Article

A new species of *Phyllocnistis* Zeller (Lepidoptera: Gracillariidae) from southern Brazil, with life-history description and genetic comparison to congeneric species

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Abstract

Male, female and immature stages of *Phyllocnistis tethys* Moreira & Vargas sp. nov. (Lepidoptera; Gracillariidae) from the Atlantic Rain Forest, coastal mountains of southern Brazil, are described and illustrated, using both optical and scanning electron microscopy. A preliminary analysis of mitochondrial (COI) DNA sequences including putative members of congeneric species is also provided. The immature stages are associated with the passion vine *Passiflora organensis* (Passifloraceae). The hypermetamorphic, endophyllous larva has four instars; the first, second and third instars are sap-feeders, associated primarily with the spongy parenchyma, and construct a blotch mine in the lower surface of the lamina; the fourth, non-feeding (spinning) instar constructs a flimsy endophyllous cocoon at the end of the mine, where pupation occurs. This is the first species of *Phyllocnistis* Zeller described from Brazil, and the first leaf-mining gracillariid associated with Passifloraceae.

Key words: leaf-mining moths, gracillariids, Neotropical region, hypermetamorphosis, passion vines

Introduction

Gracillariidae is one of the largest groups of leaf-mining Lepidoptera, with 1,885 species recognized worldwide, of which 181 are recorded in the Neotropical region (De Prins & De Prins 2012). *Phyllocnistis* Zeller, 1848 is a poorly studied genus of minute moths (wingspans generally not exceeding 5 mm) that has been assigned to different families of Gracillarioidea and only lately has been included in the Gracillariidae, within the Phyllocnistinae (Davis & Miller 1984; Kawahara *et al*. 2011; Nieukerken *et al*. 2011). The taxonomic history of the genus was reviewed recently by De Prins & Kawahara (2009), and information on the general biology was provided by Davis & Wagner (2011). The existence of subepidermal, sap-feeding instars early in the larval stage and a specialized, non-feeding last instar that spins an endophyllous cocoon prior to pupation are shared characteristics among all known species of *Phyllocnistis*. Adults show consistent differences in wing patterns (*e. g*., conspicuously colored fasciae and strigulae) at the species level, but they vary little in the structure of their genitalia compared to other gracillariids and lepidopterans in general. They are, however, relatively diverse in some pupal structures, which may provide valuable species-level differences, such as in the frontal process of the head (= cocoon cutter) and in the shape and arrangement of tergal spines present on the abdomen (Davis & Wagner 2011).

A total of 126 species have been recognized for the genus *Phyllocnistis* worldwide (De Prins & De Prins 2012). Ten species were listed for the Neotropical region by Davis & Miller (1984), with type localities in

Argentina, Colombia, Guyana, Costa Rica, Ecuador and Peru. Subsequent to this list, a new species was described from southern Chile (*P. puyehuensis*) by Davis (1994), three from Costa Rica (*P. drimiphaga*, *P. maxberryi*, and *P. tropaeolicola*) by Kawahara *et al*. (2009), and recently also from Costa Rica, *P. perseafolia* by Davis & Wagner (2011). Thus, only 16 species have so far been recorded for the genus in the Neotropics, although none for Brazil except *P. citrella* Stainton, a cosmopolitan, citrus pest species of Asiatic origin (De Prins & Kawahara 2009; De Prins & De Prins 2012). This number is supposedly greatly underestimated, probably the result of low collecting effort, in particular regarding the central and southern areas of the Neotropics where microlepidopterans in general have been historically less collected. Preliminary results from a survey of *Phyllocnistis* species for a localized area in Costa Rica conducted by Davis & Wagner (2011), for example, led to the conclusion that the tropical diversity for the genus may comprise hundreds of species.

Members of *Phyllocnistis* exploit a wide range of host plants worldwide, and are known to feed on plants from at least 20 families (De Prins & Kawahara 2009). A large proportion of species found in the Nearctic region are specialized to feed on ancient angiosperms whose origins date to the Cretaceous (*e.g.,* Lauraceae, Magnoliaceae and Hamamelidaceae) (Davis & Wagner 2011). At least one Neotropical species (*P. drimiphagha*) was recently associated with the archaic Winteraceae (Kawahara *et al*. 2009). In fact, phyllocnistine leaf mines are among the oldest known fossils of Ditrysia Lepidoptera, dating to the Late Cretaceous (Labandeira *et al*. 1994; Davis & Wagner 2011; Sohn *et al*. 2012). However, it is still uncertain whether these associations constitute evidence that *Phyllocnistis* species are ancient or that they have only recently colonized these host plants. This question will remain unanswered until more information on host-plant diversity is attained, and a consistent phylogeny is established for the genus (Davis & Wagner 2011).

Recently, as part of an ongoing study on the diversity of microlepidopterans in the Atlantic Rain Forest in southern Brazil, we found for the first time a leaf-mining gracillariid, belonging to *Phyllocnistis* and associated with Passifloraceae. Herein, we describe and illustrate all the life stages of this new species, and provide a preliminary characterization of its life history, including histological aspects of the leaf mine. We also present a preliminary analysis of mitochondrial (COI) DNA sequences including putative members of congeneric species.

Material and methods

Specimens used in the study were reared in small plastic vials under controlled abiotic conditions (14 h light / 10 h dark; 20 ± 2°C) in the Laboratório de Morfologia e Comportamento de Insetos, Departamento de Zoologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre city, Rio Grande do Sul State (RS), Brazil, from eggs, larvae and pupae collected on *Passiflora organensis* Gardner (Passifloraceae), during May 2011 and March 2012, in the Centro de Pesquisas e Conservação da Natureza (CPCN Pró-Mata / PUCRS; 29 °28'36''S, 50 °10'01''W), 900 m, São Francisco de Paula Municipality, RS.

Immature stages were fixed with Dietrich´s fluid and preserved in 75% ethanol. At least five specimens were used for the descriptions of each life stage or instar. For observations of gross morphology, the specimens were cleared in a 10% potassium hydroxide (KOH) solution and slide-mounted in either glycerin jelly or Canada balsam. Observations were performed with the aid of a Leica® M125 stereomicroscope, where structures selected to be illustrated were photographed with an attached Sony® DSC-H10 digital camera. Vectorized line drawings were then made with the software CorelDraw® X4, using the corresponding digitalized images as a guide. Adult wing-pattern nomenclature follows Kawahara *et al*. (2009), and for the general description of larvae and pupae, Davis (1987) and Davis & Wagner (2011).

For scanning electron microscope analyses, specimens were dehydrated in a Bal-tec® CPD030 critical-point dryer, mounted with double-sided tape on metal stubs, and coated with gold in a Bal-tec® SCD050 sputter coater. They were then examined and photographed in a JEOL® JSM5800 scanning electron microscope at the Centro de Microscopia Eletrônica (CME) of UFRGS.

For plant anatomical descriptions, field-collected leaf portions of *P. organensis* containing mines of *P. tethys* were fixed in FAA (37% formaldehyde, glacial acetic acid, and 50% ethanol, 1:1:18, v/v), and preserved in 70% ethanol. Under the stereomicroscope in the laboratory, leaf portions containing the last sap-feeding larval instars were later selected (n = 15). They were then progressively hydrated, immersed in 10% potassium hydroxide for 20 min, stained for 12 h with rose bengal (aqueous solution: 200 mg/liter), and then mounted whole in glycerin on

slides. Semi-permanent slides were also prepared with freehand cross sections cut with a razor blade, using additional mines containing larvae of different ages and prepared similarly. Head-capsule exuvia were located by transparency in the slide-mounted mines and measured under the stereomicroscope with an attached ocular micrometer.

Molecular analysis. Total genomic DNA was extracted from last sap-feeding larval instar specimens using the CTAB method (Doyle & Doyle 1987), in order to evaluate the phylogenetic status of *Phyllocnistis* sp. nov. using molecular characters. We surveyed four specimens to amplify part of the mitochondrial gene cytochrome oxidase I (COI—639 bp) using primers and conditions described by Folmer *et al*. (1994) (Table 1). PCR products were purified using Exonuclease I (GE Healthcare Inc.) and Shrimp Alkaline Phosphatase (SAP), sequenced with a BigDye kit and analyzed on an ABI3730XL (Applied Biosystems Inc.). Chromatograms obtained from the automatic sequencer were read and sequences were assembled using the software CodonCode Aligner (CodonCode Corporation). The COI sequences obtained in this study were deposited in GenBank (Table 1). Phylogenetic trees were constructed using maximum likelihood (ML) in the software PHYML 3.0 (Guindon *et al*. 2010). The program JMODELTEST (Posada 2008) was used to estimate the substitution model GTR + G [General Time-Reversible model (Rodríguez *et al*. 1990), with gamma distribution (G)] for ML according to the Akaike Information Criterion (AIC). Monophyly-confidence limits were assessed with the bootstrap method (Felsenstein 1985) at 60% cutoff after 1000 bootstrap iterations.

