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A new species of *Leurocephala* Davis & Mc Kay (Lepidoptera, Gracillariidae) from the Azapa Valley, northern Chilean Atacama Desert, with notes on life-history

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ABSTRACT

The Neotropical micromoth genus *Leurocephala* Davis & Mc Kay, 2011 (Lepidoptera, Gracillariidae) was originally described to include only the type species, *L. schinusae* Davis & Mc Kay, 2011, whose leaf miner larvae are associated with Anacardiaceae in Argentina, Brazil and Paraguay. An integrative analysis including morphology, life history and DNA barcode sequences revealed that specimens collected on *Schinus molle* L. (Anacardiaceae) in the coastal valleys of the Atacama Desert of northern Chile belong to a second species of this formerly monotypic genus. Adults of *Leurocephala chilensis* Vargas & Moreira **sp. nov.** are herein described and illustrated in association with the immature stages and life history, and corresponding phylogenetic relationships are assessed based on DNA barcode sequences. This finding provides the first record of *Leurocephala* from west of the Andes Range, expanding remarkably its geographic range. It is suggested that the extent of diversity within *Leurocephala* is much greater and that variation in geographic factors and host plant use may have modeled it, an evolutionary hypothesis that should be assessed in further studies.

Introduction

Gracillariidae is a highly diverse lineage of leaf-mining Lepidoptera, with 105 recognized genera and 1952 species distributed worldwide; over 180 taxa have been recorded in the Neotropical region (De Prins and De Prins, 2016; De Prins et al., 2016). Recent studies have suggested that the comparatively low diversity of the Neotropical fauna of Gracillariidae is an artifact due to the low sampling efforts in this geographic area; accordingly, further surveys should render many additional species (Lees et al., 2014; Brito et al., 2016).

The use in conjunction of distinct characters and methodologies to study taxonomical problems, since 2005 defined as integrative taxonomy, is the modern basis of delimitation and discovery of species (Dayrat, 2005). The usefulness of this approach has been widely recognized, especially in cases involving species with closely similar morphology (e.g., Schlick-Steiner et al., 2010; Barão et al., 2014; Kergoat et al., 2015; Kirichenko et al., 2015), a pattern probably widespread among genera of Neotropical Gracillariidae (Davis and Wagner, 2011).

The Neotropical micromoth genus *Leurocephala* Davis & Mc Kay, 2011 (Lepidoptera, Gracillariidae) originally included only the type species, *L. schinusae*, whose leaf miner larvae are associated with Anacardiaceae in Argentina, Brazil and Paraguay (Davis & McKay, 2011). Molecular phylogenetic analyses based on sequences of 21 nuclear protein-coding genes placed *Leurocephala* within the *Parectopa* group of Gracillariinae (Kawahara et al., 2011). This group of lineages is characterized by the placement of the ostium bursae on the VII sternum of the female, which is supposedly a highly distinct morphological apomorphy (Kumata et al., 1988; Kawakita et al., 2010).

Leurocephala remained as a monotypic genus until now. However, as part of a study of the Lepidoptera associated with native plants in the coastal valleys of the Atacama Desert of northern Chile, adults of *Leurocephala* were recently reared from leaf mines occurring on *Schinus molle* L. (Anacardiaceae). Thus, represents a novel record in terms of both geographic distribution and host plant use for such a micromoth genus. Furthermore, a preliminary analysis of the morphology of the male and female genitalia enabled us to hypothesize that these specimens were not conspecific with the

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type species. This hypothesis was subsequently supported by an integrative analysis of morphology, life history, and DNA barcode sequences.

Accordingly, the aim of this article is to provide descriptions of all the life stages and the life history of a new species of *Leurocephala* from the Atacama Desert. In addition, the first assessment of the phylogenetic relationships for the two species of this formerly monotypic genus is provided based on sequences of mitochondrial DNA.

Material and methods

Specimens used for description in this study were either dissected or reared from leaf mines collected on *S. molle* plants in the Azapa valley, Atacama Desert, northern Chile, between 2008 and 2016. They were brought to the entomology laboratory of the Facultad de Ciencias Agronómicas, Universidad de Tarapacá, Arica, where they were either dissected or reared in small plastic vials. These were maintained at room temperature and periodically inspected for emerged adults, which were pinned and dried.

Morphological analysis

Immature stages were fixed in Dietrich's fluid and preserved in 75% ethanol. For descriptions of the 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, and measurements were performed using an attached ocular micrometer (precision = 0.01 mm). Structures selected to be drawn were previously photographed with a Sony®.

Cyber-shot DSC-H10 digital camera attached to the stereomicroscope. Vectorized line drawings were then made with the software Corel Photo-Paint® X7, using the corresponding digitalized images as a guide. At least five specimens were used for the descriptions of each life stage or instar.

For scanning electron microscope analyses, additional 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 examined and photographed in a JEOL® JSM6060 scanning electron microscope at the Centro de Microscopia Eletrônica of Universidade Federal do Rio Grande do Sul (UFRGS).

For plant anatomical descriptions, field-collected leaf portions of *S. molle* containing mines of *Leurocephala chilensis* were fixed in FAA (37% formaldehyde, glacial acetic acid and 50% ethanol, 1:1:18, v/v), and preserved in 70% ethanol. Leaf portions containing the different larval instar morphotypes were selected under a stereomicroscope in the laboratory. They were then progressively hydrated, immersed in 10% potassium hydroxide for 20 min, stained for 24 h with toluidine blue (aqueous solution: 200 mg/L) and then mounted whole in glycerine 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.

Museum collections

Abbreviations of the institutions from which specimens were examined are as follows: IDEA, Colección Entomológica de la Universidad de Tarapacá, Arica, Chile; LMCI, Laboratório de Morfologia e Comportamento de Insetos, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; MNNC, Museo Nacional de Historia Natural de Santiago, Santiago, Chile.

Molecular analysis

Total genomic DNA was extracted from fresh larval tissue using the PureLink kit (Life, Invitrogen, USA) following manufacturer's instructions. Specimens from the type locality of the new taxon *L. chilensis* ($n = 2$; Azapa) and of the only species recognized in the genus, *L. schinusae* ($n = 4$; Paraná and Rio Grande do Sul, Brazil) were surveyed to generate original data (Table 1). We also incorporated from the BOLD System and Genbank databases three individuals of *L. schinusae* from Misiones, Argentina. This dataset was used to assess the monophyletic status of *L. chilensis*. Gracillarid species of the *Spinivalva* Moreira & Vargas, likely the sister lineage of *Leurocephala*, and also of *Parectopa* Clemens and *Epicephala* Meyrick, all belonging to the '*Parectopa* group' (Brito et al., 2013), were used as outgroup, the corresponding sequences being downloaded from GenBank (Table 1). We amplified the DNA barcode region (part of the mitochondrial cytochrome oxidase I) including 658 base pairs, using primers and conditions described by Folmer et al. (1994). PCR products were purified using exonuclease (GE Healthcare Inc.) and Shrimp Alkaline Phosphatase (SAP), sequenced with BigDye chemistry and analyzed in an ABI3730XL (Applied Biosystems Inc.). Chromatograms obtained from the automatic sequencer were read and sequences were assembled using the software CodonCode Aligner (CodonCode Corporation). Sequences generated in this study were deposited in the databases GenBank and BOLD System (Table 1).

Corresponding tree was constructed using maximum likelihood (ML) method in the software PHYML 3.0 (Guindon et al., 2010). The program jModelTest 2 (Darriba et al., 2012) was used to estimate the substitution model (General Time-Reversible; Rodriguez et al., 1990), following the Akaike Information Criterion. Monophyly confidence limits were assessed with the bootstrap method (Felsenstein, 1985) at 50% cut-off after 1000 bootstrap iterations. We also analyzed the pairwise genetic distance using the Kimura 2-parameter model (Kimura, 1980) procedure, with 1000 bootstrap replications, between clusters defined in the phylogeny and outgroups.

