 35 and 49) with genitalia on slides (GRPM 50-148 to 150, respectively); 28–30.VI.2017, G.R.P. Moreira, R. Brito and J. Foechezato colls., four males (LMCI 306-26, 32, 38 and 40) with genitalia on slides (GRPM 50-144 to 147, respectively); three females (LMCI 306-34, 35 and 49) with genitalia on slides (GRPM 50-148 to 150, respectively); 01–02.VII.2017, G.R.P. Moreira and J. Foechezato coll., one male (LMCI 319-69).

Additional immature specimens of *P. hemera* were deposited at LMCI, all dissected from leaf mines of *D. fasciculata* collected at the type locality: 10–13.II.2015, G.R.P. Moreira and R. Brito coll., preserved in 100% ethanol at −10°C and used for DNA extraction (LMCI 292–25); 28–30.VI.2017, G.R.P. Moreira and J. Foechezato coll. and preserved in 75% ethanol. Eight sap-feeding larvae (LMCI 319-9), three spinning larvae (LMCI 319-8) and three pupae (LMCI 319-7) were used for microscopic studies. Additionally, nine leaf mine fragments containing sections of *P. hemera* mines (LMCI 320–9 to 11) from the type locality were fixed and preserved in FAA as described above, and used in the histological sections, 01–02.VII.2017, G.R.P. Moreira and J. Foechezato coll.

**Diagnosis.** *P. hemera* adults are easily distinguished from the other Neotropical *Phyllocnistis* by a longitudinal fascia on the forewing with superior border enlarged, reaching the costal margin. Its puptal stage is similar to that of *P. drimiphaga* Kawahara, Nishida & Davis in having the cocoon-cutter divided into three processes, the central longer than the lateral ones, and by the similar arrangement of tergal spines on abdominal segments. However, *P. hemera* has lateral processes shorter and wider than *P. drimiphaga*, and two pairs of setae on the clypeus, while *P. drimiphaga* has only one pair. The spinning larva of *P. hemera* is similar to that of *P. ourea* Brito & Moreira, as both share ventral ambulatory, single callus on central meso- and metathorax. However, these species differ in the location of the ventral calli on the abdominal segments (Ab): *P. hemera* has ventral calli on abdominal segments 3 to 7 (Ab 3–7), while *P. ourea* has calli on Ab 3–6.

**Description.** *Adult* (Fig. 1). Male and female similar in size and color. Forewing length 3.51–4.17 mm (n = 5). **Head:** antennae silver, ~ the length of forewing. A pair of tufts formed by a set of scales emerging from the base of antenna are directed to the frons. Labial palp slender, silver, ~0.5 mm in length. Proboscis without scales. **Thorax:** Forewing ground color white silver, with light yellow fasciae bearing brown borders. Longitudinal fascia with well-marked border, which is much wider and convex on the basal half, reaching the costal margin; longitudinal fascia emerges from the wing base toward the median region, being completely connected to the first transverse fascia. The latter emerges on the costal margin and is slightly connected to the second transverse fascia. The second transverse fascia crosses the wing from the costal margin toward the inner margin; it is disconnected from the third and fourth fascia. Last two fasciae fused, forming a blotch on the distal region. Costal strigulae emerge from second, third and fourth transverse fasciae. Apical strigulae emerge from apical spot. Inner marginal fringes mostly light brown. Abdomen: covered with silver scales.

**Male genitalia** (Fig. 2A–D). One pair of coremata located between the intersegmentary membrane of Ab 8 and 9; the coremata are formed by a set of long, fine scales, reaching ~0.4× the size of valvae (Fig. 2B and C). Uncus absent. Tegumen narrow at base, widening toward the apex, forming a dorsal sclerotized arch; small setae occur next to the lateral borders of the tegumen; tuba analis narrow and membranous, surpassing the distal margin of tegumen. Valvae digitiform, slightly narrower and finer on base, widening toward the apex. Setae vary in size from small to medium on ventral distal region, forming a line; along the valva, setae varying in size are randomly arranged (Fig. 2A and C). Saccus U-shaped. Phallos elongated, weakly sclerotized, cylindrical and partially wrinkled, with fine apex. Cornuti absent (Fig. 2D).

