Article

Gyretes Brullé (Coleoptera: Gyrinidae) from Brazil: Morphology of eggs and early instars

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Abstract

Taxonomic information regarding Gyrinidae is mostly based on adults, especially due to the difficulty in collecting immatures and assigning them to a particular species. Association between immatures and adults is sometimes difficult because closely related species can be found in the same habitat. To solve this problem a feasible technique is rearing under laboratory conditions. However, this method is challenging because larval survival rate is usually low, and emulation of natural conditions is difficult. Molecular techniques, especially the use of the COI gene, have been applied to identify species and to associate different life stages. However, in some species groups this marker has not been successful in distinguishing closely related species. The objectives of this study are to describe the egg and the first two instars of *Gyretes nubilus* Ochs, 1965 and the egg of *G. minax* Ochs, 1967 and to evaluate the utility of COI to associate immatures and adults. The association of these immature stages with adults was done either rearing adults under laboratory conditions or by using DNA sequence data (COI), corroborating the utility of this molecular marker to associate immature and adults in *Gyretes*. These immature stages are described, including chaetotaxic analysis of larvae for the first time for the genus *Gyretes* Brullé, 1835. The eggs are described based on scanning electron microscopy. The eggs are similar to those of other Gyrinidae genera in having a micropylar region in the anterior pole and a longitudinal fissure, and by the absence of an aeropyle, but they differ mainly in characters related to chorionic structure and reticulation. Larvae of *Gyretes* can be distinguished from those of the other Neotropical Gyrinidae genera by a combination of several characters, including the frontoclypeal seta FR3 short, presence of three conspicuous additional setae on lateral region of parietal (contiguous to stemmata), and posterior margin of lacinia smooth, with apex not indented.

Key words: Aquatic insect, whirligig beetles, Gyrinidae, *Gyretes*, eggs, larvae, chaetotaxy, DNA barcoding, scanning electron microscopy

Introduction

Among Neotropical aquatic beetles, gyrinids are one of the least known groups, especially the immature stages (Archangelsky *et al.* 2009). Of the total of approximately 300 gyrinid species occurring in South America (Beutel & Roughley 2005), only eight have any of their immatures described. Of these, one species *Andogyrus seriatopunctatus* (Régimbart 1883) has all immature stages (egg, larvae and pupa) described (Bachmann 1961, 1966; Archangelsky & Michat 2007) whereas the other species only have larval instars described. Three of these larval descriptions include chaetotaxy information: *Andogyrus buqueti* (Aubé 1838) (Arndt *et al.* 1993), *Gyrinus monrosi* Mouchamps, 1957 (Michat *et al.* 2010) and *Enhydrus sulcatus* Wiedemann, 1821 (Michat *et al.* 2016). The remaining descriptions have emphasized general morphological characters [*Gyrinus gibbus* Aubé, 1838 and *Gyretes* sp. (Costa *et al.* 1988), *Gyrinus argentinus* Steinheil, 1869 (Crespo 1989a) and *Gyrinus ovatus* Aubé, 1838 (Crespo 1989b)].

Identification of immature stages of Gyrinidae at the species level is somewhat difficult due to the scarcity of

available information. Currently, identification is only possible by associating immatures with their identified adults, but sometimes this is difficult because closely related species can be found in the same habitat, and it is not possible to assign them to a particular species. Although association by rearing under laboratory conditions is a feasible and good practice, replication of natural conditions in the case of gyrinids is difficult and usually results in a low rate of larval survival.

An alternative way to succeed in this task is the use of molecular methods to associate immatures and adults. Molecular tools have been increasingly used in taxonomic studies since DNA barcoding started to be used with more than 95% resolution at the species level (e.g. Hajibabaei *et al.* 2007; Hebert *et al.* 2003, 2004) and in the association of immatures with adults (e.g. Curiel & Morrone 2012; Madaric *et al.* 2013; Miller *et al.* 2005, 2007; Zhou *et al.* 2007). However, some studies (Elias *et al.* 2007; Meier *et al.* 2006; Meyer & Paulay 2005; Whitworth *et al.* 2007; Wiemers & Fiedler 2007) indicate that, in some groups, this marker cannot distinguish closely related species, resulting in low rates of success for species identification.

The objectives of this study are to describe the egg and the first two larval instars of *Gyretes nubilus* Ochs, 1965, the egg of *G. minax* Ochs, 1967 and to evaluate the utility of DNA barcoding for associating immatures and adults of *Gyretes* species.

Material and methods

Samples were collected in two streams, one in Reserva Florestal Ducke, Manaus county (02°55'46.7"S, 59°58'22.0"W) and the other in Iranduba county (03°04'37.7"S, 60°46'14.9"W), both in Amazonas state, Brazil, from June 2012 to November 2013.

Eggs of both species were collected directly from the substrate. Substrates included branches, roots and leaves of submerged macrophytes, e.g. *Thurnia sphaerocephala*. First and second larval instars and adults of *G. nubilus* were also collected directly from the substrate (roots, leaves and sand) using a D-net. The specimens were preserved in absolute ethanol and stored at -20ºC.

Adults were identified based on original descriptions (Ochs 1965, 1967), and compared with type material deposited in the Invertebrate Collection of Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil. Vouchers of the species analyzed in this study are deposited in the above-mentioned collection.

Adult males and females of *G. nubilus* and *G. minax* were collected in the streams and were maintained alive for 30 days. Each species was maintained in separate aquarium. Adults were kept in the aquarium to allow copulation to obtain subsequent immatures. For *G. nubilus*, eggs and first-instar larvae were obtained using this method, while for *G. minax* only eggs were obtained. Immatures associated with identified adults obtained through rearing procedures were molecularly analyzed with adults collected in the field in order to evaluate whether the COI marker can successfully be used to the identify immatures through DNA sequences and, therefore, to associate the immatures and adults of both of the species analyzed.

Molecular analysis. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen®) following the manufacturer's guidelines, except for the volume of the elution buffer which was reduced to 70 µL.

Amplifications of DNA extracted from *G. nubilus* (five eggs, one second instar larva and one adult) and *G. minax* (10 eggs and two adults) were performed using specific primers for the mitochondrial COI gene developed by Folmer *et al.* (1994). Amplification conditions were 94°C (3 min); 94°C (1 min), 45°C (1 min 30 sec) and 72°C (1 min 30 sec) for 36 cycles, and 72°C (5 min). Fragments produced by PCR were examined using gel electrophoresis. The amplified samples were purified by the Exo-SAP (Exonuclease I-Shrimp Alkaline Phosphatase) enzymatic method and sent to the Centro de Estudos do Genoma Humano (Universidade de São Paulo-USP), where they were sequenced using an ABI 3730 DNA Analyzer.