Results

Leurocephala chilensis Vargas & Moreira **sp. nov.**

Type material. *Male holotype*: Azapa, Arica, Chile, August 2015, ex leaf miner larva on *S. molle*, July 2015, H.A. Vargas coll. (MNNC).

Paratypes: Two males, two females, same data as holotype (MNNC); two males and three females, same data as holotype (IDEA); one male and one female Azapa, Arica, Chile, December 2010, ex leaf miner larva on *S. molle*, July 2010, H.A. Vargas coll. (IDEA); three males Azapa, Arica, Chile, November 2011, ex leaf miner larva on *S. molle*, October 2011, H.A. Vargas coll (IDEA).

Genitalia dissected by H.A. Vargas (HAV) were deposited in IDEA, under accession numbers as follows, all from Azapa, Arica, Chile, ex leaf miner larva on *S. molle*: HAV276, one male, December 2010; HAV358, 359 and 402, three males, November 2011; HAV108, one female, December 2010; HAV1020 and 1024, two males, August 2015; HAV1021 and 1023, two females, August 2015. H.A. Vargas coll. Immature specimens of *L. chilensis* were deposited in LMCI, dissected from leaf mines on *S. molle* from Azapa, Arica, Chile, August 2012, H.A. Vargas and G.R.P. Moreira coll. as follows: preserved in 100% alcohol below −10 °C, used for DNA extraction (LMCI 191-3); preserved in 75% alcohol, used for microscopy studies, seven eggs (LMCI 191-37), six first instar larvae (LMCI 191-41), twelve last instar larvae (LMCI 43), seven pupae (LMCI 191-44).

Table 1
Gracillarid specimens used in this study to reconstruct the phylogenetic status and evolutionary relationships of *Leurocephala chilensis* based on 658 base pairs of DNA barcode (cytochrome oxidase subunit I gene) sequences.

Group	Genus	Species	Voucher	Locality	Accession number	
					Genbank	BOLD system
Ingroup						
	Leurocephala					
		chilensis	LMCI 191-3-1	Chile, Azapa	KY006921	MISA007-16.COI-5
			LMCI 191-3-5	Chile, Azapa	KY006922	MISA008-16.COI-5
		schinusae				
			DDAV-D546	Argentina, Misiones	HM382092	RDOPO 384-10.COI-5
			DDAV-D547	Argentina, Misiones	HM382093	RDOPO 385-10.COI-5
			DDAV-D576	Argentina, Misiones	HM382112	RDOPO 414-10.COI-5
			LMCI 295-19B	Brazil, Rio Grande do Sul	KY006923	MISA009-16.COI-5
			LMCI 295-19C	Brazil, Rio Grande do Sul	KY006924	MISA010-16.COI-5
			LMCI 309-01-10A	Brazil, Paraná	KY006925	MISA011-16.COI-5
			LMCI 309-01-10B	Brazil, Paraná	KY006926	MISA012-16.COI-5
Outgroup						
	Spinivalva					
		gaucha	LMCI 164-15	–	KC512112	GBGL13506-14.COI-5P
		sp.	LMCI 169-A1	–	KC512114	GBGL13508-14.COI-5P
	Parectopa	*ononidis*	CLV2269	–	KP845416	GRSLO654-11.COI-5P
	Epicephala	sp.	E312AK	–	FJ235388	–

Specimens of *L. schinusae* used for comparison were either dissected (immatures) or reared (adults) from leaf mines collected on *Schinus terebinthifolius* by C. M. Pereira (CMP) in Laranjeiras do Sul, PR, Brazil, as follows: LCMI 309-1, five larvae preserved in 100% alcohol below −10 °C, used for DNA extraction, 25.VII.2015; LMCI 309-3, one female, preserved in 70% alcohol, with genitalia in slide preparation (CMP 001-16F), 07.XI.2015; LMCI 309-4, one male, pinned, with genitalia in slide (CMP 001-18M), 07.XI.2015; LMCI 309-5, one male, in 75% alcohol, with genitalia in slide (CMP-22M), 16.VI.2016; LMCI 309-6, one female, pinned, with genitalia in slide (CMP-31F), 16.VI.2016. Also, five last instar larvae, preserved in 100% alcohol below −10 °C, used for DNA extraction, dissected from leaf mines on *Schinus* aff. *polygamus*, Coxilha das Lombas, Santo Antonio da Patrulha, Rio Grande do Sul, Brazil, April 2015, G.R.P. Moreira & S. L. Bordignon coll. (LMCI 295-19).

Diagnosis

Despite their morphological and life history resemblance, the two species of *Leurocephala* can be differentiated based on morphology of the adult and larval stages, and by the shape of the mine. The apex of the sacculus of male genitalia in *L. chilensis* is provided with a short, spine-like process that projects upwards, which is absent in *L. schinusae*; the dorsal surface of the aedeagus of *L. chilensis* is sculptured with several small tooth-like projections on the concave area, which are absent in *L. schinusae*. In the female genitalia, two signa are found on the corpus bursae of *L. chilensis*, while only one signum is found in *L. schinusae*; furthermore, the horn-like lateral extensions of the antrum are laterally projected in *L. chilensis*, while these structures are apically projected in *L. schinusae* At the larval stage, the ventral plate of the prothorax of the last instar of *L. schinusae* is uniformly sculptured by a great number of granular projections of similar size, about 12 of which are at the posterior margin of the plate, almost touching laterally, while in *L. chilensis* the greatest granular projections, almost 1.5 times the length of the smallest ones, are restricted to the posterior third of the ventral plate, with only four at the posterior margin, clearly separated from each another by a distance similar to the diameter of the respective projection. The serpentine mine constructed by the second instar of *L. schinusae* has a little blotch-like broadening a short distance from the empty chorion, while broadening is absent in the serpentine mine of the second instar of *L. chilensis*.

Description

Adult (Fig. 1)