**Female genitalia** (Fig. 2E–G). Anterior and posterior apophysae similar in shape; the posterior half the size of the anterior; the posterior apophysae reach Ab 8 and the anterior ones the posterior portion of Ab 7. Anal papillae with medium-sized setae, randomly arranged. Ostium bursae located on median region of the eighth abdominal sternum; ductus bursae long, membranous and slender; corpus bursae ellipsoid and membranous; signum wide, slightly rectangular with two spines on the proximal margin; one acute, well developed, the other of reduced size (Fig. 2E–G). Variation in these structures was found, such as: (1) one of the signa with an acute spine, the other without spines; (2) both signa bearing well developed spines; (3) one single signum containing a spine with bifurcated apex.

**Immature stages**

**Sap-feeding larva** (Figs. 3A, 4 and 7D). Leaf miner, flattened dorsoventrally (Fig. 4D), hypermetamorphic, presenting three instars. Body light yellow, 5.39–7.67 mm (min–max length); average last instar head capsule width ~0.64 mm (n = 5) (Fig. 7D). **Head:** prognathous, setae absent; labrum slightly bilobed with small hypopharyngeal spines next to lateral margin. Labium shaped like the labrum, however wider and with greater number of spines.
**Fig. 2.** *P. hemera* genitalia under light microscopy: (A–D) male genitalia; (E–G) female genitalia. (A) apex of left valva, mesal view (LMCI 319-69); (B) left corema, ventral (LMCI 306-26); (C) male genitalia, ventral; (D) aedeagus, lateral (LMCI 306-36); (E) female genitalia, ventral; (F) female last abdominal segments, lateral (LMCI 306-49) with the ostium bursae indicated by arrow; (G) signum in detail, ventral (LMCI 306-49). Scale bars: 50 (A, B, D), 100 (C, F, G), 400 μm (E).

**Fig. 3.** Larval and pupal morphology of *P. hemera* under light microscopy: (A) sap-feeding larva, dorsal and ventral views; (B) spinning larva, dorsal and ventral; (C) pupa, dorsal, ventral and lateral, respectively. Scale bars: 500 μm.

(Fig. 4A–C and E); a small aperture on labium indicating the rudimentary spinneret (Fig. 4F). Labial and maxillary palpi absent. Antenna 3-segmented; the second segment with two sensilla, the distal one with a single apical sensillum (Fig. 4G). On anterior section of lateral region of the head, one rounded stemma followed by a microseta (Fig. 4H). Thorax: without setae, legs absent; presence of one pair of latero-dorsal prothoracic spiracles, with peritreme not differentiated (Fig. 4I). Abdomen: setae, prolegs and calli absent; a pair of laterodorsal lobes from first to eighth abdominal segment (Fig. 4J and M–N). These lobes are dorsoventrally flattened; on Ab 8 there is a second pair of lateroventral lobes with fine apex (Fig. 4K and L); last abdominal segment slightly divided, with pairs of microsetae on ventral region (Fig. 4O).

**Spinning larva** (Figs. 3B, 5 and 7E). Endophyllous, cylindrical, with coloration similar to the sap-feeding larva, 5.47–6.04 mm (min–max length). Body covered with microtrichia. Head: setae absent or reduced, except for three pairs located on the clypeal region; buccal apparatus modified into an anteriorly located, trophic lobe presenting corrugated tegument (Fig. 5A, B, D and E). Labial palpi absent; maxilla represented by three pairs of small setae. The trophic lobe in ventral view is long, with functional apical aperture (Fig. 5C). Antennae short, 3-segmented; three sensilla emerge from the second segment and one, bristle-like seta from the apical segment (Fig. 5H). Thorax: setae reduced or absent. Legs absent; prothoracic shield slightly evident, represented by an irregular, corrugated, central area (Fig. 5F). Laterally on prothoracic tergum one pair of rounded spiracles, with peritreme slightly elevated (Fig. 5G). A single ambulatory callus centrally on ventral region of meso- and metathorax; slightly divided on metathorax (Figs. 3B and 5I and J). Abdomen: one pair of ambulatory calli ventrally on Ab 3–7 (Figs. 3B and 5M and N), smaller compared to those on meso- and metathorax. One pair of small lateral sensilla on Ab 4–7 (Fig. 5L), which decrease in size antero-posteriorly. Last abdominal segment partially divided into two lobes (Fig. 5K) with two pairs of microsetae on ventral region (Fig. 5O).