The sequences were aligned using the Clustal W program (Thompson *et al.* 1994) implemented in Bioedit v.7.0.8 software (Hall 1999), where they were manually edited. GenBank sequences of *Gyretes iricolor* Young, 1947 (JX478065), *Gyrinus opacu*s Cooper, 1930 (KC016847) and *Gyrinus* sp. (JX478068) were also included in the analysis, the last two species being outgroups.

The phylogram obtained using the Neighbor-Joining method and the estimate of the pairwise distance between the 10 sequences of the COI fragment included in the analyses were obtained using MEGA6 software (Tamura *et al.* 2013). In both analyses, 1000 bootstrap replicates were used in the Kimura 2-parameter (K2P) model (Kimura 1980). The COI sequences are deposited in GenBank (accession numbers: MG279352-MG279358).

COLPANI *ET AL.*

Eggs description. Eggs were analyzed under an optical microscope; measurements were performed on glass slides in gel alcohol using an Olympus DP72 camera and the Cell D program**.**

Scanning electron microscopy (SEM) of eggs. Eggs were dehydrated in an ethanol series before the critical point and dried in CO_2 for three hours. After dehydration, the eggs were mounted on double-sided adhesive tape under 32-mm stubs and labeled. Then the samples were plated with a layer of gold and photographed using a LEO 435 scanning electron microscope (LEO Electron Microscopy Ltd.) at the Laboratório Temático de Microscopia Eletrônica e Óptica at INPA.

Larval description. Larvae were macerated in warm lactic acid (85%) for 30 minutes before dissection. They were then washed in distilled water, placed in 80% alcohol and mounted on glass slides using Hoyer's as mounting medium. Observation (at magnifications up to 1000x) were made using Leica DMLB and Olympus CX31 composite microscopes.

Measurements and images of the larvae were obtained using a Leica DFC420 camera coupled to a Leica M165C stereomicroscope using a LED illumination dome (Kawada & Buffington 2016), and the Leica Application Suite V3 Digital Image Processing program. To describe and illustrate structures, larvae were photographed using an Olympus BX51 optical microscope coupled to an Olympus DP72 photographic camera, with processing using the Cell D program. Drawings were made from photographs and edited using Adobe Illustrator CS6 software, free trial version, and photographs were edited with Adobe Photoshop CS6, free trial version. Terminology used in the larval description follows Archangelsky & Michat (2007) and Michat *et al.* (2010, 2016, 2017).

Morphometric analysis. The following measurements were taken: total length (excluding terminal tracheal gills) (TL); maximum width (excluding tracheal gills) (MW); head length (HL) (total head length including frontoclypeus, measured medially along the epicranial stem); maximum head width (HW); length of frontoclypeus (from anterior margin to the joint of frontal and coronal sutures) (FRL); coronal suture length (COL); length of mandible (MNL) (measured from laterobasal angle to apex); width of mandible (MNW) (maximum width measured at base); length of maxillary palpifer (PPF); length of galea (GA). Length of antenna (A), maxillary (MP) and labial (LP) palpi were derived by adding the lengths of individual segments (for example, LP1, first segment of the labial palpus). The maxillary palpus was considered as being composed of three segments and a palpifer (Michat and Gustafson 2016). Length of leg, including the longest claw (CL), was derived by adding the lengths of the individual segments. Lengths of terminal hooks of abdominal segment X were separated into medial hook (MH) and lateral hook (LH).

Chaetotaxy analysis. The chaetotaxy analysis (setae and pores) in first- and second-instar larvae of *Gyretes* was based on comparison with previous studies of several genera of Gyrinidae (Archangelsky & Michat 2007, Michat *et al.* 2010, 2016, 2017; Michat & Gustafson 2016), and other families of the suborder Adephaga (e.g. Bousquet & Goulet 1984). The setae and pores were coded by two capital letters, in most cases corresponding to the first two letters of the name of the structure on which they are located, followed by a number (setae) or a lower case letter (pores).

Results

Eggs, first- and second-instar larvae of *G. nubilus* and eggs of *G. minax* were obtained from the laboratory-rearing procedures and therefore could be directly identified to species. Molecular analysis of these identified immatures obtained by the rearing procedure and of the adults collected in the field allowed their association with adults of the corresponding species.

In the philogram (Fig. 1), the adult, egg and larva of *G. nubilus* formed a strongly supported clade, whereas the adults and eggs of *G. minax* were grouped in another robustly supported clade. The mean interspecific variation observed for *Gyretes* was 15.6% (14.5–15.9%), similar to other genera of Gyrinidae (*Gyrinus* and *Aulonogyrus*) where an interspecific distance of 14% was found (Xi *et al.* 2008).

The mean genetic distance between *Gyretes* and *Gyrinus* was 17% (14,0–18.0%) (Table 1). Miller *et al.* (2005, 2007) reported a mean distance of 13% for different genera of Dytiscidae. The egg, larva and adult of *G. nubilus* showed no genetic variation between the sequences, differing from *G. minax,* in which a genetic variation of 0.15% was observed only for egg and adult (Table 1).

TABLE 1. Pairwise genetic distance (%) between specimens of *Gyretes nubilus* and *Gyretes minax* (in bold) and interspecific distance between species of *Gyrinus* and *Gyretes*, estimated on the basis of a fragment of the mitochondrial gene cytochrome oxidase subunit I (COI), using the K2P model. Note: Egg (Eg), larva (Lv) and adult (Ad). Code: Genbank accession numbers.

	Species	Code	1	2	3	4	5	6	7	8	9
1.	*Gyretes minax* Eg	MG279352									
2.	*Gyretes minax* Eg2	MG279353	0.0%								
3.	*Gyretes minax* Ad	MG279354	0.15%	0.15%							
4.	*Gyretes minax* Ad2	MG279355	0.15%	0.15%	0.0%						
5.	*Gyretes nubilus* Eg	MG279356	14.7%	14.7%	14.5%	14.5%					
6.	*Gyretes nubilus* Ad	MG279357	14.7%	14.7%	14.5%	14.5%	0.0%				
7.	*Gyretes nubilus* Lv	MG279358	14.7%	14.7%	14.5%	14.5%	0.00%	0.00%			
8.	*Gyretes iricolor*	JX478065	15.9%	15.9%	15.6%	15.6%	15.9%	15.9%	15.9%		
9.	*Gyrinus* sp.	JX478068	16.9%	16.9%	16.7%	16.7%	17.2%	17.2%	17.2%	16.2%	
10.	*Gyrinus opacus*	KC016847	17.6%	17.6%	17.4%	17.4%	17.6%	17.6%	17.6%	16.2%	11.9%