TABLE 1. Specimens used in this study to reconstruct the phylogenetic status of *Phyllocnistis tethys* based on cytochrome oxidase subunit I sequences.

Family	Species	Voucher number	GenBank accession number
Bucculatricidae	*Bucculatrix canadensisella*	UBC-2007-0541	FJ412220
Gracillariidae	*Acrocercops astericola*	08-JDWBC-1948	HQ682752
Gracillariidae	*Caloptilia stigmatella*	BIOUG(CAN): 04HBL007525	GU438783
Gracillariidae	*Phyllocnistis labyrinthella*	MM00041	GU828587
Gracillariidae	*P. citrella*	SK-013	AB614513
Gracillariidae	*P. hyperpersea*	DDAV-D557	HQ971045
Gracillariidae	*P. perseafolia*	DDAV-D555	HM382097
Gracillariidae	*P. populiella*	08-JDWBC-2658	HQ683340
Gracillariidae	*P. saligna*	SK-011	AB614511
Gracillariidae	*P. gracilistylella*	SK-010	AB614510
Gracillariidae	*P.* sp. 1 (BOLD AAF6349)	DDAV-D565	HM382102
Gracillariidae	*P.* sp. 2 (BOLD AAF6349)	DDAV-D566	HM382102
Gracillariidae	*P.* sp. *'longipalpus'*	DDAV-D562	HM382099
Gracillariidae	*P. tethys* sp. nov.	LMCI 174-55-1	JX272049
Gracillariidae	*P. tethys* sp. nov.	LMCI 174-55-3	JX272050
Gracillariidae	*P. tethys* sp. nov.	LMCI 174-55-5	JX272051
Gracillariidae	*P. tethys* sp. nov.	LMCI 174-55-9	JX272052
Gracillariidae	*P. vitegenella*	2VE	JQ412575

Museum collections. Abbreviations of the institutions from which specimens were examined are:

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

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

MCNZ   Museu de Ciências Naturais, Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

MCTP   Museu de Ciências e Tecnologia da Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

Results

Phyllocnistis tethys **Moreira & Vargas, sp. nov.**
Figs. (1–8)

Type material. BRAZIL: Centro de Pesquisas e Conservação da Natureza Pró-Mata (CPCN Pró-Mata; 29º28'36'S, 50º10'01'W; 900 m), São Francisco de Paula Municipality, Rio Grande do Sul State, Brazil. All adults were preserved dried and pinned, and reared by the senior author from larvae and pupae collected on 05-11.V.2011 by G.R.P. Moreira, R. Brito & K. Barão, on *Passiflora organensis* Gardner (Passifloraceae). HOLOTYPE: ♂ (LMCI 155-58), deposited in DZUP (22.623). PARATYPES: 2 ♀♀ (LMCI 155-41 and 155-43), deposited in DZUP (22.633 and 22.643); 1 ♂, 1 ♀ (LMCI 155-31 and 155-26), deposited in MCNZ (81901 and 81902); 1 ♂, 1 ♀ (LMCI 155-35 and 155-30), deposited in MCTP (28635 and 28636).

    Other specimens examined. Adults, dried and pinned, 4 ♂♂, with the same collection data, deposited in LMCI (155-25, 27, 32, 33); 2 ♀♀, fixed in Dietrich's fluid and preserved in 70% ethanol, with the same collection data, deposited in LMCI (155-20). Genitalia preparations, mounted in Canada balsam on slides, with the same collection data, deposited in LMCI under the following accession numbers: 5 ♂♂ (GRPM 50-10, 13, 14, 15 and 16); 4 ♀♀ (GRPM 50-8, 17, 18, and 19). Immature stages, fixed in Dietrich's fluid and preserved in 70% ethanol, with the same collection data, deposited in LMCI under the following accession numbers: 3 eggs (LMCI 155-14), 2 first-instar (sap-feeding) larvae (LMCI 155-3 and 4), 5 third-instar (sap-feeding) larvae (LCMI 155-12 and 13), 4 fourth-instar (spinning) larvae (LMCI 155-16), and 8 pupae (LMCI 155-18 and 19). Mature leaf mines (n = 24) containing exuvia of all instars, mounted in glycerin on slides and stained with rose bengal, with the same collection data, 26.III.2012, deposited in LMCI, under accession numbers LMCI 174-1 to 24.

    Diagnosis. Adults of *P. tethys* can be readily distinguished from all other known species of Neotropical *Phyllocnistis* in the forewing pattern, primarily by the absence of longitudinal and costal fasciae. Of the five species of *Phyllocnistis* known from neighboring Argentina and Chile (Davis & Miller 1984, Davis 1994), only two (*P. abatiae* Hering and *P. puyehuensis* Davis) lack the basal longitudinal fascia. However, *P. abatiae* possesses a pair of small, isolated costal fasciae; and *P. puyehuenis* has a single, broad, isolated pale-gold costal fascia that crosses the wing. In addition, in these species the presence of yellowish-orange scales on the subapical part of the forewing is restricted to a small circular area adjacent to the black spot. Also, in contrast to *P. tethys*, in these species the tornal fringes are uniform in color.

    Adult (Figs. 1, 2). Male and female similar in size and color (Fig. 1). Forewing length 2.41–2.72 mm (n = 5). *Head*: Vestiture moderately smooth, with a pair of latero-dorsal light-gray scale tufts that curve forward to the frons. Eyes medium in size (interocular index ranging from 0.51 to 0.72; n = 4). Antenna mostly dark gray, ~ equal to length of forewing, covered with lanceolate scales; a single row of scales encircling each flagellomere. Labial palpus slender, ~ 0.3 mm in length, covered with dark-gray scales. Proboscis without scales, slightly longer than labial palpus. *Thorax*: Forewing light gray; longitudinal and costal fasciae absent; transverse fascia C-shaped, with faint dark border filled in with sparse light-gray scales; apical to subapical area bright yellowish orange, medially interspersed on costal strigulae and transverse fascia, and with large black spot; three slender, dark costal strigulae, three slender dark apical strigulae, and one dark tornal strigula arising from the apical black spot; fringe along tornal margin light gray with a wide dark basal band of scales; ventral surface dark gray. Hindwing dark gray. Legs light gray; foretibia and tarsomeres mostly dark gray. *Abdomen:* Length ~ 1.7 mm, covered with dark-gray scales. *Male genitalia:* Tergum VIII small, semicircular; sternum VIII reduced to a narrow transverse band. A pair of coremata present meso-laterally on segment VIII, consisting of inflatable tubular extensions bearing a terminal cluster of long, wide and flat scales (Fig. 2D). Tegumen formed by a basal, narrow transverse band that continues caudally up to approximately the length of the valvae, as an elongate, mostly membranous, basally spinose cylinder that encloses the anal tube (Fig. 2A); saccus well developed, ~ 0.3 length of valve, U- shaped with rounded anterior end and sinuous posterior margin having pronounced concavity medially; valvae digitiform, slightly curved medially and long, ~2.0 length of saccus, with moderately broad base formed by two wide dorsal and ventral