**Pupa** (Figs. 3C, 6 and 7G). Dark brown (Fig. 7G), 3.92–4.08 mm (min–max length). Cocoon-cutter with three projections; the central one lanceolate, longer and wider, with serrated border; the lateral ones shorter, hook-shaped (Fig. 6A–B and D). Clypeus slightly bilobed, with two pairs of small setae (Fig. 6B). Antenna long...
Fig. 4. Scanning electron micrographs of *P. hemera* sap-feeding larva: (A–D) head under dorsal, ventral, anterior and lateral views (arrow indicates stemma); (E) labrum, dorsal; (F) labium, ventral (arrow indicates spinneret aperture); (G) antenna, ventral; (H) stemma in detail (indicated by arrow in D), lateral; (I) prothoracic spiracle, dorsal; (J) segment A7, ventral; (K) segments A8-10, ventral; (L) detail of latero-ventral lobe indicated by arrow in K, ventral; (M, N) latero-dorsal lobe, highlighted by the red rectangle in K, lateral and dorsal; (O) last abdominal segment, ventral. Scale bars: 200 (A, B, D, K), 70 (C), 100 (E, F, O), 30 (G), 20 (H), 10 (I), 250 (J), 25 (L), 50 (M), 40 μm (N).

Fig. 5. Scanning electron micrographs of *P. hemera* spinning larva: (A, B) head, dorsal and ventral views; (C) spinneret, antero-lateral (arrow indicates functional aperture); (D) head, lateral; (E) detail of trophic lobe, dorsal; (F) prothoracic shield, dorsal; (G) prothoracic spiracle, lateral; (H) antenna, anterior; (I) meso- and metathoracic calli, ventral; (J) mesothoracic callus in detail (indicated by rectangle in I), ventral; (K) abdominal segments Ab 7-10, dorsal; (L) latero-sensillum indicated by arrow in K, dorsal; (M) abdominal segment Ab 7, ventral (arrow indicates one of the calli); (N) callus in detail, ventral (indicated by arrow in M); (O) last abdominal segment, ventral. Scale bars: 200 (A, B, D, E, K), 150 (C, F), 10 (G, N), 20 (H, I), 250 (J), 80 (J, O), 100 μm (M).
Fig. 6. Scanning electron micrographs of *P. hemera* pupa: (A) head, lateral view; (B) setae over clypeus, ventral; (C, D) cocoon-cutter, ventral and dorsal; (E) terga of abdominal segments Ab 3-4, dorsal; (F) detail of segment Ab 3, dorsal; (G) lateral seta with fine apex, adjacent to spiracle on abdominal segment Ab 4, dorsal; (H) lateral seta of Ab 7 with clavate apex, dorsal; (I) detail of tergum of Ab 3, lateral; (J–L) last abdominal segments, lateral, dorsal and ventral. Scale bars: 200 (A), 80 (B), 100 (C, D, G, K, L), 400 (E), 150 μm (F, H, I, J).

...and filiform, reaching the last abdominal segments (Fig. 3C); proboscis extending to anterior margin of Ab 2; anterior, median and posterior legs reaching Ab 3, Ab 4 and Ab 7, respectively; forewings extending to the posterior portion of Ab 5 (Figs. 3C and 6E). A set of small, posteriorly directed spines, dorsally at the center from second to seventh abdominal segments (Fig. 6I); on tergum of the...
same abdominal segments, also one pair of stout spines and one pair of posteriorly directed small setae (Fig. 6E, F and I). A pair of medium-sized setae laterally on meso- and metathorax. One pair of setae with fine apex on pleura from Ab 2–5 (Fig. 6G); from Ab 6–7 the setae have clavate apex (Fig. 6H). Open spiracles present on Ab 2–7 (Fig. 6G). The eighth abdominal segment presents one pair of microsetae dorsolaterally on tergum, one pair of spiracles partially closed and one pair of medium-sized setae posteriorly directed (Fig. 6J and K). One pair of posteriorly directed, digitiform caudal processes on last abdominal segment (Fig. 6L).