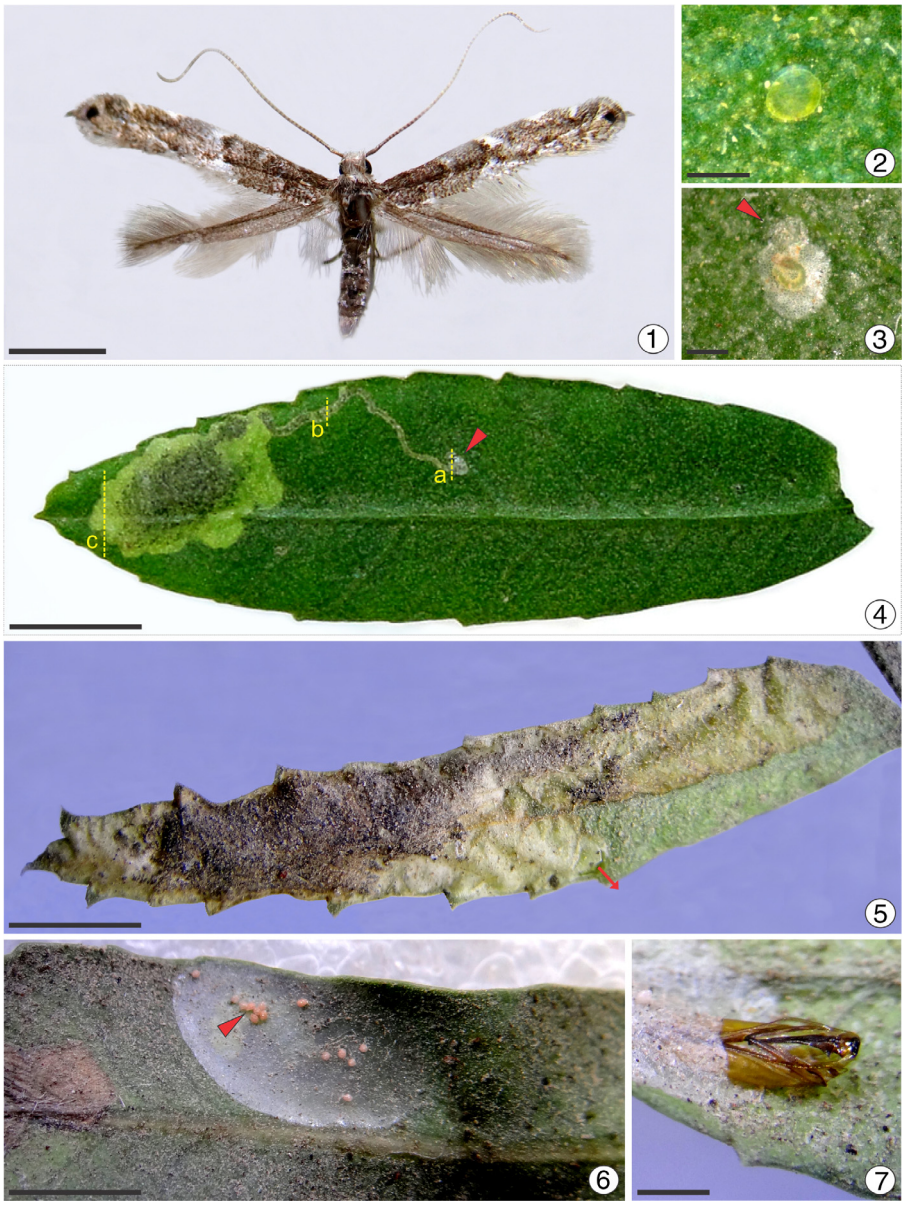
Male. Head. Front mostly whitish gray with brownish gray spots close to the compound eyes; vertex whitish gray; maxillary palpus whitish gray; labial palpus mostly whitish gray, second segment with brownish-gray spots distally; proboscis short, naked; antenna filiform, slightly shorter than forewing; scape elongated, brownish gray dorsally, whitish gray ventrally; with two narrow longitudinal stripes on the medial surface, one brownish gray in contact with the ventral area, the other whitish gray in contact with the dorsal area; pedicel and two first flagellomeres with coloration similar to the scape, remaining flagellomeres brownish gray.

Thorax. Mostly brownish gray dorsally with a few whitish gray scales; tegula dorsally brownish, ventrally whitish gray with a tuft of long scales at tip; lateral and ventral surfaces whitish gray. Foreleg mostly whitish gray, medial surface of femur and tibia brownish gray, tibial epiphysis whitish gray, a brownish gray ring at base of each tarsomere. Middle leg mostly whitish gray, tibia with two brownish gray rings, tibial spurs whitish gray, tarsomeres similar to those of the foreleg. Hindleg mostly whitish gray, a brownish gray ring at base of the femur, two brownish gray rings on the tibia, proximal tibial spurs brownish gray, distal tibial spurs mostly whitish gray with a brownish gray ring at middle, long whitish gray hair-like scales on the anterior and posterior surface of the tibia, tarsomeres whitish gray.

Forewing. Length: 4.0–4.5 mm ($n = 10$). Mostly brownish gray; a distinctive white transverse stripe arises in the middle of the coastal and hind margins, slightly projected apically at the longitudinal axis of the wing, sometimes interrupted by ground color scales; a short oblique, apically projected stripe arises from 3/4 of the hind margin reaching the longitudinal axis of the wing; a distinctive blackish gray dot subapically; fringe around apex short, concolor with the wing, a small apical tuft of plain scales; fringe on hind margin with long hair-like brownish gray scales. Venation as described by Davis et al. (2011) for *L. schinusae*.

Hindwing. Length: 3.2–3.4 mm ($n = 10$). Uniformly brownish gray with concolorous fringe of long hair-like scales. Venation as described by Davis et al. (2011) for *L. schinusae*.

Abdomen. Mostly brownish gray dorsally, with oblique segmental stripes of whitish gray ventrally until segment VI, completely whitish gray ventrally at apex. Segment VII with tergum and sternum reduced to fine transversal stripes. Segment VIII with sternum as a hood-like slightly sclerotized plate; sternum VIII as a slightly



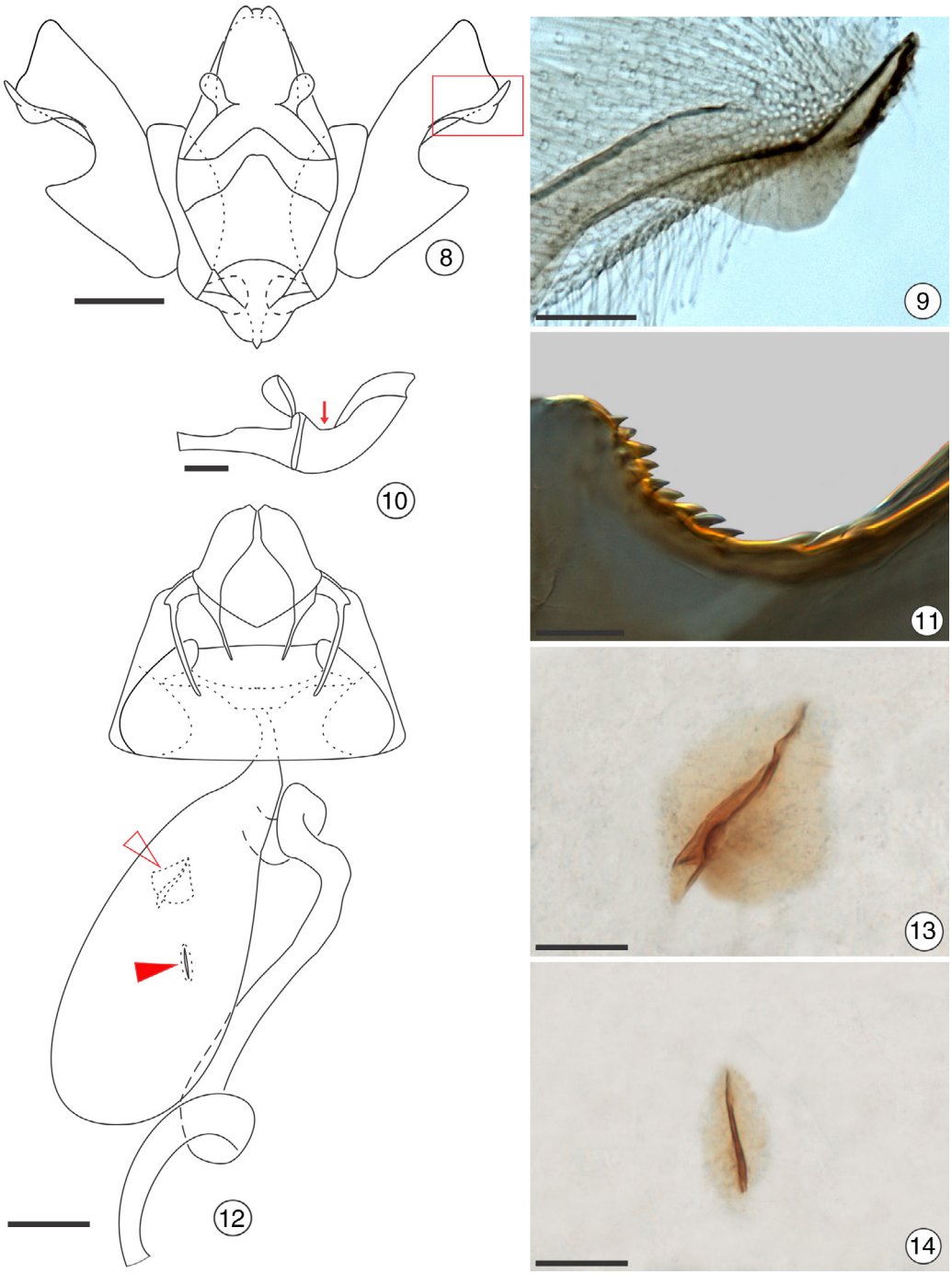
Figs. 1–7. Adult and life-history of *Leurocephala chilensis* on the abaxial surface of *Schinus molle* leaves: (1) pinned-dried male, dorsal view; (2) egg; (3) freshly hatched first instar larva, seen by transparence within the mine (empty chorium is indicated by arrow aside); (4) middle age mine (arrow indicates the egg-chorium at the beginning of the mine; letters and associated dashed-lines correspond to the locations of tissue sections, presented in transversal view in Figs. 33–35); (5) old, empty mine (seta indicates exit of last instar larva); (6) pupal cocoon, ornamented with bubbles (arrow); (7) pupal exuvium, partially protruding from the cocoon. Scale bars = 1, 0.5, 0.5, 5, 5, 3 and 1 mm, respectively.

sclerotized fine transversal stripe. Membranous area between segment VII and VIII laterally with a pair of hair-like coremata at the apex of a finger-like mostly membranose lobe provided with a rod-like sclerite. Segment VIII with a second pair of hair-like coremata on the lateral apex of the sternum.