projections that converge, reaching each other medially; setae of medium size are scattered found on median surface of valve, and short setae distally. Aedeagus (Fig. 2B) subcylindrical, weakly sclerotized, ~ equal to length of valva, having basal 2/3 portion slightly dilated and with subapical, dorsally located concave aperture. Vesica with several short spiniform cornuti (Figs. 2B, E). *Female genitalia*: Sternum VII subrectangular, with concave anterior margin more heavily sclerotized, and posterior margin slightly concave (Fig. 2C); tergum VIII reduced to narrow transverse band, with large subtriangular, latero-ventral projections; anterior apophysis similar in length to subtriangular projections of sternum VIII; anal papillae connected dorsally, covered with long piliform setae and microtrichia (Figs. 2C, F); posterior apophyses similar in length to anterior ones; ostium bursae broad , located on posterior margin of sternum VII; ductus bursae membranous, broader at base and narrow distally; corpus bursae membranous, pear-shaped, ~ twice length of ductus bursae, with a conspicuous, proximal, diagonally oriented, and hook-shaped signum that is directed posteriorly into the lumen (Figs. 2C, G); ductus seminalis membranous, narrow, inserted in apex of corpus bursae.
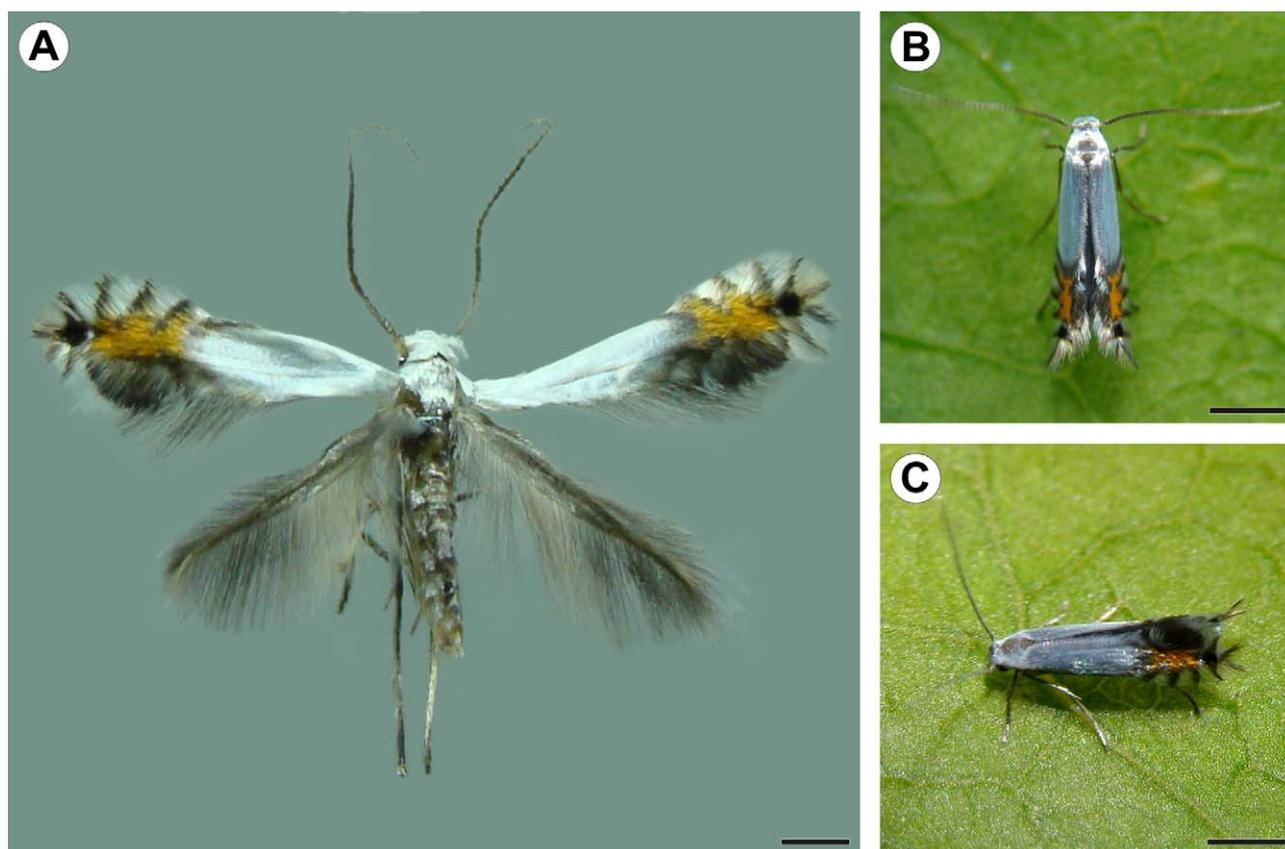

FIGURE 1. *Phyllocnistis tethys* adult: wings spread, pinned, dorsal view (A); wings folded, on *Passiflora organensis* leaf, in dorsal (B) and lateral (C) views. Scale bars = 0.5, 1.0 mm, respectively.

Immature stages. Egg (Fig. 4A; 7C). Flat, slightly ellipsoid; chorion translucent, without external ornamentation, and white at deposition; larva can be seen by transparency before emergence; aeropyles and micropylar area were not observed.

Larva (Figs. 3A–C; 4B–I; 5; 7B, E, G). Leaf-miner, with hypermetamorphic development and four instars, all endophyllous. The first three instars are sap feeders, prognathous and apodous, with highly modified buccal apparatus and depressed body; maximum length of larvae examined 4.79 mm. The prothorax and mesothorax of first-instar larvae are somewhat longer than the metathorax, which is not the case in the following instars. However, we found no stable differences either in shape or coloration among the sap-feeding instars of *P. tethys*. Instars can be correctly identified through measurements of the head capsule, since there is no overlap between the head-capsule size of succeeding instars (Table 2). For the three sap-feeding instars, the following exponential growth equation was adjusted for the head-capsule width: $y = 0.073e^{0.504x}$; n = 45; r = 0.98; p < 0.0001. The fourth instar (= non-feeding, "spinning") is also prognathous and apodous, but has the mouth parts either reduced

or absent, except for the functional spinneret; maximum length of larvae examined 4.17 mm. Body color uniformly white in all instars.

TABLE. 2. Variation in size among head capsules of sap-feeding instars of *Phyllocnistis tethys* (n = 15 per instar).