Moreover, the intraspecific genetic distances between stages for both analyzed species are within the limits proposed for considering them to be conspecific (Zhou *et al.* 2007). These values are also in line with other studies, where intraspecific genetic distances ranged from 0.04% to 0.82% (Čiampor & Ribera 2006; Čiamporova-Zaťovičová *et al.* 2007; Curiel & Morrone 2012; Miller *et al.* 2005, 2007). The average nucleotide composition of the COI sequences for *Gyretes* was T = 38.9%, A = 30.0%, C = 15.9% and G = 15.2%. These values are close to those obtained for other gyrinids (Xi *et al.* 2008) and for other groups of insects (Simon *et al.* 1994). Our study provides the first data on the genetic distance pattern for *Gyretes*. These values are similar to those obtained in the study of other groups of insects (Hebert 2004).
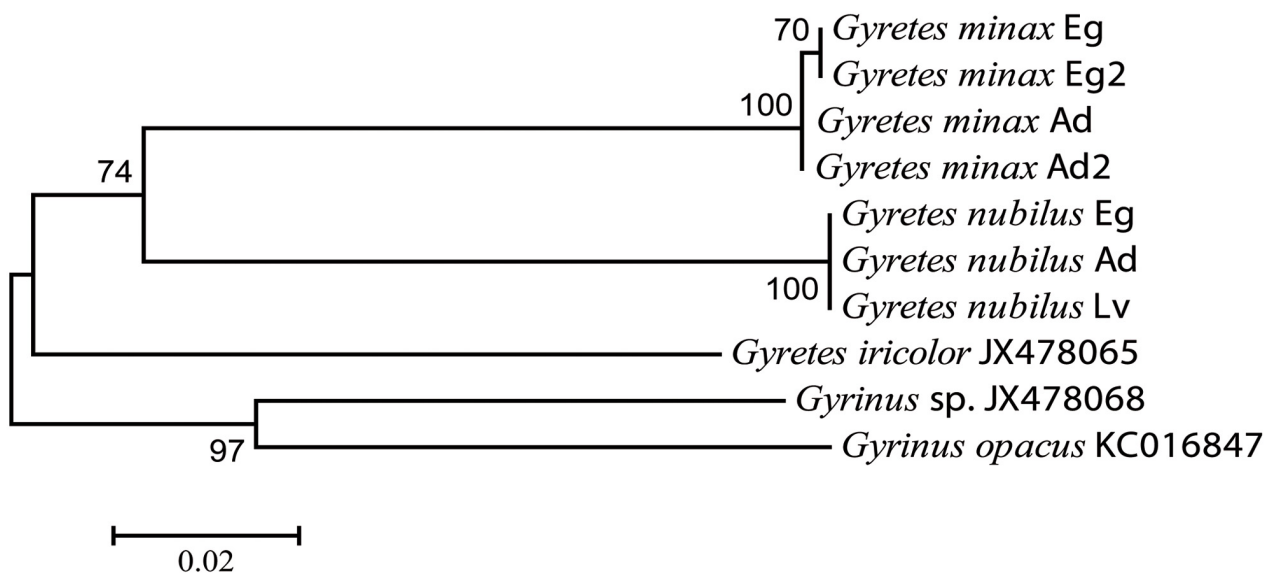
FIGURE. 1. Neighbor-Joining tree using partial COI sequences of eggs, larvae and adults of species of *Gyretes*, and adults of *Gyrinus*. Species from GenBank: *Gyretes iricolor* (JX4780651), *Gyrinus* sp. (JX4780681) (Miller & Bergsten 2012) and *Gyrinus opacus* (KC0168471) (unpublished data), the latter two being included as outgroups. Numbers above branches indicate bootstrap support values (> 50%). Note: Egg (Eg), larva (Lv) and adult (Ad). Scale bar = nucleotide substitutions per site.

COLPANI *ET AL.*

Description of eggs

Gyretes nubilus **Ochs, 1965**
(Figs. 2–5)

Description. Eggs. Cylindrical, length 1.12–1.69 mm, width 0.28–0.48 mm (n = 10); dark brown (just before eclosion) (Fig. 2). As the embryo develops, the egg becomes wider from the anterior pole toward the posterior pole. The chorion is thick, sculptured in most of the egg, except for the ventral surface, which is flat and smooth (Figs. 2–5). The chorion consists of rounded or quadrangular reticulation with irregular borders (Fig. 4). The micropylar projection is inserted into a depression in the center of the anterior pole and is connected to the depression by four branches (Figs. 3, 5).

Gyretes minax **Ochs, 1967**
(Figs. 6–9)

Description. Eggs. Cylindrical, length 1.16–1.18 mm, width 0.29–0.31 mm (n = 10); dark brown (just before eclosion) (Fig. 6). The chorion is sculptured, except for the ventral surface, which is smooth and flat. The chorion consists of reticulation with variable shape (triangular, rounded, cylindrical, rectangular); the edges of these cells are smooth, without layers (Figs. 6–9). The micropylar projection is inserted into the anterior pole, is stem shaped and has a round apex (Figs. 7, 9).

Comparative notes. Generally, Gyrinidae eggs have the chorion very strongly sculptured, usually ribbed or sculptured in honeycomb format (Hinton 1981).

The general structure of the eggs of both species of *Gyretes* agrees with previous descriptions for the family (Baker & Wai 1987; Komatsu & Kobayashi 2012; Saxod 1964) such as length, width, shape, color, presence of sculpture on the chorion, longitudinal splitting-slit and micropylar projection. Newly laid eggs are white, long ellipsoid, and loosely fixed to the substrate by a mucous-like substance.

The eggs of the two species analyzed have morphological differences. Although both species have a sculptured chorion, it is of different shapes. In *G. nubilus* the reticulation of the chorion is rounded or quadrangular with uneven edges along its height and, in some of them, the lower portion of the edge covers part of the cell bottom, whereas in *G. minax* the reticulation is more variable and its edges are smooth, without layers. Both species also differ in the apex of the micropyle, which is pointed in *G. nubilus* and rounded in *G. minax*.

There are only three descriptions of whirligig beetle eggs in the literature based on scanning and transmission electron microscopy: *Gyrinus substriatus* Stephens, 1829 (Saxod 1964), *Dineutus mellyi* Régimbart, 1883 (Komatsu & Kobayashi 2012) and *Dineutus hornii* Roberts, 1895 (Baker & Wai 1987). Comparing the morphology of the eggs of *Gyretes* and those of other genera, several differences are found. For example, *Dineutus* eggs are much larger, reaching 1.5–2.0 mm; also, chorionic sculpture is well differentiated in *D. hornii* and *D. mellyi*, formed by conical projections that can be either little or well developed, differing from *Gyretes* and *Gyrinus*, in which the sculpture of the chorion forms cells that are similar to a honeycomb (Hinton 1981). The sculpture of the chorion of *G. substriatus* differs from those of *G. minax* and *G. nubilus* in having hexagonal-shaped cells (Sadox 1964). Other features, such as the presence of a micropylar region in the anterior pole and presence of a longitudinal fissure, are common to all gyrinid genera. As noted in the literature, gyrinid eggs do not have aeropyle. According to Hinton (1981), these openings are commonly found in eggs of aquatic insects and are probably related to respiration, facilitating the exchange of gases between the egg and the external environment.