Male genitalia (Figs. 8–11). Uncus absent. Tegumen as two fine stripes touching dorsally. Saccus U-shaped in posterior view, ventral area with the anterior margin slightly projected forward, posterior margin slightly convex. Gnathos (Fig. 8) as two short, slightly sclerotized finger-like lobes. Valva (Fig. 8) broadly joined basally to the posterior margin of the lateral arms of the saccus; costal margin straight; cucullus mostly membranous, ventral margin parallel to the costa; sacculus well sclerotized, basal part broad, triangle-like, delimited by a broad concavity on the ventral margin, with distal part straight, down-curved, slightly dilated subapically, bearing a short spine-like projection at apex (Fig. 9). Transtilla as a slightly sclerotized transversal band joining the base of the costal margin of the right and left valvae. Juxta absent. Aedeagus (Fig. 10) a bit shorter than valva, with insertion of the ductus ejaculatorius dorsally, close to the middle; basal half forward directed, tip blunt, diameter increasing toward the middle; distal half upcurved, with lateral sides slightly asymmetrical at apex, dorsal surface sculptured by several small tooth-like projections on the concave portion (Fig. 11). A small sclerite joined dorsally on the middle of the aedeagus. Cornuti absent.

Female. Similar to male in size and color.

Female genitalia (Figs. 12–14). Anterior and posterior apophyses well sclerotized, with length similar to the sternum VII. Ostium bursae broad, covering completely the posterior margin of the sternum VII. Antrum broad, sclerotized, trapezoid-like in ventral view, length about half of the sternum VII, cephalic side about half of the posterior side, with two laterally directed horn-like extensions



Figs. 8–14. Genitalia morphology of *Leurocephala chilensis* under light microscopy: (8) male, posterior view; (9) distal end of valva, in detail (enlarged, rectangular area marked in Fig. 8); (10) aedeagus, lateral view; (11) dorsal spines of aedeagus, in detail (enlarged area indicated by seta in Fig. 10); (12) female, ventral view; (13, 14) signa, in detail (indicated by open and closed arrows in Fig. 12). Scale bars = 200, 50, 100, 20, 200, 50 and 50 μm, respectively.

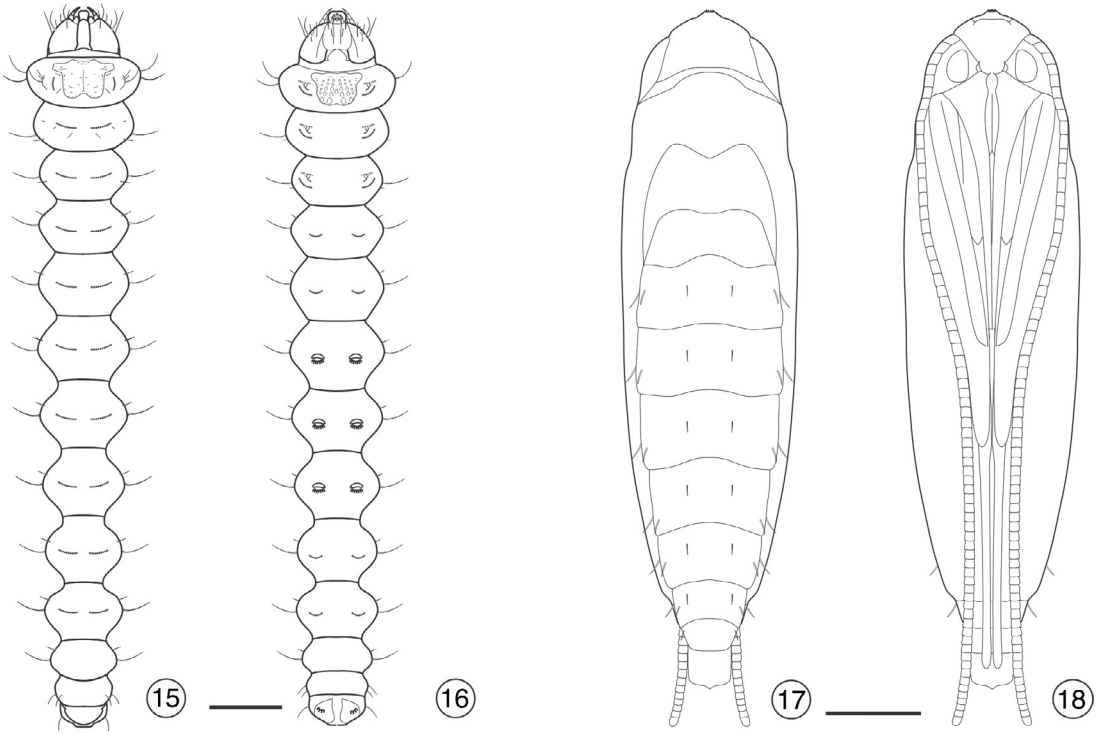
on the middle of the lateral sides. Ductus bursae membranous, with similar length to the antrum. Corpus bursae elliptical, elongated, mostly membranous, with two signa (Fig. 12); the larger (Fig. 13) one on the ventral surface, triangle-like, with a narrow sclerotized longitudinal stripe with the cephalic tip projected forward as a short spine into the corpus bursae; the smaller (Fig. 14) one on the dorsal surface, elliptical, elongated, length about 3/4 of the larger, with a longitudinal sclerotized stripe. Ductus seminalis basally inserted on the ventral surface of the corpus bursae (Fig. 12).

Egg (Fig. 2).

Round and flat, with a translucent chorion, allowing by transparence visualization of the embryo under development within.