**Etymology.** Hemera, in Greek mythology is the daughter of the night and represents the divinity that personifies the daylight, here making an allusion to the light and bright color of the forewings of the species.

**Distribution.** *P. hemera* is known only from its type locality, the Dense Umbrophilous Forest (= Atlantic Forest sensu stricto), CPCN Pró-Mata, São Francisco de Paula municipality, Rio Grande do Sul, Brazil.

**Host plant** (Fig. 7A). *D. fasciculata* (Meisn) Neveling (Thymelaeaceae). The host plant of *P. hemera* occurs as either a shrub or a small tree, endemic to Brazil and occurring in the following regions: Midwest (Distrito Federal), Southeast (Espírito Santo, Minas Gerais, Rio de Janeiro and São Paulo) and South (Paraná, Santa Catarina and Rio Grande do Sul) (Rossi, 2017).

**Life history** (Fig. 7B–H). Eggs of *P. hemera* are deposited on the adaxial leaf surface. After eclosion, the sap-feeding larva (Fig. 7D) penetrates the leaf blade starting the mine construction. In the beginning the mine is narrow and serpentine-shaped, increasing in width during development (Fig. 7B). Centrally along the mine a path of black feces left by the larva can be seen by transparency (Fig. 7B). Later the larva goes deeper into leaf tissues and install within palisade parenchyma cells, also by cutting the anticlinal cell walls (Fig. 8C). The epidermal cells remain intact over the intermediary mine (Fig. 8C–E), while fragments of the anticlinal cell walls of the palisade parenchyma cells remain in the lateral portions of the mine (Fig. 8D) but are totally consumed in the central portion (Fig. 8E).

The spinning larva (Fig. 7E) does not feed and is responsible for the construction of the cocoon. This is endophylous and constructed at the final portion of the mine, and completely covered by whitish silk that provokes a slight leaf wrinkling (Fig. 7F). The pupal cocoon...
is ruptured by the pupa’s cocoon-cutter (Fig. 7G) during adult emergence. Later, the pupal exuvia is seen partially protruding from the cocoon (Fig. 7H). More than one mine were found in most of the mined leaves. A few mines were found on full grown D. fasciculata trees. The greatest density of leaves mined by P. hemera was found on young plants, especially those located in humid sections of trail borders existing in the type locality. The larvae were found active in the field from February to August, suggesting the species is multivoltine.

**Phylogenetic inference**

A total of 660 nucleotide sites were analyzed, of which 268 (40%) were variable. In accordance with our phylogenetic hypothesis, the monophyly of the new species was recovered in both methods of inference (distance and model-based), with high node support values (Fig. 9). Since the topologies were slightly different, all are presented. The sister relationship of P. hemera was not well resolved: node supports were quite low in all trees (NJ, Bayesian and ML). In the NJ and ML inferences, P. hemera clustered with P. saligna (Fig. 9). In the Bayesian analysis, the closest related lineage was P. pheobus; however, the node support (BPP) was very low. The genetic distance estimated between P. hemera and other taxa ranged from 14% to 20% (+2%) (see Supplementary Material; Fig. S1). The distance of the new species to the outgroups was 24% (+3%).

**Discussion**

P. hemera is described here based on morphological and molecular characters, showing enough stable characters in both types of analysis to separate it clearly from other congeneric species. Phylogeny showed a monophyletic status for the new species, but did not resolve close relationships. Different methods of reconstruction retrieved different results for sister taxa of P. hemera, although with low support. In the NJ and ML trees it was close to P. saligna, whereas in Bayesian inference it clustered with P. pheobus, a sympatric species from the same region of Atlantic Forest (Brito et al., 2017a). A comparative assessment of genetic distance to other Neotropical Phylllocnistis indicates a minimum of 12% for P. hemera to P. citrella and P. vitegenella, which indicates a great amount of divergence sampled in Phylllocnistis. Such high diversity is likely reflected in the evolutionary history reconstructed for the genera, e.g. by the absence of unknown lineages in the phylogeny, suggested by the putative long-branch attraction apparently in P. hemera and P. saligna relationship (NJ and ML trees).