Description of larvae

Gyretes nubilus **Ochs, 1965**
(Figs. 10–24)

Diagnosis. Larvae of *Gyretes* can be distinguished from those of other described Gyrinidae genera by the following

combination of characteristics: cephalic capsule not constricted at the level of the occipital region (slightly constricted in instar II) (Figs. 12–13, 24); occipital suture absent (Figs. 12, 24); anterior margin of frontoclypeus lacking teeth (Figs. 12, 24); seta FR3 short, hair-like (Fig. 12); parietal with three additional setae on lateral region, contiguous to stemmata (Fig. 12); lacinia not serrate on posterior margin, not indented apically (Figs. 16–17); last maxillary palpomere in dorsal view with six pore-like additional structures near apex (Fig. 16), in ventral view with one additional seta on distal portion (Fig. 17); pore MXg proximal (Fig. 17); cardo and stipes each with one additional seta on ventral region (Fig. 17); maxillary and labial palpomeres without additional pores (Figs. 16–19); pore LAc located medially (Fig. 18); mandible with six spines on inner margin and several additional setae on dorsal surface (Fig. 20); seta CO12 proximal (Fig. 22); coxa lacking additional setae (Figs. 21–22); setae FE2, FE3 and TI3 bifid (Fig. 21); seta TR2 present (Fig. 21); claws lacking basoventral spinulae (Figs. 21–22); abdominal segment X lacking ventral spinulae (Fig. 23); tracheal gills bearing long spinulae.

Description. *First instar larva.* **Color** (Figs. 10–11): Cephalic capsule with an irregular brown spot that extends from basal region of coronal suture to of frontal sutures, and one brown macula on each laterobasal angle. Antenna, maxilla, and labium white to light brown. Thoracic sclerites light brown, meso- and metathorax whitish with light brown spots arranged at random. Legs and abdominal segments testaceous, terminal hooks brown.

Body. (Fig. 10): Elongate, head and pronotum strongly sclerotized, rest of body weakly sclerotized. Measurements and ratios are presented in Table 2.

Head. *Cephalic capsule* (Figs. 10–13): Subrectangular, 1.7 times longer than pronotum, parallel sided, coronal suture short; frontal sutures V-shaped, extending to antennal bases (Fig.12). Frontoclypeus subtriangular, with anterior margin straight (without teeth) and anterior corners rounded (Fig. 12). Stemmata present, forming a cluster where each stemma could not be individualized (Fig. 11). Egg bursters present, formed by a single cuticular spine on each posterolateral surface (Fig. 12).

Antenna (Figs.14–15): Long, slender, composed of four articles, first antennomere shortest, second and third antennomeres 3.0 times more elongated in relation the first, fourth antennomere longest in relation to the other antennomeres, narrowed at base and at apex. Third antennomere with two minute spinulae on ventrodistal surface, and two subapical flat plates on inner margin. Fourth antennomere with a subapical flat sensorial plate on distal inner margin, accompanied by one spinula.

Maxilla (Figs. 16–17): Long, slender, cardo subrectangular, stipes subglobular, broad, about as long as cardo. Lacinia slender, slightly curved, apically tapered. Galea with two articles, first galeomere short, globose, second galeomere longer, narrowing to apex. Palpifer short, subrectangular, maxillary palpus composed of three articles, first palpomere 3.5 times longer than palpifer, second palpomere shorter than third palpomere (0.07–0.08 mm), third palpomere longest, curved and slightly widened.

Labium (Figs. 18–19): Well developed, prementum longitudinally subdivided in two halves, first palpomere 1.4 times longer than second palpomere, second palpomere curved, narrow at base and widened towards the apex.

Mandible (Fig. 20): Mandibles of both sides symmetrical, elongate, robust, curved, broadest in basal region, apex sharp, inner margin with six small spines on central portion, mandibular channel present.

Thorax (Fig. 10): Long, narrow, pronotum with about same width as head, mesonotum and metanotum subequal in length. Protergite well developed, membrane between pronotum and mesonotum with single narrow transverse sclerite, both sclerites with sagittal line; spiracles absent.

Legs (Figs. 21–22): Long, slender, longest coxa 1.4 times longer than trocanther, trocanther shortest, profemur, protibia and protarsus slender, profemur (1.5 times) longer than protibia, and protibia 1.4 times longer than protarsus, preprotarsus with two slightly curved claws subequal in length.

Abdomen (Fig. 10): Long, narrow, with 10 segments, subcylindrical, segments I–V of similar width, segments VI–X progressively narrowing towards the apex. Segments I-VIII with a pair of lateral tracheal gills at posterior angles, segment IX narrower, with two pairs of tracheal gills at posterior angles; segment X narrowest, with four strongly sclerotized terminal hooks (Figs. 10, 23).

Chaetotaxy. *Frontoclypeus* (Fig. 12): Medial region of anterior margin with two spine-like setae (FR10, FR11). Anterior (submarginal) region with three short hair-like setae (FR5, FR7, FR8), two minute setae (FR6, FR9) and one pore (FRe). Central portion with one short hair-like seta (FR4). Lateral margin with two short hair-like setae (FR1, FR2) and two pores (FRa, FRc) on distal third, and one hair-like seta (FR3) at base.

Parietal (Figs. 12–13): Dorsal surface (Fig. 12) with one short hair-like seta (PA10) near antennal base, three conspicuous additional setae external to stemmata, four short and two elongate setae (PA4, PA5, PA6, PA7, PA8,

PA9) posterior to stemmata, and three short spine-like setae (PA1, PA2, PA3) and two pores (PAa, PAb) near posterior margin. Ventral surface (Fig. 13) with two short hair-like setae (PA18, PA19) and one pore (PAo) near anterior margin; two short hair-like setae (PA11, PA12) and four pores (PAf, PAg, PAh, PAi) on anterolateral angle; two short hair-like setae (PA16, PA17) on central portion, two short hair-like setae (PA13, PA14) and one pore (PAk) near lateral margin at about mid length, and one relatively elongate hair-like seta (PA15) and one pore (PAm) on basal third. Some inconspicuous additional setae present on dorsal and to a lesser extent ventral surface.

Antenna (Figs. 14–15): First antennomere with three pores (ANa, ANb, ANc) on dorsodistal portion and two pores (ANd, ANe) on ventrodistal portion. Second antennomere with one pore (ANi) on ventromedial portion. Third antennomere with one pore (ANf) on dorsomedial region and two short hair-like setae on distal portion. Fourth antennomere with two short spine-like setae (AN4, AN5) and one pore (ANg) at apex.