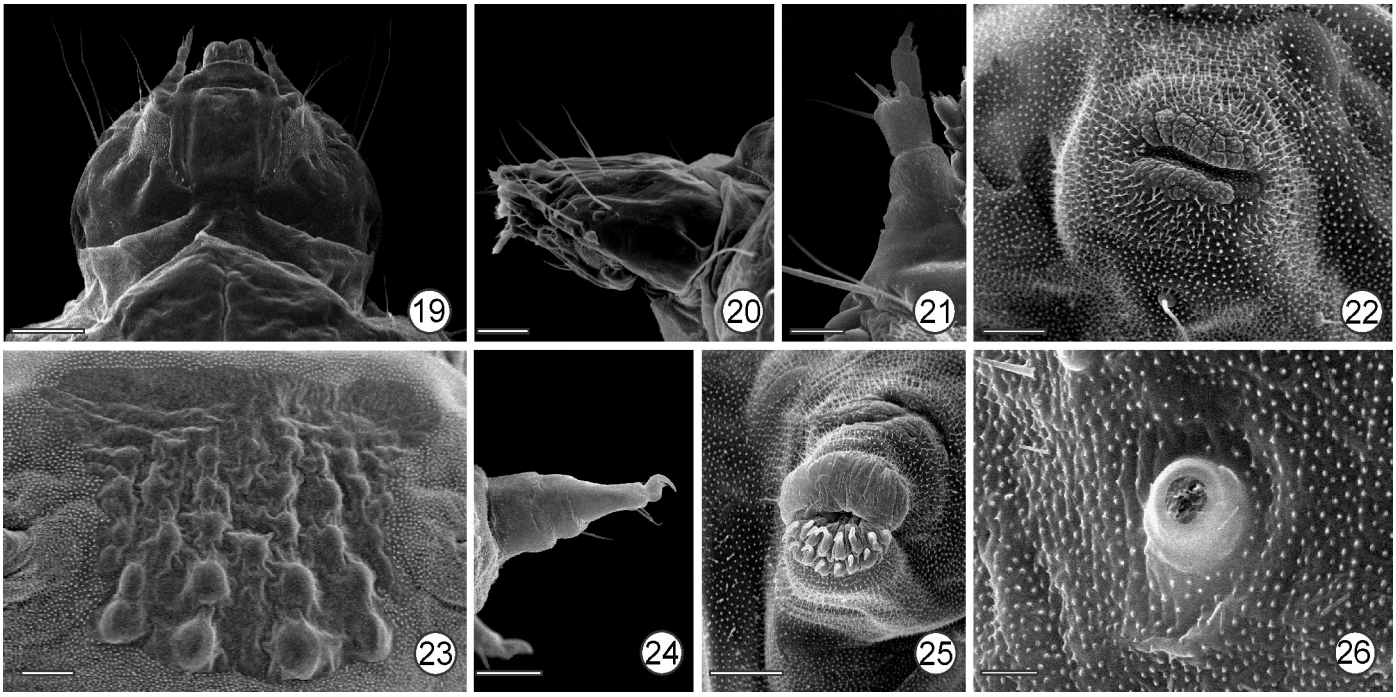
Larva (Figs. 15, 16, 19–26).

There are three morphotypes and five intars, which are similar in morphology to those described by Davis et al. (2011) for the type species, except for the last instar described below. The first instar is of a "sap-feeding" type, having mandibles modified for slicing the epidermis cells, differing from the remaining four instars that are tissue-feeders, which have mandibles used for chewing the leaf parenchyma. These can be identified by measuring their head capsule width, which do not overlap in size (Table 2). Corresponding exponential growth curve for the four tissue-feeding instars of *L. chilenis* reared on *L. molle* was: $y = 0.0494e^{0.4787x}$; $n = 50$; $r = 0.98$; $p < 0.0001$.



Figs. 15–18. Last instar (15, 16) and pupal (17–18) morphologies of *Leurocephala chilensis* under light microscopy, in dorsal and ventral views, respectively. Scale bars = 0.5 mm.

Last instar (Figs. 15, 16, 19–26). Maximum body length = 5.5 mm (n = 10). Head (Figs. 15, 16, 19, 20). Brown, semicircular in dorsal view, vertex partially covered by the prothorax, slightly depressed dorsoventrally, mostly smooth with a rhomboid-like area covered by short spine-like microtrichia between the frontoclypeous and the stemmata; epicranial notch U-shaped, broad and deep; frontoclypeous rectangle-like, about two times longer than wide; six circular stemmata, with stemmata 1 and 2 close to seta A3, stemmata 3–5 in a diagonal line ventral to seta A1, stemma 5 slightly displaced to the ventral surface of the head, stemma 6 isolated on the ventral surface of the head, almost equidistant to setae S2 and SS2. Antenna three-segmented; first segment annular, second segment cylindrical, about two times longer than the first segment, with sensillae distally, and third segment cylindrical, similar in



Figs. 19–26. Last larval instar of *Leurocephala chilensis* under scanning electron microscopy: (19–20) head, under dorsal and lateral views, respectively; (21) antenna, dorsal; (22) callum of first abdominal segment, ventral; (23) sternal prothoracic plate, ventral; (24) mesothoracic leg, lateral; (25) pseudopodium of fifth abdominal segment, ventral; (26) spiracle of eighth abdominal segment, lateral. Scale bars = 100, 50, 20, 50, 25, 50, 50 and 10 μm, respectively.

Table 2
Variation in size of head capsule width among instars of *Leurocephala chilensis* reared on *Schinus molle* ($n = 10$ per instar).

Instar	Head capsule width (mm)		
	Mean ± standard error	Range	Growth rate
I	0.127 ± 0.026	0.117–0.143	–
II	0.127 ± 0.003	0.117–0.143	–
III	0.211 ± 0.009	0.182–0.260	1.661
IV	0.340 ± 0.007	0.312–0.377	1.611
V	0.534 ± 0.005	0.507–0.559	1.571

length to second segment, about a half the diameter the second segment, with sensillae at apex. Mouthparts of the chewing type; labrum bilobed, four short hair-like setae on the external surface; epipharyngeal spines close to the distal margin of the labrum, one pair of plain epipharyngeal sclerites close to the each group of epipharingeal spines; mandible well-developed, with five distal cusps; maxilla with well-differentiated galea and palpus; labium with a well-developed cylindrical spinneret at apex and a pair of bi-segmented palpi laterally to the spinneret; hypopharynx provided with long hair-like projections. Chaetotaxy. AF group bisetose, AF1 and AF2 as microsetae close to the dorso-median apex of the patch of microtrichiae. A group bisetose, A1 close to antenna, A3 dorsal to stemma, about two times the length of A1. CD group of microsetae trisetose. C group unisetose, C1 as a microseta. F group of setae absent, Fa pore present. L group unisetose, L1 as a short hair-like seta posteroventral to A3. MG group of microsetae bisetose. P group bisetose, P1 at middle of the patch of microtrichiae, size similar to A1, P2 greatly reduced, slightly greatest CD setae. S group bisetose, S1 about halfway between stemmata 1 and 3, S2 about halfway between stemmata 1 and 6. SS group trisetose, SS1 ventromedial to stemma 5, SS2 about halfway between stemmata 4 and 6, SS3 posteromedial to stemma 6. Thorax and abdomen sculptured by short spine-like microtrichiae.

Thorax (Figs. 15, 16, 22–24). Prothorax. Dorsal shield grayish brown, smooth, in the form of two subtriangular plates separated medially by a narrow membranous stripe; each plate with the anterior margin slightly convex, medial margin straight, lateral margin widely concave close to the anterior margin and almost parallel to the medial margin on the distal 2/3, posterior vertex widely rounded. An ellipsoid shield postero-ventral to SV group. Ventral shield square-like, lateral sides slightly concave close to the anterior margin, posterior margin slightly convex; sculptured by granular projections variable in size, the largest restricted to the posterior third of the plate, clearly separated from each other by a distance similar to the diameter of the respective projection. A circular spiracle with slightly elevated peritrema laterally close to the posterior margin of the segment. A longitudinally oriented callus-like structure between the lateral margin of the dorsal shield and the SV group; another callus-like structure postero-lateral to the coxa. Chaetotaxy: D group bisetose, D1 greatly reduced, on the posterior half of the dorsal shield close to the lateral margin, D2 about three times the length of D1 between the dorsal shield and the callus-like structure. XD group bisetose, XD1 anterior to D2, XD2 ventral to XD1. SD group bisetose, lateroventral to the callus-like structure, SD1 similar in size to D2, SD2 about 4–5 times longer SD1. L group bisetose, similar to SD2 in size, clearly anterior to the spiracle. SV group bisetose, antero-dorsal to the ellipsoid shield. V group unisetose, V1 between the coxa and the ventral shield.

Meso and metathorax without dorsal, lateral or ventral shields. A transversally oriented callus-like structure anterior to D2; another callus-like structure postero-lateral to the coxa. Chaetotaxy: D group bisetose, D1 anterior and D2 posterior to the callus-like structure. SD group bisetose, ventro-lateral to the callus-like structure. L group bisetose. SV and V groups unisetose. Legs moderately well developed, bearing large tarsal claws.