The forewing pattern of P. hemera resembles those described for congeneric species in the Neotropical region (Brito et al., 2017a), regarding number of fasciae and strigulae, presenting one well-marked longitudinal fascia, two visible transversal fasciae, three costal and four apical strigulae, the last emerging from the apical spot. Comparing P. hemera to the other congeneric Neotropical species the greatest similarity is found with P. bourquini Pastrana, a species described for Argentina; both species share forewing light yellow fasciae, but they can be contrasted by the morphology of the third and fourth fasciae, which are separated in P. bourquini and united in P. hemera. Another character that differentiates P. hemera from P. drimiphaga is the male valva; in P. drimiphaga the valva is divided into two lobes (Kawahara et al., 2009), the dorsal being more prominent than the ventral, while P. hemera has the distal portion of the valva undivided.

As already mentioned, the presence of ambulatory calli on the ventral region of meso- and metathorax in the spinning larva has already been described for P. ourea, also a species from the Atlantic forest. Ambulatory calli are not exclusive characters of Phylllocnistis, and they also occur in representatives of the Oecophyllaenidae – Angelabella Vargas & Parra, Eumetrochroa Kumata, Metrochroa Busck and Phylllocnistis Davis, a gracillariid subfamily closely related to the Phylllocnistinae (Vargas and Parra, 2005; Kumata, 1998; Busck, 1900; Davis, 1994; Kawahara et al., 2017). Stemmata have already been described for the sap-feeding larvae of some Phylllocnistis species of the Neotropical region, such as P. ourea Brito & Moreira and P. selee Brito & Moreira, which have two stemmata on the lateral region of the head. Thus P. hemera is the only species known so far with a single stemma followed by a microseta (Brito et al., 2017b).

The species described here is the first gracillariid associated with a Thymelaeaceae plant. As already described, D. fasciculata has a broad distribution in southeast Brazil (Rossi, 2017), suggesting that P. hemera might be distributed in other regions not evaluated in this study, which should be further explored. Interestingly, results presented here regarding the histology of the mine give further support for the existence of a broad feeding habit in relation to use of leaf tissues within Phylllocnistis. In other words, our data suggest that although highly species-specific to a given type of leaf tissue, species within this genus may use any kind of tissue, including the epidermis (e.g. P. citrella, Archor et al., 1997), spongy parenchyma (e.g. P. theys, Brito et al., 2012), and palisade parenchyma (e.g. latter instars of P. hemera, as demonstrated here). The ultimate factors that lead to this variation in tissue usage remain to be determined. P. hemera uses the epidermis initially, and moves to the palisade parenchyma in the intermediary phase of the mine, which may indicate a search for better nutritional resources. Epidermal cells may function as lenses for capturing sunlight and as a consequence have large water-filled vacuoles and scarce cytoplasm. Palisade parenchyma cells, on the other hand, are commonly cytoplasm-rich, and may accumulate energetic molecules (Evert, 2006; Bowes and Mauseth, 2008).

The Atlantic forest is known for its extreme diversity with approximately 50% of the species considered endemics (Stemann et al., 2009). However, only five species of Phylllocnistis are known for the region (Brito et al., 2012, 2017a). As suggested by Brito et al. (2016), the vast majority of Neotropical gracillariid species remain to be discovered. Data presented in this study regarding a new species of Phylllocnistis for the Atlantic forest support further the hypothesis proposed by such authors in the sense that the scarcity of species described for the region in largely due to a lack of sampling, associated with a taxonomic impediment.

**Conflicts of interest**

The authors declare no conflicts of interest.

**Acknowledgements**

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rbe.2017.11.001.

References

Supplementary Material:

Fig. S1. Graph depicting pairwise genetic distance (in percentage) between *P. hemera* and other congeners, as well as outgroups (*Angelabella* and *Marmara*) based on 660 base pairs of the ‘DNA barcode’ region of the cytochrome oxidase I (COI) gene, using the Kimura 2-parameter model.
CAPÍTULO III

Descrição dos estágios imaturos e história de vida de uma nova espécie da Oecophyllembiinae (Lepidoptera: Gracillariidae) da Mata Atlântica.
CAPÍTULO IV