Instar	Head capsule width (mm)		
	Mean ± standard error	Range	Growth rate
I	0.121 ± 0.003	0.116–0.158	-
II	0.197 ± 0.005	0.179–0.242	1.63
III	0.333 ± 0.004	0.305–0.368	1.69
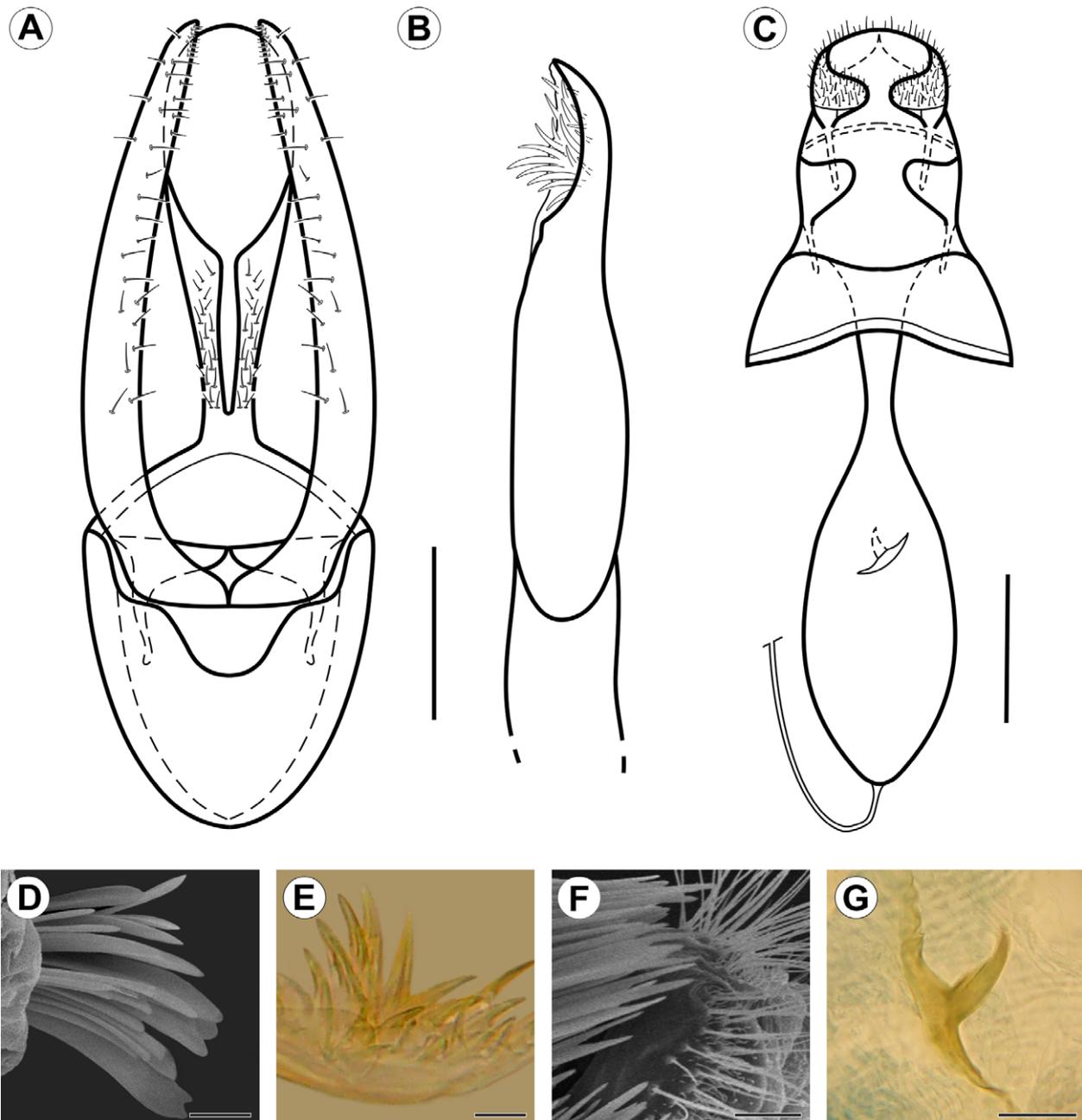
FIGURE 2. Genital morphology of *Phyllocnistis tethys* under light and scanning electron microscopy: (A) male genitalia, ventral view (aedeagus omitted); (B) aedeagus, lateral view; (C) female genitalia, ventral view ; (D) male coremata, lateral view; (E) male cornuti in detail, lateral view; (F) female papilla annalis in detail, latero-dorsal view; (G) female signum in detail, lateral view. Scale bars = 100, 200, 25, 50, 25, 25 μm, respectively.
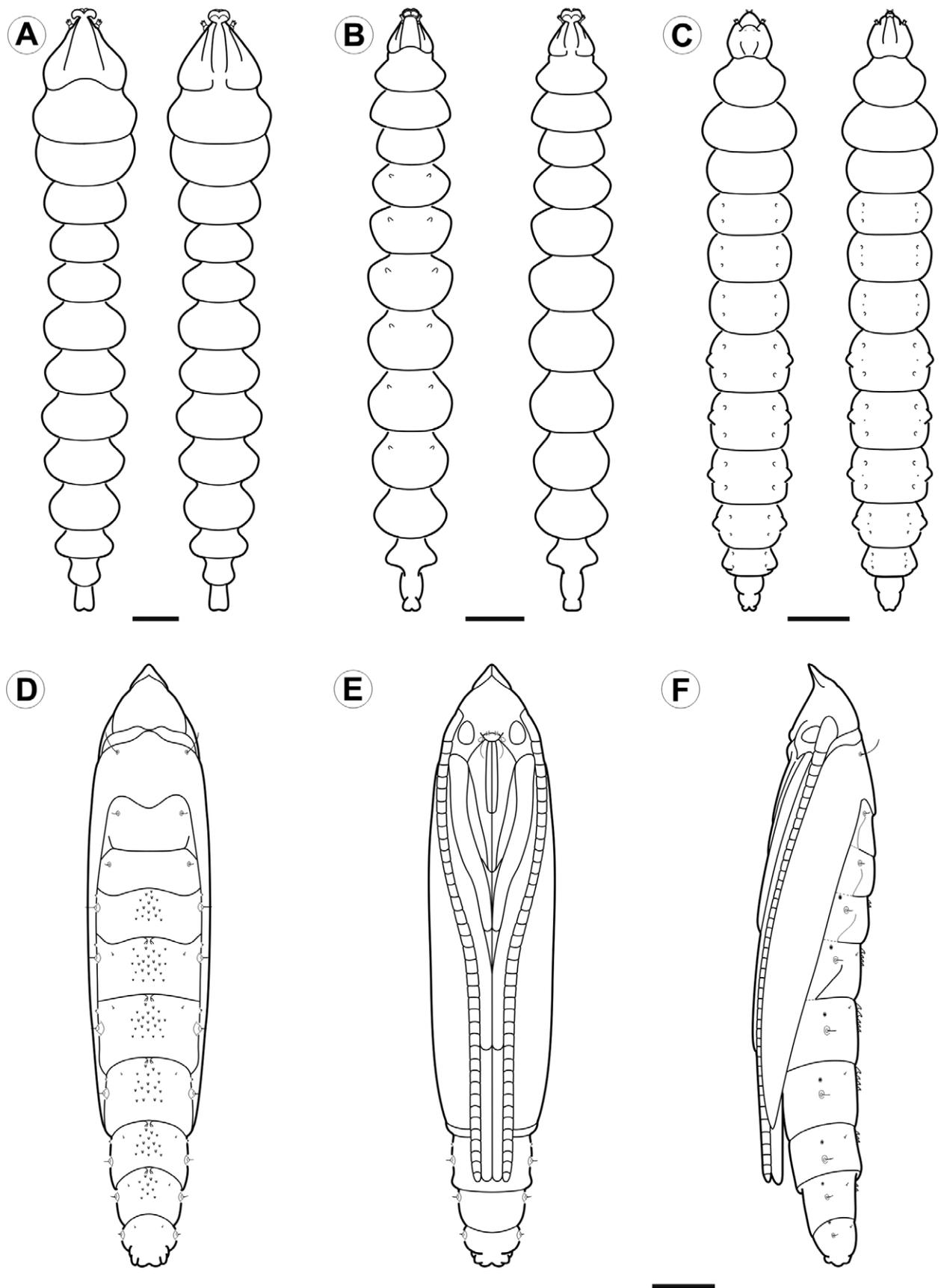
FIGURE 3. Larval and pupal morphology of *Phyllocnistis tethys* under light microscopy: (A) first larval ("sap-feeding") instar, dorsal and ventral views; (B) third larval ("sap-feeding") instar, dorsal and ventral views; (C) fourth larval ("cocoon-spinning") instar, dorsal and ventral views; (D–F) pupa, dorsal, ventral and lateral views. Scale bars = 100, 400, 400, 300 µm, respectively.
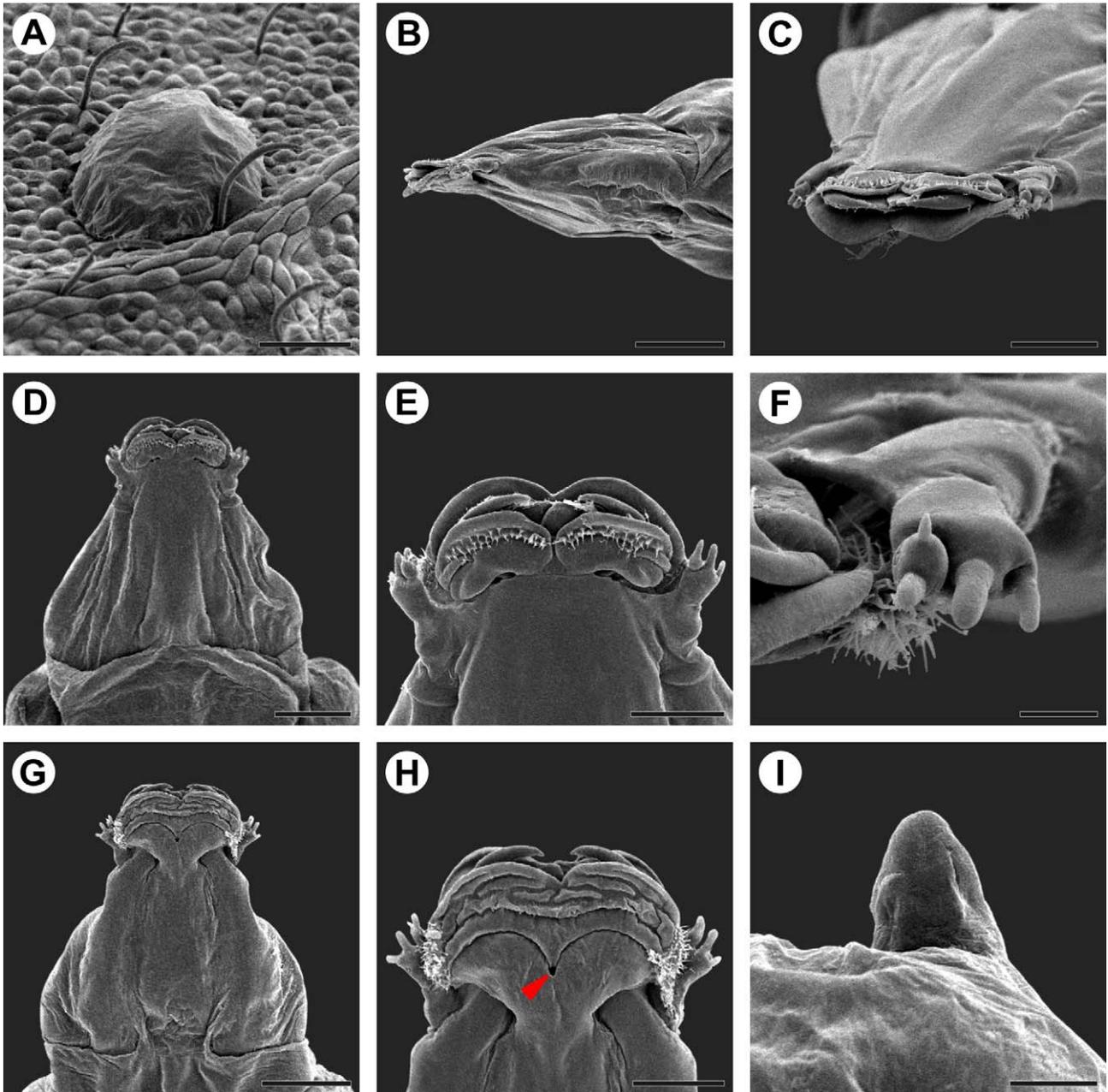
FIGURE 4. Scanning electron micrographs of *Phyllocnistis tethys* egg and third larval "sap-feeding" instar: (A) egg, on abaxial surface of a *Passiflora organensis* leaf; (B–D) head, lateral, anterior and dorsal views; (E) labrum and mandibles, dorsal view; (F) antenna, anterior view; (G) head, ventral view; (H) labium, ventral view (arrow indicates the spinneret); (I) abdominal lobe, dorsal view. Scale bars = 100, 100, 50, 100, 50, 10, 100, 50, 20 µm, respectively.