Abdomen (Figs. 15, 16, 25, 26). All segments bearing dorsal and ventral smooth shields varying in shape. Dorsal shield of A1–6 in the form of a small irregular plate, those of A7 and A8 little developed, in the form of a small dot; dorsal shield of A9 ellipsoidal, well-developed, transversally arranged; dorsal shield of A10 in the form of two widely separated plates close to the posterior margin of the segment. Ventral shield of A1–2 and A8 circle-like, little evident on A3–5, between the respective prolegs; those of A6–7 similar in size and shape to those of A3–5; ellipsoidal on A9, transversally arranged, smaller than the dorsal shield of the same segment; absent on A10. Chaetotaxy: A1–2, 6–7: D group bisetose, D1 anterior and D2 posterior to the callus-like structure. SD group bisetose, SD1 latero-ventral to the callus-like structure, SD2 greatly reduced, dorsal to the spiracle. L group unisetose, L1 postero-ventral to the spiracle. SV bisetose, dorso-lateral to the callus like structure. V group unisetose, V1 between the callus-like structure and the ventral shield. A3–5: similar to the preceding segments; SV dorso-lateral to the proleg. A8 similar to preceeding segment, except that SV group unisetose. A9 similar to preceeding segment, except that SD group unisetose. A10 with D and SD groups bisetose; D1 anterior and D2 posterior to the dorsal shield, SD1, SD2 on the margin of the lateral part of the dorsal shield; L, SV and V groups unisetose. Spiracles round, with moderately elevated peritreme. Prolegs present on A3–5 and A10; crochets arranged in a staggered caudal varying from 10 to 16 hooks on A3–5 (Fig. 25); A10 with crochets reduced to 4 hooks.

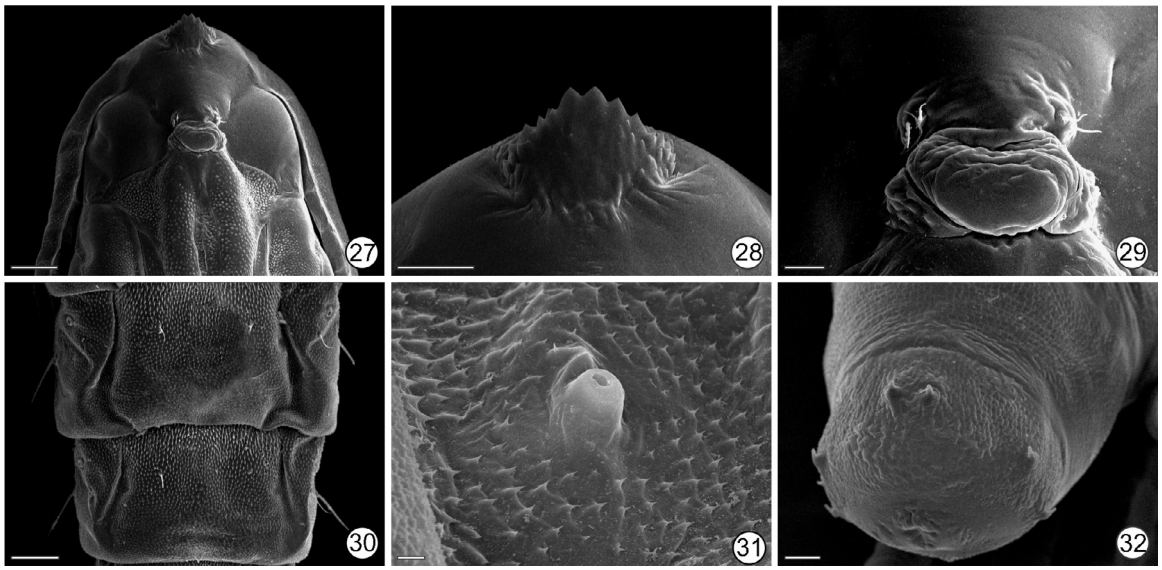
Pupa (Figs. 17, 18, 27–32).

Maximum body length: 4 mm ($n = 10$). Similar in color and general appearance to *L. schinusae*, as described by Davis et al. (2011) for *L. schinusae*. Minutely and densely spinose, particularly the dorsal abdomen. Cocoon cutter subtriangular, with outer ridge having numerous minute teeth, the central three teeth the largest. Antennae long, surpassing the abdomen in length. Labial palpi ca. 1/3 the length of proleg. Proboscis as long as the proleg. Forewings narrow, well separated, extending to abdominal segment A7. Hindlegs extending to abdominal segment 9 + 10. Setae D1, SD1, and L1 present on A1–7; only SD1 present on A8. Abdominal spiracles round, with elevated peritreme (Fig. 31). Cremaster formed by three pairs of slightly curved spines, two lateral and one dorsal (Fig. 32).

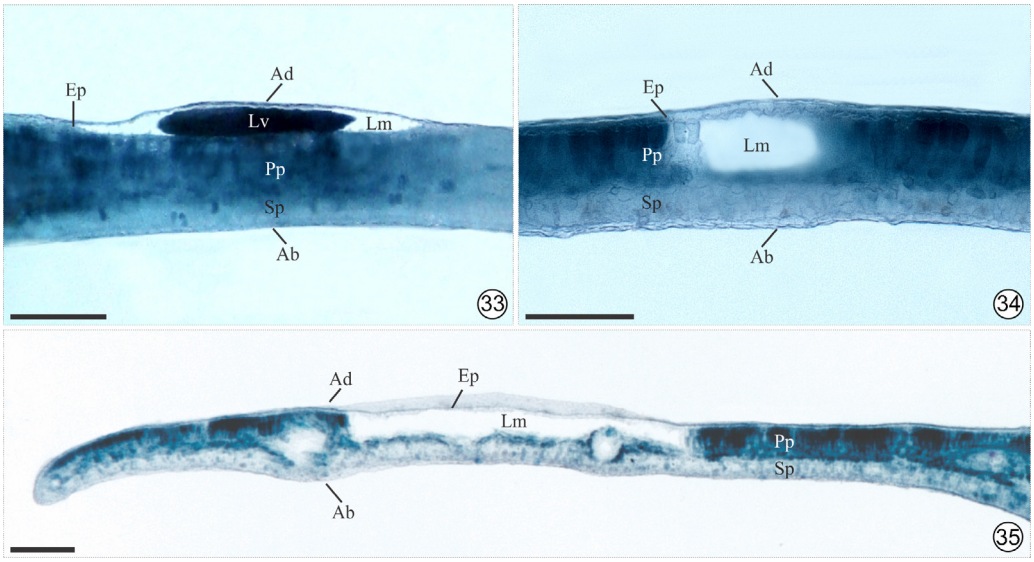
Etymology. The specific epithet is derived from the country of the type locality.

Distribution. *L. chilensis* is known only from Azapa (type locality) Valley in the Atacama Desert of northern Chile.

Life history (Figs. 2–7). Eggs are laid individually (Fig. 2) on the adaxial surface of the leaflet, mostly close to the main leaflet vein. After hatching, the first, sap-feeder instar introduces itself into the leaflet, constructing a small, superficial, blotch-like mine a short distance from the empty chorion (Fig. 3). The feces are deposited into the lumen of the chorion during the time that the anal apex of the first instar remains there. The second instar constructs a narrow serpentine mine (Fig. 4) on the adaxial surface of the leaflet. Subsequent instars construct a conspicuous blotched mine on the adaxial surface whose diameter increases with the sequence of the instars, generally covering more than 50% of the leaflet when fully



Figs. 27–32. Pupa of *Leurocephala chilensis* under scanning electron microscopy: (27) head, ventral view; (28) "cocoon-cutter" in detail, ventral; (29) labrum in detail, ventral; (30) sixth and seventh abdominal segments, dorsal, (31) spiracle of eighth abdominal segment, lateral; (32) last abdominal segments, dorso-posterior. Scale bars = 100, 25, 100, 100, 5 and 100 μm, respectively.