CONSIDERAÇÕES FINAIS

Neste trabalho foram descritas, de forma inédita, os estágios imaturos de duas novas espécies de microlepidópteros minadores foliares de Gracillariidae encontradas no sul do Brasil, os quais tem como hospedeiras plantas das famílias Thymeleaceae e Rubiaceae. A descrição dos estágios adultos de ambas bem como a caracterização taxonômica correspondentes constam de trabalho em colaboração, sendo em relação a primeira já publicado (Phyllocnistis hemera Brito & Fochezato), e da segunda, em andamento. A primeira espécie pertence a Phyllocnistinae e ao gênero Phyllocnistis e foi encontrada se alimentando em folhas Daphnopsis fasciculata (Meisn.) Nevling. Já a segunda, pertencente provavelmente a um novo gênero de Oecophyllembiinae, foi encontrada se alimentando em folhas de Psychotria suturella, ambas no sul do Brasil.

Phyllocnistis hemera é uma das nove espécies do gênero descritas para o Brasil e o primeiro gracilarídeo associado a uma planta da família Thymeleaceae. Ela compartilha com as demais espécies do grupo o padrão de coloração como as fásicas e estrígulas nas asas anteriores. Além disso, destacam-se na genitália, a forma das valvas nos machos. Outra exclusividade desta espécie é a presença de uma única estema seguida por uma microcerda nas larvas do tipo sap-feeding. Em relação a mina foliar, pode-se verificar que a mesma se encontra na face adaxial, com formato serpenteante, característica comum do grupo, extensivo ao casulo que é construído na porção final da mina, causando um dobramento na folha. Foi demonstrado através da análise de cortes histológicos que a alimentação inicia no tecido subepidérmico da folha, ficando restrita posteriormente ao parênquima paliçádico ao longo do desenvolvimento da larva.

O terceiro capítulo contemlou a descrição dos imaturos de uma espécie de Oecophyllembiinae, a primeira a ser assinalada para a subfamília no Brasil, sendo o segundo representante dessa associado a Psychotria. O padrão de coloração correspondente é escuro com manchas prateadas, considerado comum para os representantes dessa subfamília. Quanto a mina foliar, é adaxial e serpenteante não sendo formado rastros de fezes, que são depositadas de forma desordenada ao longo do desenvolvimento. O casulo pupal é construído no final da mina e também causa um dobramento na folha, porém no sentido dorsal. Já a alimentação dessa espécie se restringe
ao parênquima paliçádico durante todos os estágios larvais. De forma atípica para a família, foi verificada na larva sap-feeding desta espécie uma hipofaringe modificada, do tipo escavadora/raspadora, o que deve ser melhor explorado.

As espécies ora descritas para o estado do Rio Grande do Sul quanto aos estágios imaturos, dentro do bioma Mata Atlântica, evidenciam a grande diversidade gracilarídeos, de plantas hospedeiras usadas e respectivos hábitos alimentares nesse bioma, porém até então desconhecidos, cuja exploração deve ser continuada.
1) Normas para publicação na Revista Brasileira de Entomologia:

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Author’s responsibility: Page proofs are sent to the corresponding author and should be returned, with the necessary corrections, at the indicated deadline. Authors are entirely responsible for the scientific content of the paper, as well as for proper use of grammar. Authors are encouraged to look at the latest issues of the RBE to check current format and layout. When submitting the manuscript, the author may suggest potential reviewers. Please, include the complete name, mailing and electronic addresses of suggested reviewers. The choice of reviewers, however, remains with the Editors.
2) Normas para publicação na Revista Austral Entomology:

4. PREPARING YOUR MANUSCRIPT

Style and Formatting

For submission, the manuscript should preferably be submitted as a single file, with the figures embedded as low resolution files. Tables and figures should be inserted at the end of the manuscript. Name the manuscript file as: authornamedoc.

- Submissions should be typed in 12 pt Times New Roman and have 1.5 line spacing.
- All margins should be set to 2.5 cm.
- The first paragraph under each heading is not indented; indent following paragraphs, with no blank line between paragraphs.
- Ensure that all mark-up (‘Track Changes’) done during manuscript preparation is removed (‘Accept All Changes’ on Reviewing Toolbar) so that reviewers have a clean copy on which to insert suggested changes and comments.