Sap-feeding instars (Figs 3A, B; 4B–I; 7B, E). Head prognathous, greatly depressed (Figs. 4B–D, G); primary setae either lost or reduced; stemmata absent. Antenna 3-segmented (Fig. 4F); second segment more slender than first, with 2 moderately stout sensilla; third segment less than 1/3 the length of second, with 2 apical sensilla. Labrum (Figs. 4D, E) with well-developed lateral lobes; antero-lateral margins rounded; anterior submargin densely spinose; posterior margins slightly concave. Mandibles large, rounded, flattened plates; anterior surface smooth, lateral area with single tooth, and mesal area with minute serrations. Labium with well-developed lateral lobes, conspicuous rugose cuticular band extending across anterior margin, and cluster of short hypopharyngeal spines laterally. Spinneret rudimentary (Fig. 4H), without extension of cuticle covering aperture. Maxillary and labial palpi absent. Thorax and abdomen without setae. Legs and prolegs absent; one latero-dorsal pair of rounded lobes on each of terga A1–6 (Fig. 3B, 4I).

Spinning instar (Figs. 3C; 5; 7G). Body cylindrical, with all appendages and setae greatly reduced. Head capsule weakly sclerotized, with anteriorly pronounced trophic lobe (Figs. 5A–D); integument finely corrugated. Stemmata absent. Antenna short (Fig. 5F), one-segmented, nearly flush with head capsule, with 4 short sensilla. Maxilla rudimentary (Fig. 5E), flush with head capsule, represented by one moderately long and a pair of short sensilla chaetica. Spinneret short, with simple terminal opening (Fig. 5E). Legs and prolegs absent. Two pairs of weakly differentiated, ventral and dorsal callosities (Fig. 5G) on A1–8; pair of microsetae laterally between the ventral callosities; pair of ventral and dorsal lobes laterally on A4–8. Pleural region of body and last two abdominal segments partly covered by microtrichia (Figs. 5H, I).
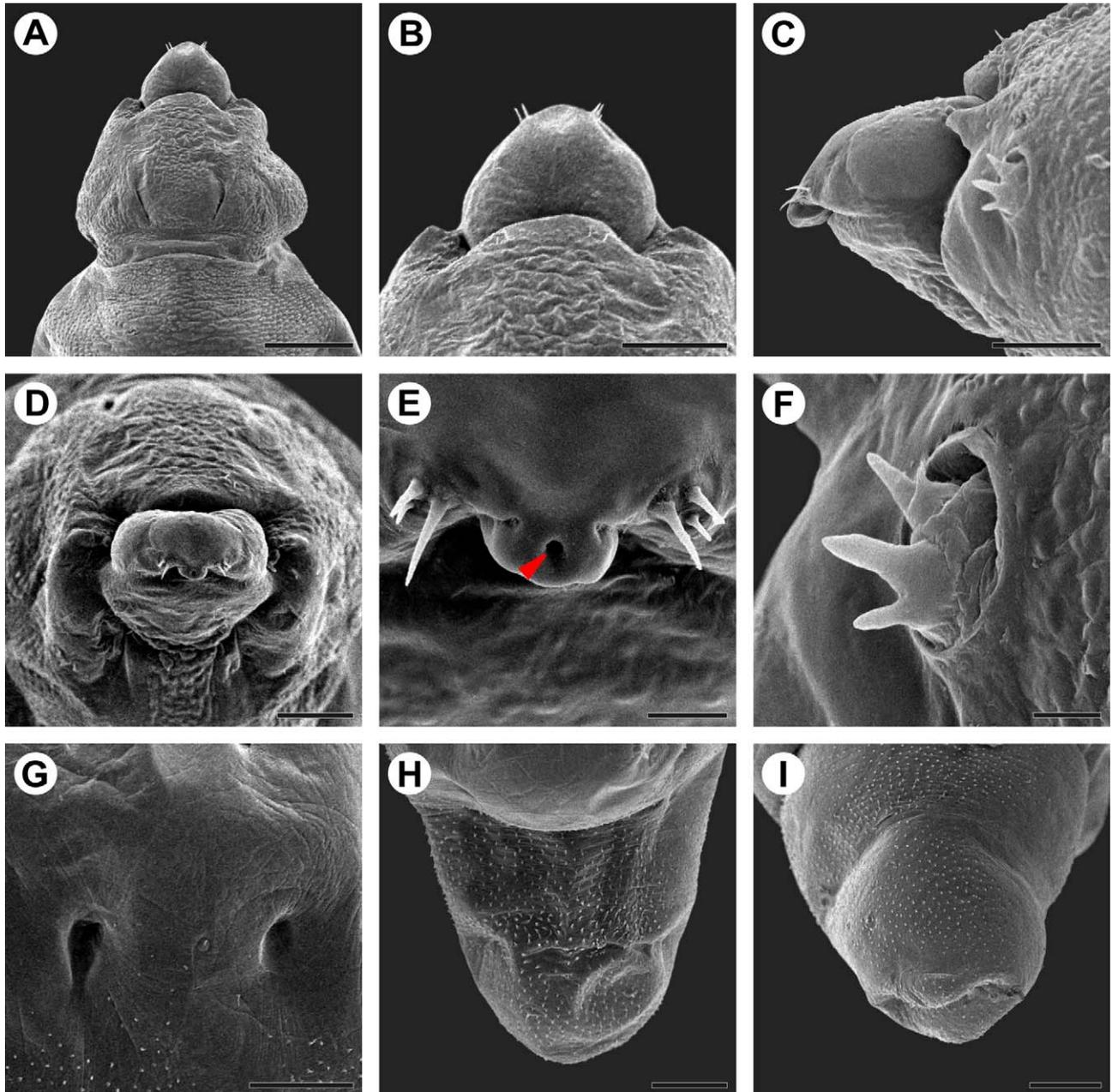

FIGURE 5. Scanning electron micrographs of *Phyllocnistis tethys* fourth larval (cocoon-spinning) instar: (A) head, general, dorsal view; (B–C) detail of head, dorsal and lateral views; (D) head, general, anterior view; (E) spinneret (indicated by arrow), anterior view; (F) antenna, lateral view; (G) invaginations of integument on abdominal sterna, ventral view; (H) caudal end of abdominal , ventral view; (I) dorsal view of fig. H (last segment retracted). Scale bars = 100, 50, 50, 50, 10, 10, 50, 50, 50 µm, respectively.