Figs. 33–35. Variation in transversal histological sections of *Leurocephala chilensis* on *Schinus molle* leaf, according to larval ontogeny; (33) first, *sapfeeding* instar (position indicated by letter "a" in Fig. 4); (34) early *tissue-feeding* instar (letter "b" in Fig. 4); (35) last *tissue-feeding* instar (letter "c" in Fig. 4). Ab, abaxial surface of epidermis; Ad, adaxial surface of epidermis; Lm, leaf mine; Ep, epidermis; Pp, palisade parenchyma; Sp, spongy parenchyma. Scale bars = 0.2 mm.

developed (Fig. 5). A large number of feces are glued on the internal side of the "epidermal" surface of the blotch mine. The fully developed fifth instar makes a short slit on the margin of the mine to exit from it to search for a site for pupation. Pupation mostly occurs on the abaxial surface of a leaflet of the same plant; previously the fifth instar constructs an ellipsoidal smooth cocoon, generally with one of the lateral margins touching the lateral margin of the leaflet, and externally deposits silk bubbles (Fig. 6) secreted by the anus. When metamorphosis is completed, the pupa makes a slit on the cocoon using the cephalic cocoon-cutter to enable the adult to emerge, after which the pupal exuvium typically appears protruded with the posterior body portion remaining in the cocoon (Fig. 7). The first, sap-feeding instar feeds only on the epidermis cells (Fig. 33), while the other four tissue-feeding instars feed on the palisade parenchyma, leaving the spongy parenchyma intact (Figs. 34, 35).

Molecular analyses. Two reciprocally monophyletic lineages were found within the genus *Leurocephala*: the currently recognized *L. schinusae* and the new species *L. chilensis* (Fig. 36). Genetic divergence estimated between these lineages was 12% (±1%) and of both species together versus the outgroups (*Spinivalva*, *Parectopa* and *Epicephala*), varied from 16 to 18% (±1%, for any comparison).

Discussion

Morphological resemblance with the type species enables us to include this new gracillariid species in the formerly monotypic Neotropical genus *Leurocephala*. No differences were found in body color or wing venation in the adult stage for the two species. In addition, we were unable to separate *L. chilensis* from *L. schinusae* based on the external morphology of the pupa; also, no differences were found between their last larval instar chaetotaxy. However,

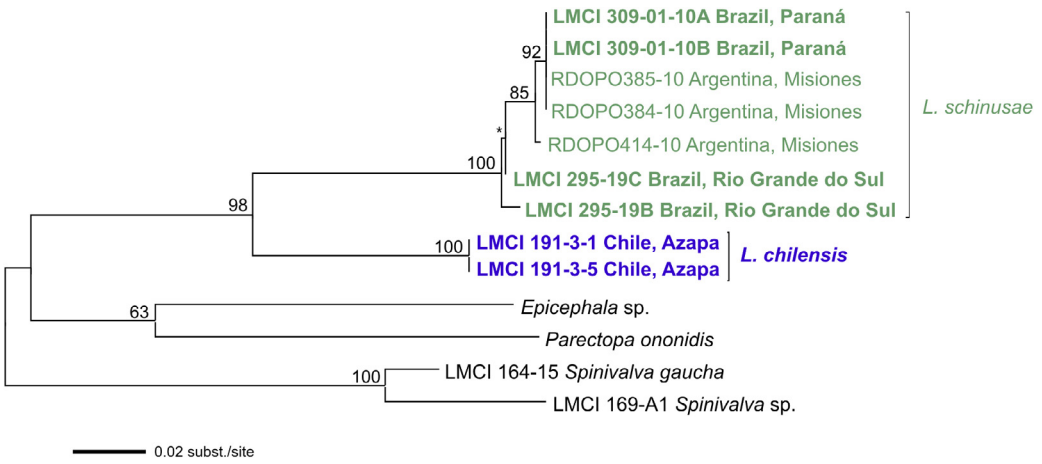


Fig. 36. Maximum likelihood phylogenetic tree of *Leurocephala* inferred based on 658 bp of DNA barcode sequences (cytochrome oxidase subunit I gene). Asterisk above branch indicate bootstrap support lower than 50%. Species of *Spinivalva, Parectopa* and *Epicephala* were used as outgroup; see Table 1 and text for further description.

as already mentioned, highly constant differences were found to separate the two species in the adult genitalia, and in the ventral prothoracic plate of larvae. In addition, subtle differences were found in the shape of the mine built by the second, tissue feeding instar. These morphological differences were reinforced by the molecular phylogenetic analysis, as the two species were grouped as closely related, but reciprocally monophyletic taxa that diverge from each other by ca. 12% (±1%) in DNA barcode sequences.

The known distribution of *Leurocephala* was previously restricted to the southern part of the Atlantic Forest, including portions of Argentina, Brazil and Paraguay (Davis et al., 2011). Thus the discovery of *L. chilensis* in the Atacama Desert provides the first record of *Leurocephala* from west of the Andes Range, expanding remarkably the geographic range of the genus. Additional surveys will be needed to characterize adequately the host(s) and geographic range of *L. chilensis*. It is very likely to find this species in other coastal valleys of the Atacama Desert, either in northern Chile or southern Peru, in the same way that other Gracillariidae were found in this hyperarid area (Maita-Maita et al., 2015), since the host plant *S. molle* is widespread in the region. The type species *L. schinusae* is able to breed on several species of *Schinus*, and also on species of two other genera in Anacardiaceae (Mc Kay et al., 2012). Adults of *Leurocephala* specimens were recently reared from leaf-mines collected on *S. polygamus* (Cav.) Cabrera, and mines typical of *Leurocephala* were also found on leaves of *Schinus latifolius* (Gill. ex Lindl.) Engler in two localities of Central Chile not included in the present study. These new findings suggest that all the species of *Schinus* distributed in south-central Chile (see Rodríguez et al., 1983) should be surveyed to know the effective range and diversity of *Leurocephala* west of the Andes. In our opinion, further taxonomic decisions on this regard should wait until comparative, fine scale analyses with an integrative taxonomic approach are performed, and after a broader survey for this micromoth genus been conducted throughout its range in South America. According to Davis et al. (2011), the type material used in the original description of *L. schinusae* was reared from mines collected on *S. terebinthifolius* Raddi, but similar mines were also found by them on other *Schinus* species, and also on an additional anarcadeacean, *Astronium balansae* Engl.

Additional monospecific genera of Neotropical Gracillariidae have been described during the last decades (e.g., Davis, 1994; Vargas and Landry, 2005; Vargas and Parra, 2005; Mundaca et al., 2013a,b), whose monotypic status might be associated with low sampling effort. The discovery of a second species of *Leurocephala* with morphology very close to the type species but with relative high level of genetic divergence, and with larvae feeding on a plant of the same family as the type species suggests that analogous studies should be carried out also for these other apparently monotypic genera. In other words, host-plant shifts between closely related plants and the existence of cryptic species in association should be further explored in such Neotropical gracillariid genera, as already suggested by Brito et al. (2013).

Finally, our results illustrate the existence of wide diversity of feeding habits at fine scale throughout ontogeny and among gracillariid lineages, which should be better explored. As far we are aware, feeding confined to single-celled epidermis, as demonstrated here for the first sap-feeding instar of *L. chilensis* has not been described for any species within the *Parectopa* group. This very specialized feeding behavior was demonstrated for the sap-feeding larvae of *Phyllocnistis citrella* Stainton (Achor et al., 1997) and *Marmara arbutiella* Busck (Wagner et al., 2000). Sap-feeding instars of other gracillariids are supposedly associated primarily with the outer layers of parenchyma (e.g. Brito et al., 2012). Feeding on palisade parenchyma is restricted to tissue-feeding instars in *L. chilensis*, which is also the case for all tissue-feeding instars of *Spinivalva gaucha* Moreira & Vargas, as described by Brito et al. (2013). Our data give further support in the sense that the three larval morphs (sap-feeder, apodal and legged) *Leurocephala* found (see Davis et al., 2011) might be associated with the three mining types described here (the epidermic, serpentine and blotch types, respectively).