Maxilla (Figs. 16–17): Cardo with one short hair-like seta (MX1) and one short additional seta on ventral surface. Stipes with one hair-like seta (MX3) on laterodorsal region, one short hair-like seta (MX2), one very short hair-like seta (MX4), one short additional seta and two pores (MXa, MXb) on ventral surface, and two spine-like setae (MX5, MX6) at base of lacinia. First galeomere with one short hair-like seta (MX7) on ventral margin. Second galeomere with two pores (MXd, MXh) on ventral surface, one spine-like seta (MX8) on dorsal surface, and one short hair-like seta (MX9) at apex. Palpifer with one short hair-like seta (MX10) on ventral surface. First palpomere with one pore (MXf) on dorsodistal portion and one minute seta (MX13) and one pore (MXe) on ventroproximal portion. Second palpomere with one short hair-like seta (MX11) on dorsodistal portion, one short hair-like seta (MX12) on ventrodistal portion, and one pore (MXg) on ventroproximal portion. Third palpomere with one pore (MXj) on dorsomedial portion, one short hair-like seta (MX14) and one short additional seta on ventrodistal portion, and a longitudinal row of four minute pore-like additional structures on dorsodistal portion.

Labium (Figs. 18–19): Prementum with three short hair-like setae (LA3, LA4, LA5) and one pore (LAa) near anterior margin, one minute spine-like seta (LA9) on ventrodistal surface, one short hair-like seta (LA2) on ventrodistal portion, and one minute seta (LA1) on ventroproximal surface. First palpomere with one pore (LAb) on ventroproximal portion. Second palpomere with one short hair-like setae (LA12) ventrally close to apex and one pore (LAc) on dorsomedial portion.

Mandible (Fig. 20): Dorsal surface with one pore (MNa) on proximal portion, two hair-like setae (MN1, MN2) and two pores (MNb, MNc) on distal portion, and numerous minute additional setae.

Legs (Figs. 21–22): Anterior surface of coxa with six short spine-like setae (CO1, CO2, CO3, CO4, CO5, CO17) and one short hair-like seta (CO18) on proximal portion, one short hair-like seta (CO7), three relatively more elongate hair-like setae (CO6, CO8, CO9) and one pore (COa) on central portion, and one short hair-like seta (CO10) on distal portion. Posterior surface of coxa with four short spine-like setae (CO13, CO14, CO15, CO16) on proximal portion, one short hair-like seta (CO12) and one pore (COd) on central portion, and one short spine-like seta (CO11) on distal portion. Anterior surface of trochanter with two short hair-like setae (TR1, TR2), two relatively more elongate hair-like setae (TR3, TR4), and five pores (TRa, TRb, TRc, TRd, TRe). Posterior surface of trochanter with three short hair-like setae (TR5, TR6, TR7) and two pores (TRf, TRg). Anterior surface of profemur with one hair-like seta (FE1) and one pore (FEb) on proximal portion, and two short spine-like setae (FE2, FE3) on distal portion. Posterior surface of profemur with one short spine-like seta (FE4) on central portion and two short spine-like setae (FE5, FE6) on distal portion. Anterior surface of protibia with one long hair-like seta (TI1) on proximal portion and three short spine-like setae (TI2, TI3, TI4) on distal portion. Posterior surface of protibia with one pore (TIa) on central portion and one long hair-like seta (TI7) and two short spine-like setae (TI5, TI6) on distal portion. Anterior surface of protarsus with one pore (TAa) on central portion, and three short spine-like setae (TA2, TA3, TA4), one minute seta (TA7) and two pores (TAc, TAd) on distal portion. Posterior surface of protarsus with one short spine-like seta (TA1) on central portion, and two short spine-like setae (TA5, TA6) and three pores (TAb, TAe, TAf) on distal portion. Anterior surface of preprotarsus with one relatively elongate spine-like seta (PT1) on basoventral portion. Posterior surface of preprotarsus with one short spine-like seta (PT2) on basoventral portion.