Pupa (Figs. 3D–F; 6B–O; 7H–K). Maximum length of specimens examined ranging from 2.59 to 3.20 mm. Coloration changing from light yellowish during early stage of pupation to yellowish brown (Fig. 7J) later in development. Vertex with large, subtriangular acute process (= cocoon cutter; Figs. 6B–E) with serrated anterior edge. Frons with 2 pairs of short frontal setae (Fig. 6F). Antenna long and straight, extending almost to abdominal segment A7; forewing extending almost to A6 (Figs. 3E, F). A pair of relatively long setae, latero-dorsally on meso-, metathorax and A1–8, those of A2–8 on chalaza (Fig. 6J); a second pair of micro-setae, meso-dorsally on anterior margin of A3–8; spiracles (Figs. 6K, L) on prothorax and from A1–8, anterior to latero-dorsal setae (Fig. 6J). Six mid-dorsal spine clusters, arranged in V-shaped pattern (Figs. 6G–I) on anterior margin of A2–7; each cluster with row of similar, low, posteriorly curved spines. Tenth abdominal segment with two pairs of relatively short, stout, digitate caudal projections located latero-dorsally and latero-ventrally (Figs. 6M–O). Pleural region of body and last two abdominal segments partly covered by microtrichia (Figs. 6J, M–O).

Pupal cocoon (Figs. 6A; 7I). Endophyllous, constructed at the end of the mine; spherical, covered by sparse silk threads (Fig. 6A), and without external ornamentation (Fig. 7I). Spun by the non-feeding (spinning) fourth-instar larva prior to molting.

Etymology. *Phyllocnistis tethys* is named after Tethys, a Titan goddess in the Greek mythology; the wife of Oceanus, and the mother of rivers, springs, streams, fountains and clouds. Thus, the name also alludes to the cloudy and humid nature of the area of the Brazilian Atlantic Rain Forest where the new species was first found. Proposed as a noun in apposition.

Host plant (Fig. 7A). The only host plant known for the immature stages of *P. tethys* is the passion vine *Passiflora organensis* Gardner (Passifloraceae) (Fig. 7A). This passion vine is found mainly on forest edges in the coastal mountains of southern Brazil, where it is endemic, ranging in distribution from the states of Minas Gerais to Rio Grande do Sul (details of the biology and distribution of *P. organensis* were given by Mondin *et al*. 2011 and Moreira *et al*. 2011, respectively).

Distribution. *Phyllocnistis tethys* is known only from the type locality, the Dense Umbrophilous Forest (= Brazilian Atlantic Rain Forest *sensu stricto*) portions of the CPCN Pró-Mata, São Francisco de Paula Municipality, Rio Grande do Sul, Brazil.

Life history. *Phyllocnistis tethys* eggs (Figs. 4A, 7C) are deposited mostly on the abaxial leaf surface, adhered by a cement substance, usually on the secondary veins. Eclosion occurs through the surface of the egg adhered to the leaf; the first-instar larva enters progressively into the leaf, loading frass to the outside, empty space covered by the chorion (Fig. 7D), since initially the posterior part of the body remains within the chorion. Larvae are sap-feeding leaf miners during the first three instars. By feeding in circles, they form a blotch mine that widens as the larvae develop (Figs. 7B, D). The feeding paths of a larva can be traced by following the dark-green, non-granular frass lines left and head capsule exuvia shed in the mine (Figs. 7F, 8A, B). The three sap-feeding instars are specialized in the abaxial spongy parenchyma, leaving the two epidermis layers and generally the palisade parenchyma intact (Figs. 8C–E). In conditions of low larval density, the adaxial palisade parenchyma may be partly used by later instars (Fig. 7F), and in this case the feeding damage appears as white scars visible through the transparent upper leaf surface (Fig. 7A). However, if a leaf is intensively attacked, at the end of development the palisade parenchyma can be almost completely consumed; leaves then appear mostly deprived of green color (Fig. 7H, I). We could not find a distinct weaving pattern for the flimsy endophyllous cocoon constructed at the end of the mines by the last larval (spinning) instar (Fig. 7I). During adult emergence, the pupal cocoon is ruptured by the frontal process of the pupa (cocoon cutter). Generally after the adult emerges, the anterior half of the pupal exuvium (head and thorax) protrudes outside, while the posterior half remains in the pupal cocoon (Fig. 7K).

At the type locality, *P. tethys* mines are common in *P. organensis* plants. One to several mines may be present per leaf (up to 13 young mines have been found in a single leaf) and may cover almost the entire lamina later in development (Figs. 7A, H). Our field collection data indicate that the species may have more than one generation per year, with adults emerging primarily in summer and autumn.