Abbreviations and Units

SI units (metre, kilogram etc.), as outlined in the latest edition of Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors (Royal Society of Medicine Press, London), should be used wherever possible. Give statistics and measurements in figures; that is, 10 mm, except where the number begins the sentence. When the number does not refer to a unit measurement, it is spelt out, except where the number is greater than nine. Use only standard abbreviations. Shorten the word ‘Figure’ to Fig. unless starting a sentence.

The journal uses Australian spelling and authors should therefore set the Language in MS Word to English (Australia) (accessible under the Tools menu in MS Word) and follow the latest edition of the Macquarie Dictionary. Manuscripts that do not conform to this requirement and the following format will be returned to the author prior to review for correction.

Parts of the Manuscript
Title page

The title page should contain:
(i) an informative title that contains the major key words. The title should contain the scientific name of the insect, with the order and family placed in parentheses;
(ii) the full names of the authors;
(iii) the author's institutional affiliations at which the work was carried out;
(iv) a short running title of less than 50 characters including spaces.
(iv) the email address of the author to whom correspondence about the manuscript should be sent.

Abstract

All manuscripts must include a brief but informative abstract intelligible without reference to the main text. It should not exceed 350 words and should describe the scope of the work and the main findings. Both common and scientific names of the insect should be included. Authorities to species names are not required except for taxonomic papers. References to the literature should not be included. Use the passive voice in the Abstract. DO NOT use the uninformative phrase ‘Results are discussed.’

Key Words

Up to 10 additional key words should be provided below the Abstract.

Main Text Sections

- Introduction: This section should include sufficient background information to set the work in context. The aims and goals of the manuscript should be clearly stated. The introduction should not contain findings or conclusions.

- Materials and Methods: This should be concise but provide sufficient detail to allow the work to be repeated by others.

- Results: This should be presented in a logical sequence in the text, tables and figures; repetitive presentation of the same data in different forms is not permissible. The results should not contain material appropriate to the Discussion.
• **Discussion:** This should consider the results in relation to any hypotheses advanced in the Introduction and place the study in the context of other work.

Details of sections required in taxonomic papers are set out here: Template for Taxonomic Papers.

**Acknowledgements**

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**References**

The Harvard (author, date) system of referencing is used.

• In the text give the author’s name followed by the year in parentheses: Sago (2000).
• When reference is made to a work by three or more authors, the first name followed by *et al.* should be used: Powles *et al.* (1998).
• Within parentheses, groups of references should be cited in chronological order.
• Personal communication, unpublished data and publications from informal meetings are not to be listed in the reference list but should be listed in full in the text (e.g. Smith A, 2000, unpublished data).
• Titles of journals should be given in full.
• If several manuscripts by the same author(s) and from the same year are cited, a, b, c etc. should be put after the year of publication.
• ‘In press’ should only be used to cite manuscripts actually accepted for publication in a journal. Citations such as ‘manuscript in preparation’ or ‘manuscript submitted’ are not permitted. Data from such manuscripts can only be mentioned in the text as ‘unpublished data’.
• References should be listed in alphabetical order at the end of the manuscript.
• Cite the names of all authors when there are six or fewer; when seven or more cite the
first three plus et al.

• Authors are responsible for the accuracy of the references.

References should be listed in the following form:

**Journal articles**


**Books**


**Chapters in books**


**Website**


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Tables must be constructed using the ‘Table’ function of your word processor and must not have the Enter key used in any cell. Tables should be self-contained and complement, but not duplicate, information contained in the text. Tables should be numbered consecutively in Arabic numerals. Each table should be presented on a separate page at the end of the text with a comprehensive but concise legend above the table. Tables should be double-spaced and vertical lines should not be used to separate columns. Column headings should be brief, with units of measurement in parentheses; all abbreviations should be defined in footnotes. Use superscript letters (not numbers) for footnotes and keep footnotes to a minimum. *, **, *** should be reserved for $P$-values. The table and its legend/footnotes should be understandable without reference to the text.
Figure Legends

Legends should be concise but comprehensive – the figure and its legend must be understandable without reference to the text. Include definitions of any symbols used and define/explain all abbreviations and units of measurement.

Figures

Only scientifically necessary illustrations should be included. Magnifications should be indicated using a scale bar on the illustration. Figures should be cited in consecutive order in the text.

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