Abdomen (Fig. 23): Segments I–IX with short hair-like setae on dorsal and ventral surfaces. Segment X: dorsal surface with two short spine-like setae; ventral surface with four setae; lateromedial portion with two pores; laterodistal portion with one seta and one pore. Terminal hooks with two pores on ventral margin at about mid-length (Fig. 23).
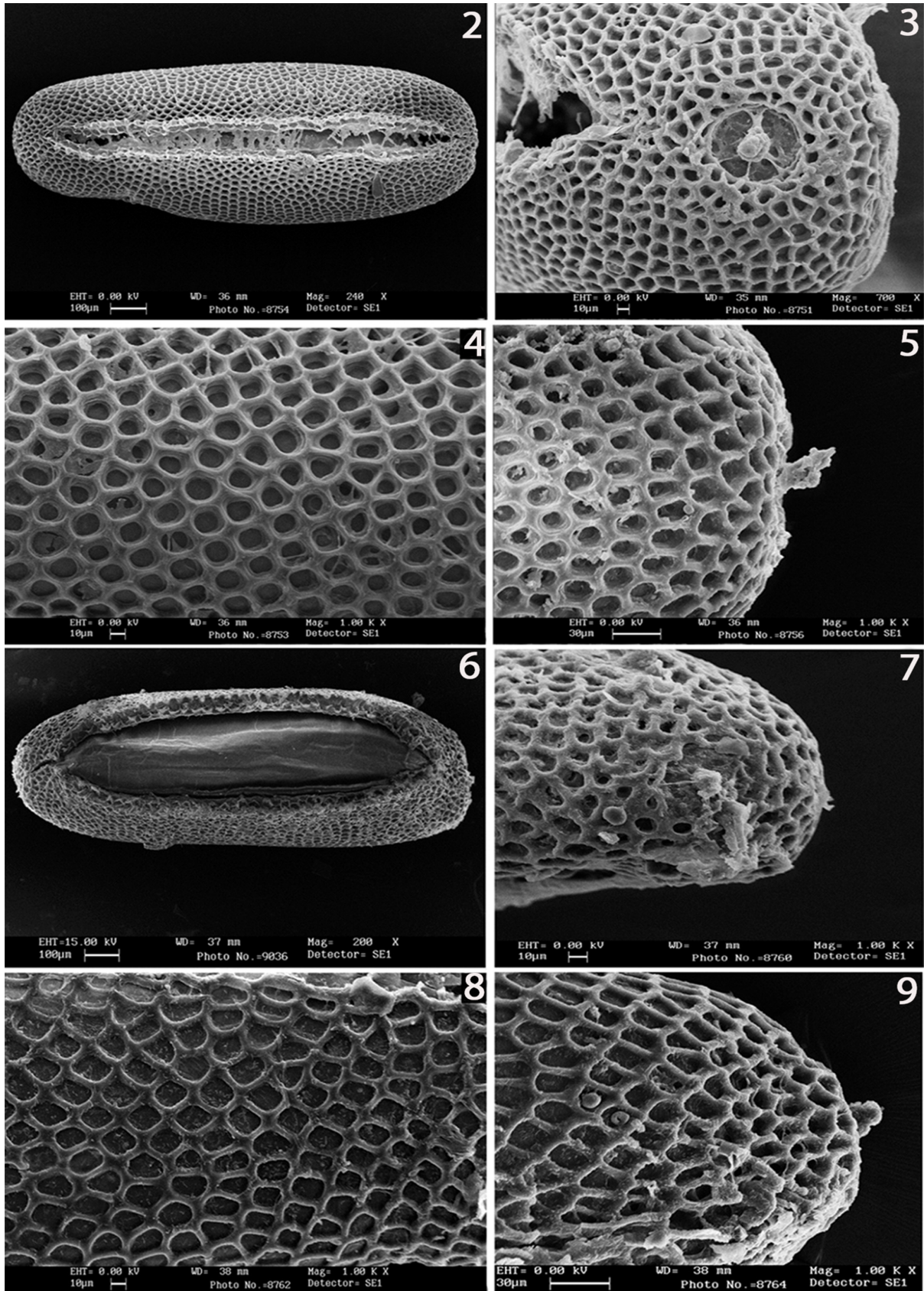
FIGURES. 2–9. Eggs of *Gyretes* analyzed under scanning electron microscopy. *Gyretes nubilus*: (2) Dorsal view, (3) Micropyle (anterior pole), (4) Lateral region amplified showing chorion details, (5) Lateral view of micropyle. *Gyretes minax*: (6) Dorsal view, (7) Micropyle (anterior pole), (8) Lateral region amplified showing chorion details, (9) lateral view of micropyle.
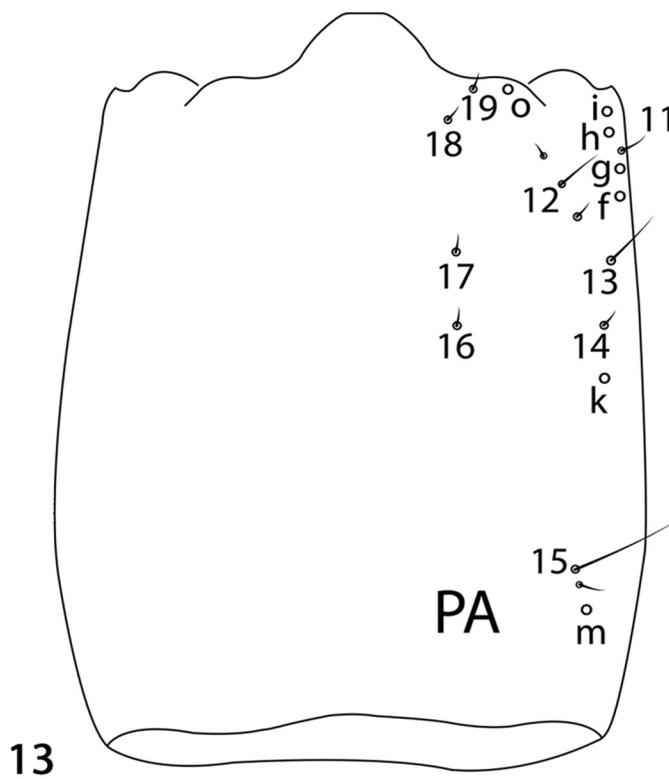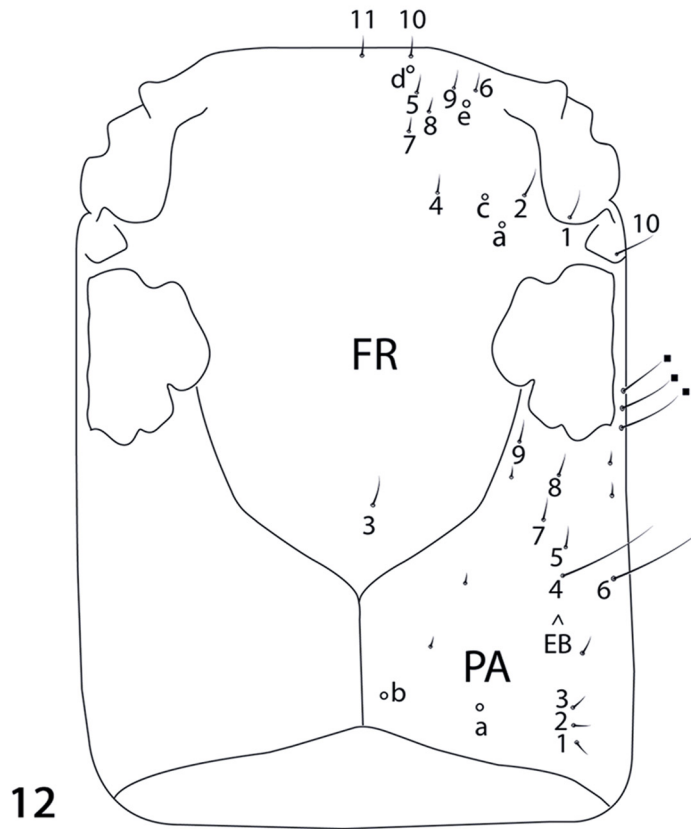
FIGURES. 10–11. *Gyretes nubilus*, first-instar larva. (10) Habitus, dorsal view, (11) Head and pronotum, dorsal view, Scale bars 1.0 mm (Figure 10), 0.2 mm (Figure 11).

Description. *Second-instar larva.* Similar to first-instar larva except for the following features: **Color** (Fig. 11): Cephalic capsule and pronotum yellowish with light brown pigmentation, abdominal segments darker, meso- and metanotum with light brown maculae.

Body (Fig. 24): Measurements and ratios in Table 2.

**Head** (Fig. 24): Stemmata well differentiated, four dorsal and two ventral.

Chaetotaxy. Cephalic capsule with numerous secondary setae, which can be differentiated from the other setae by their shorter length and slender shape. Second antenomere with four small cuticular spines randomly distributed. Cardo and stipes with short secondary spine-like setae. Palpifer with cuticular spines covering all its extension. Mandible with secondary setae on dorsal surface.