Molecular phylogeny. A total of 639 nucleotide sites were analyzed, in which 231 were variable and 173 parsimony-informative. ML and MP analyses showed identical topology and similar bootstrap supports, and we therefore show only the former (Fig. 9). According to our phylogenetic hypothesis, *P. tethys* was strongly supported as a monophyletic clade, showing high branch length in relation to the other 11 species surveyed. Additionally, it was placed as the most basal lineage within *Phyllocnistis*.
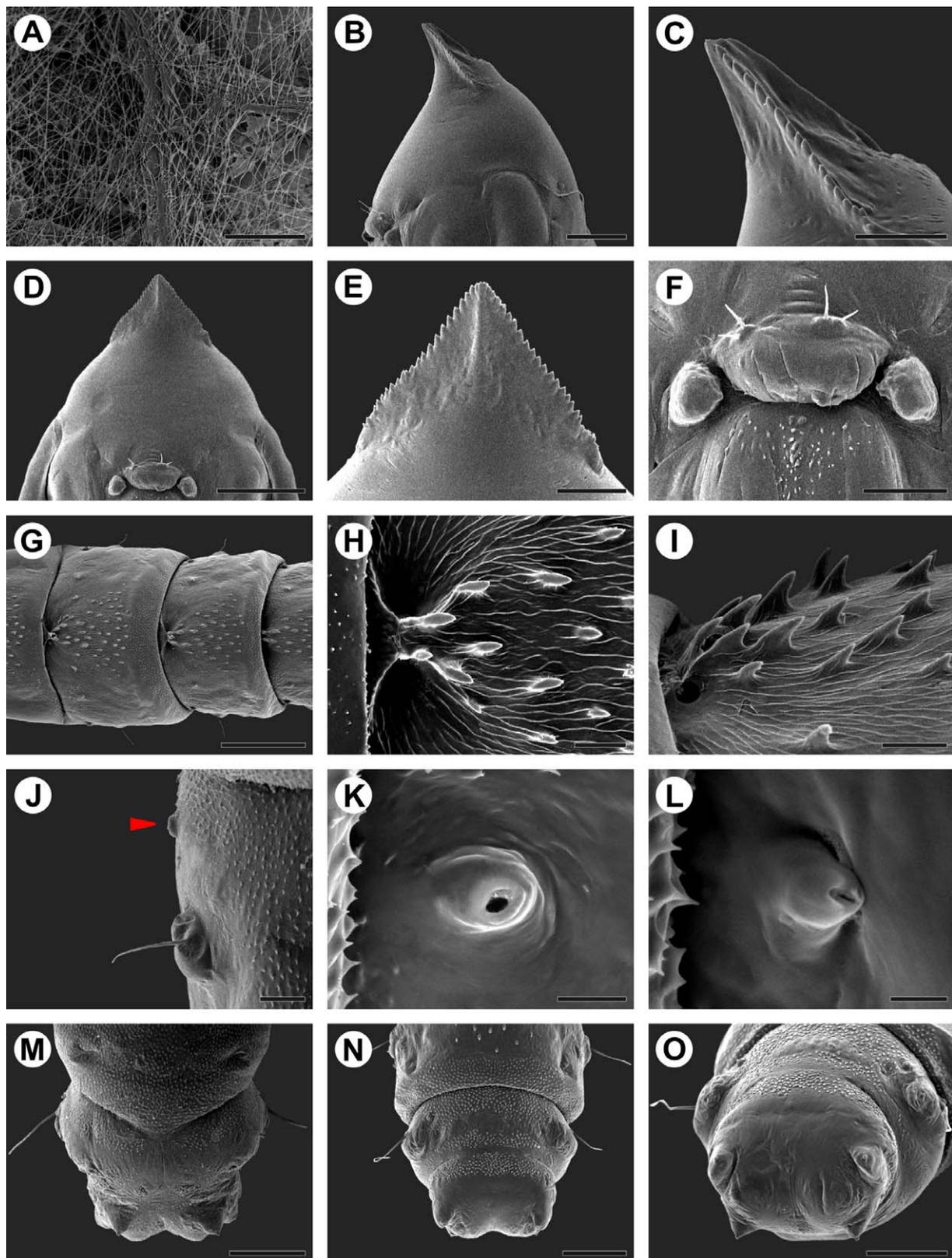
FIGURE 6. Scanning electron micrographs of *Phyllocnistis tethys* pupal cocoon and pupa: (A) weaving pattern of lower surface of pupal cocoon; (B) head, lateral view; (C) cocoon-cutter, lateral view; (D) head, ventral view; (E) cocoon-cutter, ventral view; (F) frons, ventral view; (G) abdominal segments 4 and 5, dorsal view; (H) spines on tergum 5, dorsal view; (I) spines on tergum 4, lateral view; (J) spiracle (arrow) and lateral seta on A5, dorsal view; (K) spiracle A3, lateral view; (L) spiracle A8, lateral view; (M–O) last abdominal segments in ventral, dorsal and posterior views, respectively Scale bars = 100, 100, 50, 150, 50, 50, 200, 25, 25, 25, 10, 5, 100, 100, 100 μm, respectively.
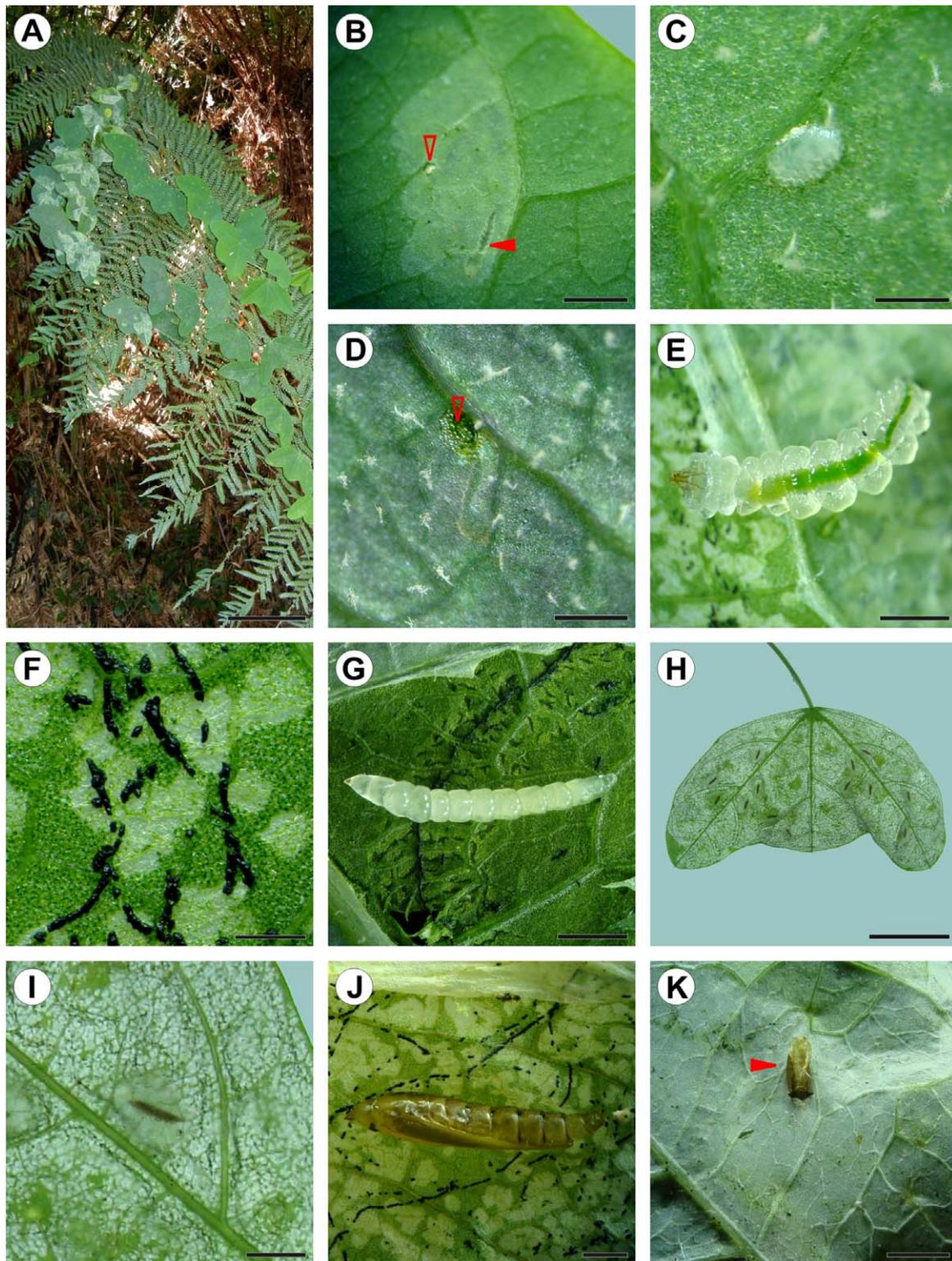
FIGURE 7. Life history of *Phyllocnistis tethys*: (A) *Passiflora organensis* shoot twining around on a fern at the type locality, showing several leaves with leaf mines at different development stages; (B) leaf mine on abaxial leaf surface (open and closed arrows, respectively, indicate empty chorion on leaf surface, and sap-feeding larva seen through transparent mine); (C) egg containing developing embryo; (D) freshly hatched larva (indicated by closed arrow; open arrow indicates green frass lines left within the egg chorion); (E) third-instar (sap-feeding) larva; (F) detail of frass lines and damage on leaf parenchyma, left by the larva within the mine; (G) fourth-instar (spinning) larva; (H) *Passiflora organensis* containing several pupae, seen by transparency (indicated by arrows); (I) a pupal chamber in detail, showing a pupa by transparency; (J) pupa, lateral view; (K) pupal exuvium protruded (arrow) from mine exit hole, just after the adult emergence. Scale bars = 100, 1, 0.2, 0.3, 1, 1, 1, 20, 5, 0.5, 2 mm, respectively.
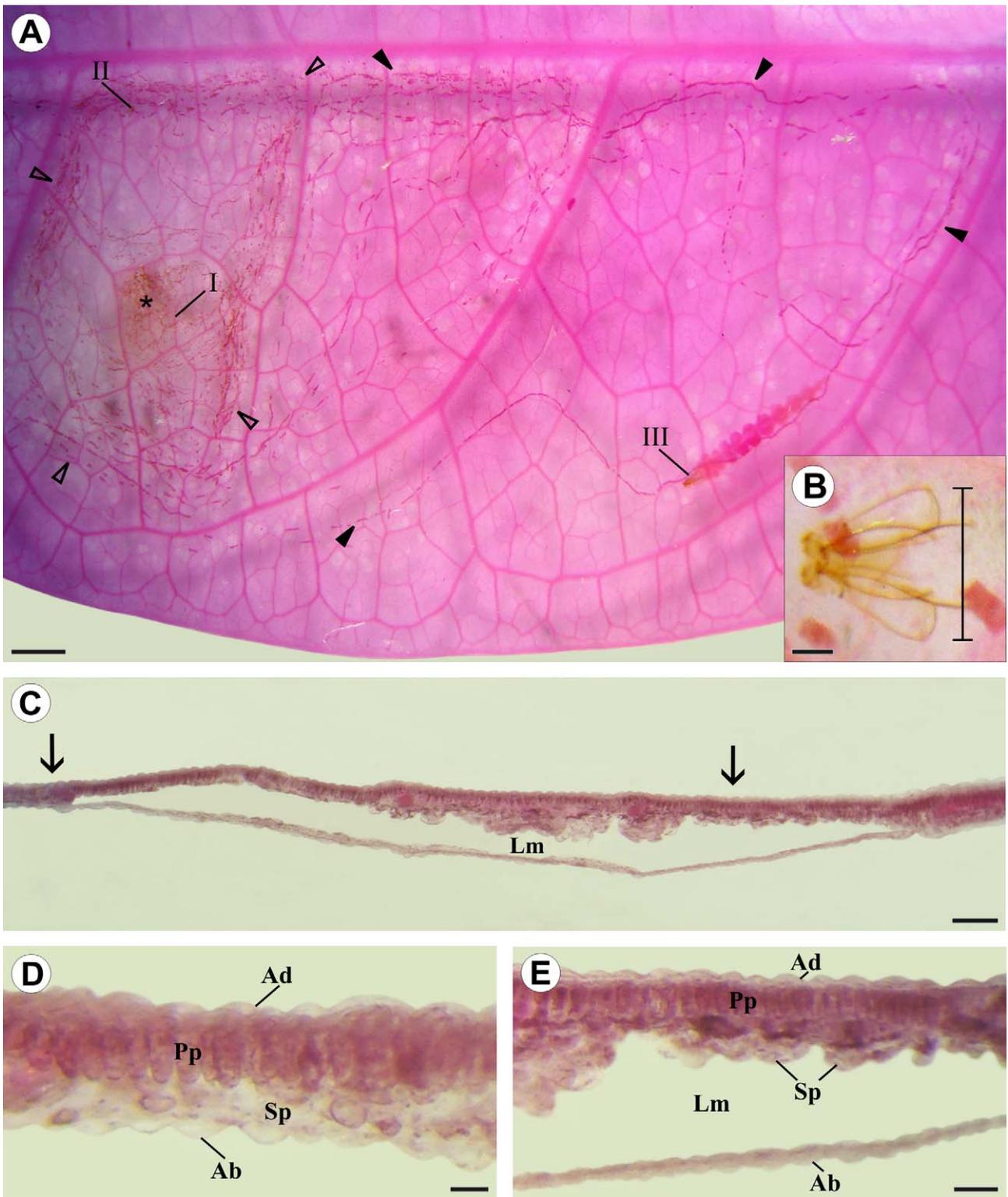
FIGURE 8. Diaphanized portion and histological sections of a *Passiflora organensis* leaf, showing by transparency the organization levels of a *Phyllocnistis tethys* mine in relation to larval ontogeny: (A) general aspect of the mine, containing a third-instar "sap-feeding" larva; asterisk indicates feeding area of the first instar; Roman numerals indicate larval instar numbers and corresponding positions of head capsules in the mine; open and closed arrows indicate the limit areas of non-granular frass lines left by second- and third-instar larvae, respectively; (B) detail of head capsule shed by the second-instar larva (bar indicates position for measurement of head-capsule width); C) transverse section of a mine; (D) transverse section of intact portion of leaf lamina (indicated by left arrow in C); (E) transverse section of mined portion of leaf lamina (indicated by right arrow in C). **Ab**, abaxial surface of epidermis; **Ad**, adaxial surface of epidermis; **Lm**, leaf mine; **Pp**, palisade parenchyma; **Sp**, spongy parenchyma. Scale bars = 1 mm, 50, 100, 25, 50 µm, respectively.
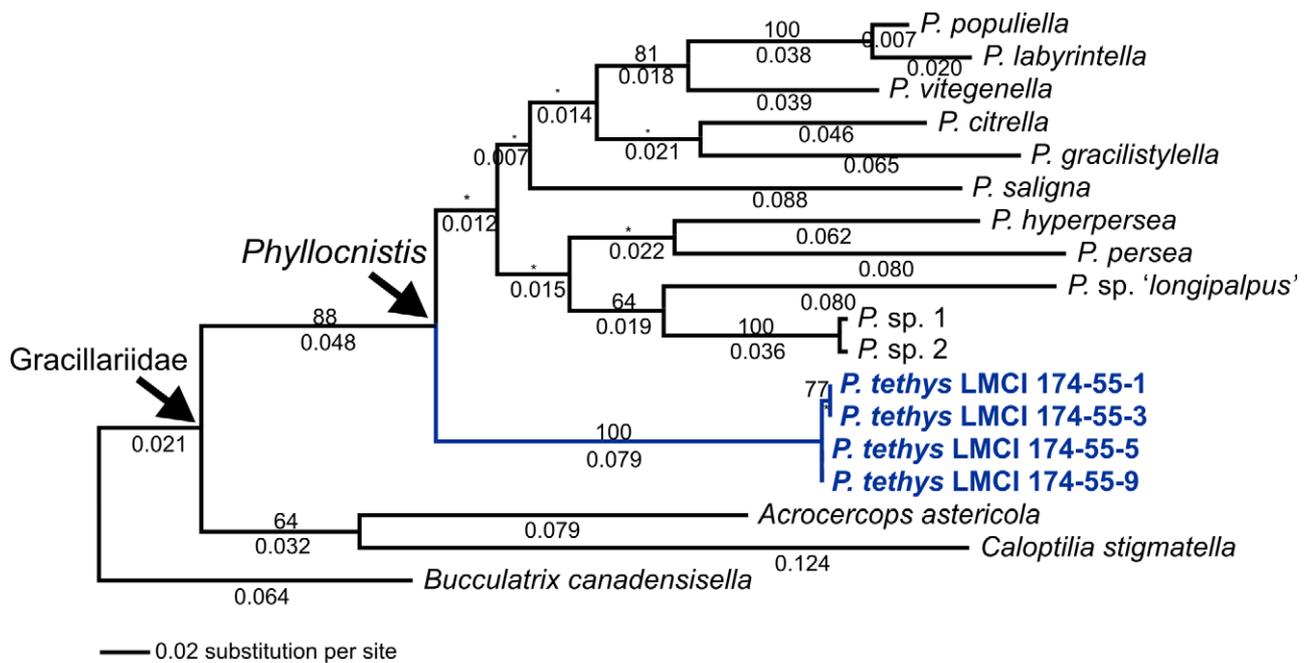
FIGURE 9. Maximum-likelihood tree of *Pyllocnistis* species based on 639 bp of the mitochondrial gene cytochrome oxidase subunit I (COI). Numbers above branches indicate bootstrap support higher than 60%; branch lengths are indicated below. Asterisks indicate bootstrap values < 60% and branch lengths < 0.001. Species of Bucculatricidae (*Bucculatrix canadensisella*) and Gracillariidae (*Acrocercops* and *Caloptilia*) were used to root the tree, according to the phylogeny proposed by Kawahara *et al*. (2011); see Table 1 and text for further description.