Conflicts of interest

The authors declare no conflicts of interest.

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References

Achor, D.S., Browning, H., Albrigo, L.G., 1997. Anatomical and histochemical effects of feeding by *Citrus* leafminer larvae (*Phyllocnistis citrella* Stainton) in *Citrus* leaves. J. Am. Soc. Hortic. Sci. 122, 829–836.

Barão, K.R., Gonçalves, G.L., Mielke, O.H.H., Kronforst, M.R., Moreira, G.R.P., 2014. Species boundaries in *Philaethria* butterflies: an integrative taxonomic analysis based on genitalia ultrastructure, wing geometric morphometrics, DNA sequences, and amplified fragment length polymorphisms. Zool. J. Linn. Soc. 170, 690–709.

Brito, R., Gonçalves, G.L., Vargas, H.A., Moreira, G.R.P., 2012. A new species of *Phyllocnistis* Zeller (Lepidoptera: Gracillariidae) from southern Brazil, with life-history description and genetic comparison to congeneric species. Zootaxa 3582, 1–16.

Brito, R., Gonçalves, G.L., Vargas, H.A., Moreira, G.R.P., 2013. A new Brazilian *Passiflora* leafminer: *Spinivalva gaucha*, gen. n., sp. n. (Lepidoptera, Gracillariidae, Gracillariinae), the first gracillariid without a sap-feeding instar. ZooKeys 291, 1–26.

Brito, R., De Prins, J., De Prins, W., Mielke, O.H.H., Gonçalves, G.L., Moreira, G.R.P., 2016. Extant diversity and estimated number of Gracillariidae (Lepidoptera) species yet to be discovered in the Neotropical region. Rev. Bras. Entomol. 60, 275–283.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772.

Davis, D.R., 1994. New leaf-mining moths from Chile, with remarks on the history and composition of Phyllocnistinae (Lepidoptera: Gracillariidae). Trop. Lepid. 5, 65–75.

Davis, D.R., Mc Kay, F., Oleiro, M., Vitorino, M.D., Wheeler, G.S., 2011. Biology and systematics of the leaf mining Gracillariidae of Brazilian Pepper Tree, *Schinus terebenthifolius* Raddi, with descriptions of a new genus and four new species. J. Lepid. Soc. 65, 61–93.

Davis, D.R., Wagner, D.L., 2011. Biology and systematics of the New World *Phyllocnistis* Zeller leafminers of the avocado genus *Persea* (Lepidoptera, Gracillariidae). ZooKeys 97, 39–73.

Dayrat, B., 2005. Towards integrative taxonomy. Biol. J. Linn. Soc. 85, 407–415.

De Prins, J., De Prins, W., 2016. Global Taxonomic Database of Gracillariidae (Lepidoptera), Available at: http://www.gracillariidae.net (accessed 09.07.16).

De Prins, J., Brito, R., Moreira, G.R.P., 2016. An annotated taxonomic checklist of the Neotropical Gracillariidae (Lepidoptera) with links to the information on host plants and parasitoids. Zootaxa 4158, 1–51.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321.

Kawahara, A.Y., Ohshima, I., Kawakita, A., Regier, J.C., Mitter, C., Cummings, M.P., Davis, D.R., Wagner, D.L., De Prins, J., Lopez-Vaamonde, C., 2011. Increased gene sampling strengthens support for higher-level groups within leaf-mining moths and relatives (Lepidoptera: Gracillariidae). BMC Evol. Biol. 11, 182, http://dx.doi.org/10.1186/1471-2148-11-182.

Kawakita, A., Okamoto, T., Goto, R., Kato, M., 2010. Mutualism favours higher host specificity than does antagonism in plant–herbivore interaction. Proc. R. Soc. B 277, 2765–2774.

Kergoat, G.J., Toussaint, E.F.A., Capdevielle-Dulac, C., Clamens, A.L., Ong'amo, G., Conlong, D., Van Den Berg, J., Cugala, D., Pallangyo, B., Mubenga, O., Chipabika, G., Ndemah, R., Sezonlin, M., Bani, G., Molo, R., Ali, A., Calatayud, P.A., Kaiser, L., Silvain, J.F., Le Ru, B., 2015. Integrative taxonomy reveals six new species related to the Mediterranean corn stalk borer *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera, Noctuidae, Sesamiina). Zool. J. Linn. Soc. 175, 244–270.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kirichenko, N., Huemer, P., Deutsch, H., Triberti, P., Rougerie, R., Lopez-Vaamonde, C., 2015. Integrative taxonomy reveals a new species of *Callisto* (Lepidoptera, Gracillariidae) in the Alps. ZooKeys 473, 157–176.

Kumata, T., Kuroko, H., Ermolaev, V.P., 1988. Japanese species of the *Acrocercops*-group (Lepidoptera: Gracillariidae). Part 1. Ins. Matsum. 38, 1–111.

Lees, D.C., Kawahara, A.Y., Rougerie, R., Ohshima, I., Kawakita, A., Bouteleux, O., De Prins, J., Lopez-Vaamonde, C., 2014. DNA barcoding reveals a largely unknown fauna of Gracillariidae leaf-mining moths in the Neotropics. Mol. Ecol. Res. 14, 286–296.

Mc Kay, F., Oleiro, M., Vitorino, M.D., Wheeler, G., 2012. The leafmining *Leurocephala schinusae* (Lepidoptera: Gracillariidae): not suitable for the biological control of *Schinus terebinthifolius* (Sapindales: Anacardiaceae) in continental USA. Biocontrol Sci. Technol. 22, 477–489.

Maita-Maita, J., Huanca-Mamani, W., Vargas, H.A., 2015. First remarks on genetic variation of the little known leaf miner *Angelabella tecomae* Vargas & Parra (Gracillariidae) in the Atacama Desert of northern Chile. J. Lepid. Soc. 69, 192–196.

Mundaca, E.A., Parra, L.E., Vargas, H.A., 2013a. A new genus and species of leaf miner (Lepidoptera, Gracillariidae) for Chile associated to the native tree *Lithraea caustica*. Rev. Bras. Entomol. 57, 157–164.

Mundaca, E.A., Parra, L.E., Vargas, H.A., 2013b. A replacement name for *Hualpenia* Mundaca, Parra & Vargas (Lepidoptera, Gracillariidae). Rev. Bras. Entomol. 57, 353–353.

Rodriguez, F., Oliver, J.L., Marin, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitution. J. Theor. Biol. 142, 485–501.

Rodríguez, R., Matthei, O., Quezada, M., 1983. Flora arbórea de Chile. Universidad de Concepción, Chile.

Schlick-Steiner, B.C., Steiner, F.M., Seifert, B., Stauffer, C., Christian, E., Crozier, R.H., 2010. Integrative taxonomy: a multisource approach to exploring biodiversity. Annu. Rev. Entomol. 55, 421–438.

Vargas, H.A., Landry, B., 2005. A new genus and species of Gracillariidae (Lepidoptera) feeding on flowers of *Acacia macracantha* Willd. (Mimosaceae) in Chile. Acta Entomol. Chil. 29, 47–57.

Vargas, H.A., Parra, L.E., 2005. Un nuevo género y una nueva especie de Oecophyllembiinae (Lepidoptera: Gracillariidae) de Chile. Neotrop. Entomol. 34, 227–233.

Wagner, D.L., Loose, J.L.T.D., Fitzgerald, J.A., Debene, D., Davis, D.R., 2000. A hidden past: the hypermetamorphic development of *Marmara arbutiella* (Lepidoptera: Gracillariidae). Ann. Entomol. Soc. Am. 93, 59–64.