FIGURES. 12–13. *Gyretes nubilus*, first-instar larva, cephalic capsule. (12) Dorsal view. (13) Ventral view. Numbers and lowercase letters indicate primary setae and pores. Note: frontoclypeus (FR), parietal (PA) and egg bursters (EB). Scale bar 0.5 mm. black square = Conspicuous additional setae. Inconspicuous additional setae not labeled.
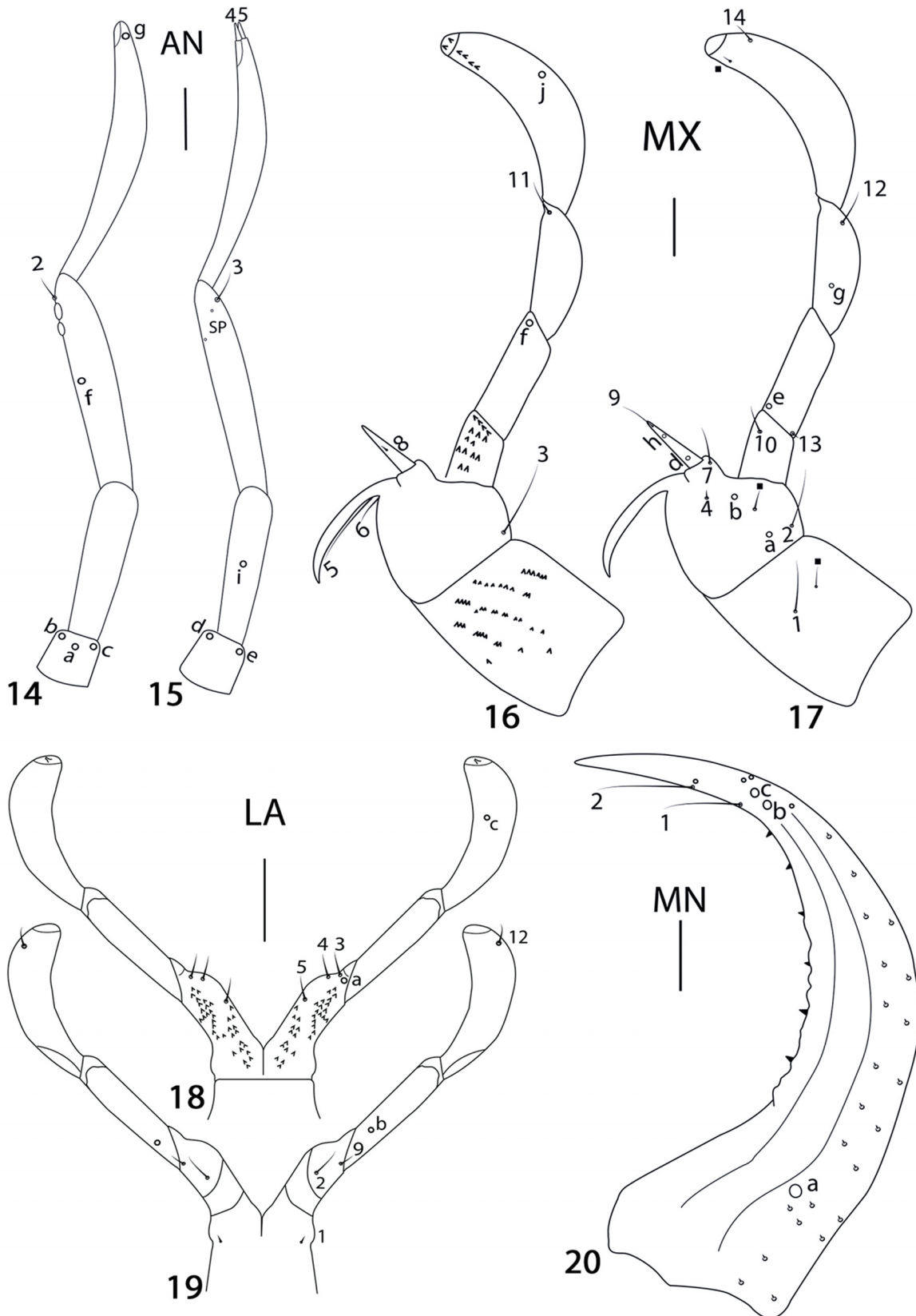
FIGURES. 14–20. *Gyretes nubilus*, first-instar larva. (14). Right antenna, dorsal view. (15). Left antenna, ventral view. (16) Right maxilla, dorsal view. (17) Left maxilla, ventral view. (18) Labium, dorsal view (19) Labium, ventral view. (20). Right mandible, dorsal view. Numbers and lowercase letters indicate primary setae and pores. Note: antenna (AN), labium (LA), mandible (MN), maxilla (MX). Scale bars 0.1 mm (Figs. 16, 17, 20), 0,3 mm (Figs. 14, 15, 18, 19). Black square = Additional setae (except for minute additional setae on mandible which are not labelled).
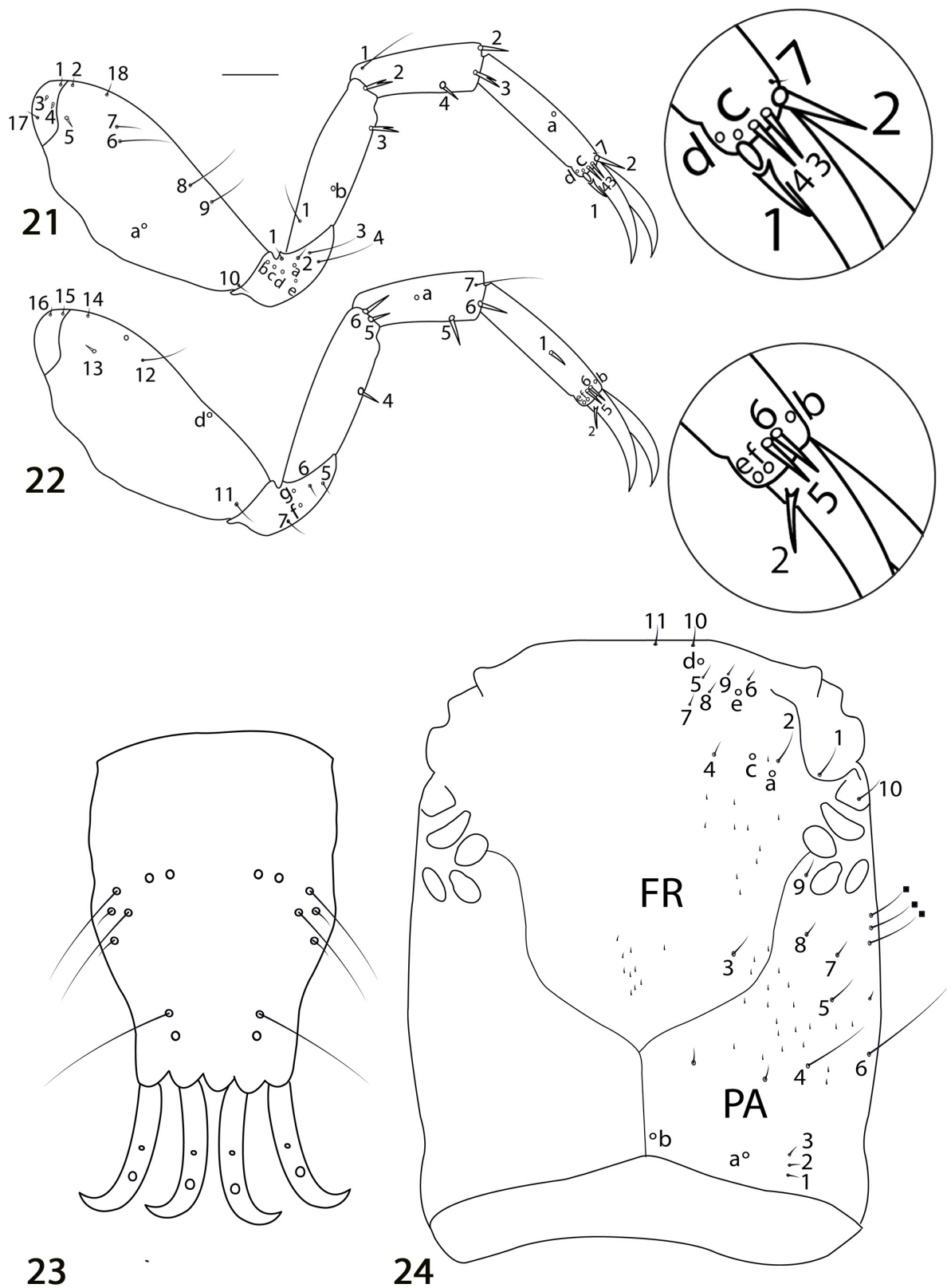
FIGURES. 21–24. *Gyretes nubilus*. 21–23. First-instar larva. (21) Left metathoracic leg, anterior view. (22) Right metathoracic leg, posterior view. (23) Abdominal segment X, ventral view. (24) Second-instar larva, cephalic capsule, dorsal view. Numbers and lowercase letters indicate primary setae and pores. Note: frontoclypeus (FR), parietal (PA). Scale bars 0,3 mm (Figure. 23), 0,5 mm (Figures. 21, 22, 24). Black square = Additional setae.