Discussion

Adults of *P. tethys* can be readily distinguished from all other known species of Neotropical *Phyllocnistis* by the forewing pattern, and also by differences in the genitalia and in the morphology and life history of the immature stages. For example, the cornuti of *P. tethys* are conspicuous, whereas their existence is not mentioned for other congeneric Neotropical species (*e.g*., Hering 1958; Davis 1994; Kawahara *et al*. 2009; Davis & Wagner 2011). In most female *Phyllocnistis*, the ostium bursae is a slender duct and the corpus bursae contains a pair of fusiform signa, bearing a short median projection (Davis & Wagner 2011); in the case of *P. tethys*, the ostium bursae is broad and there is only one fusiform signum in the corpus bursae. All Neotropical *Phyllocnistis* where the pupal stage has been described in detail have a pair of large recurved spines on the abdominal terga, and between these spines is a concentration of smaller spines arranged in a V-shaped pattern (Kawahara *et al*. 2009; Davis & Wagner 2011). Corresponding pairs of large spines are absent on all abdominal segments of *P. tethys*. Furthermore, all known phyllocnistine larvae construct long, serpentine leaf mines (Davis 1994; De Prins & Kawahara 2009). This is not the case for *P. tethys*, whose mines are clearly of the blotch type for all larval instars. Thus, most life stages of *P. tethys* show conspicuous differences in biology compared to other species of *Phyllocnistis* known for the Neotropical region. Also, phylogenetic reconstruction based on CO-I sequences indicated that this species is the basal lineage within *Phyllocnistis*, with higher differentiation (i.e., branch length) in relation to all other taxa in the genus for which sequences are known. However, we retain here the traditional nomenclatural status of the genus, until further evidence becomes available. This is the first species of *Phyllocnistis* described from Brazil. Future fieldwork in neighbouring Neotropical areas may reveal other undescribed congeneric species that are more closely related to *P. tethys*, and in that case the current taxonomic status should be re-evaluated.

According to our knowledge, this is the first gracillariid that has been found in association with a member of Passifloraceae. Our preliminary observations suggest this association is not occasional, because at least two additional undescribed leaf-mining gracillariid species are found on these plants in southern Brazil. Our discovery raises several questions regarding such a peculiar insect-plant association. Passion vines are toxic to most herbivorous insects, which is the reason that they have been successfully used as a food resource by only a few

ectophagous lepidopteran lineages, for example the Heliconiini (Nymphalidae) (for reviews, see Benson *et al.* 1975; Brown 1981; Gilbert 1991). Cyanogenic glycosides present in their leaves, for example, can negatively affect the feeding of some species, but on the other hand indirectly benefit other herbivorous insects. The heliconians in particular may either sequester or modify, or alternatively synthesize *de novo*, and use these substances for their own defense against vertebrate predators (Nahrstedt & Davis 1983; Spencer 1988; Engler-Chaouat & Gilbert 2007). The mechanisms by which these gracillariid larvae deal with the chemical compounds existing in the leaf parenchyma of their *Passiflora* host plants, where they are confined and feed throughout the larval stage, are of primary interest to be explored in the near future. The corresponding consequences, if any, for the adult stage of these gracillariids should also be investigated.

Acknowledgements

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