TABLE 2. Measurements and ratios of larval structures of the first (I) and second (II) instars of *Gyretes nubilus*. See Material and methods for abbreviations.

Measure (mm)	Instar		Measure (mm)	Instar	
	I (n= 43)	II (n= 4)		I (n= 43)	II (n= 4)
TL	1.57–4.58	6.35–8.25	GA/MP1	0.15–0.54	0.52–0.57
MW	0.17–0.42	0.75–0.98	PPF/MP1	0.15–0.34	0.41–0.42
HL	0.30–0.56	0.57–0.74	MP1/MP2	1.50–2.16	1.41–1.46
HW	0.28–0.40	0.41–0.63	MP3/MP2	0.80–0.84	1.00–1.15
FRL	0.15–0.39	0.42–0.50	MP/LP	0.94–1.09	1.04–1.10
COL	0.10–0.13	0.15–0.17	LP2/LP1	0.63–0.85	0.80–0.87
HL/HW	1.07–1.40	1.17–1.39	L3 (mm)	0.40–0.65	1.17–1.48
COL/HL	0.23–0.33	0.22–0.26	L3/L1	0.59–0.85	0.76–0.80
FRL/HL	0.50–0.69	0.67–0.84	L3/L2	0.17–0.29	3.28–3.90
A/HW	0.30–0.61	0.41–0.63	L3/HW	1.42–1.62	2.34–2.85
A1/A3	0.18–0.40	0.25–0.33	L3 (CO/FE)	1.25–1.36	0.71–0.81
A2/A3	0.81–1.20	1.65–1.66	L3 (TI/FE)	1.0–1.18	2.12–2.14
A4/A3	1.25–2.00	1.00–1.08	L3 (TA/FE)	0.87–1.00	1.07–1.25
MNL/MNW	3.25–3.33	3.20–4.00	L3 (CL/TA)	0.57–1.00	0.65–0.66
MNL/HL	0.44–0.54	0.56–0.70	MH/LH	0.90–1.00	1.00–1.10
A/MP	0.95–1.57	1.17–1.25			

Discussion

Few studies of gyrinid larvae are available in the literature. Regarding *Gyretes*, there is only a brief description of an unidentified species (Costa *et al.* 1988), including general morphological characters of the head, mouthparts, thorax and abdomen. The genus was also included in a key to larvae of Nearctic Gyrinidae (Leech & Chandler 1956).

Recent taxonomic studies of larvae of aquatic beetles have included analyses of the chaetotaxy in the descriptions, since the inclusion of this character set is important for the distinction between larval instars and the discovery of new characters for the separation of species (e.g. Archangelsky & Michat 2007; Michat *et al.* 2010, 2016, 2017; Michat & Gustafson 2016). Our study represents the first detailed treatment of larvae of *Gyretes*, including chaetotaxic analysis.

Larvae of *Gyretes* share with those of other genera of the tribe Orectochilini (*Orectochilus* Dejean, 1833, *Orectogyrus* Régimbart, 1884, *Patrus* Aubé, 1838) the absence of teeth on the anterior margin of frontoclypeus (see also Costa *et al.* 1988; Leech & Chandler 1956). This character has been previously proposed as a synapomorphy of the Orectochilini by Beutel & Roughley (1994). Unfortunately, chaetotaxic characters are difficult to evaluate within this tribe because descriptions of larvae of *Orectochilus*, *Orectogyrus* and *Patrus* Aubé, 1838 are either non-existent or have not emphasized this character system.

Five gyrinid genera are currently recognized in the Neotropics: *Gyretes*, *Gyrinus* Geoffroy, 1762, *Dineutus* Macleay, 1825, *Enhydrus* Laporte, 1834 and *Macrogyrus* Régimbart, 1883 (Gustafson & Miller 2017; Miller & Bergsten 2012). Larvae of *Gyretes* can be distinguished from those of the other Neotropical genera by several characters. The cephalic capsule of *Gyretes* is not constricted at the level of the occipital region (Costa *et al.* 1988). This is similar to *Gyrinus*, whereas a constriction is present in *Dineutus*, *Enhydrus* and *Macrogyrus* (subgenus *Andogyrus* Ochs, 1924). The anterior margin of frontoclypeus lacks teeth in *Gyretes*, whereas it has teeth (which may be either more or less developed) in the other Neotropical genera. The frontoclypeal seta FR3 is short in *Gyretes* and the remaining Neotropical genera except *Gyrinus* in which it is elongate. The parietal has three conspicuous additional setae on the lateral region (contiguous to stemmata) in *Gyretes*.

In *Gyrinus* and *Dineutus* these setae are absent, whereas in *Enhydrus* and *Macrogyrus* (*Andogyrus*) there is

only one. The posterior margin of the lacinia is smooth in *Gyretes*. This combination is not observed in the other genera, in which either the posterior margin is dentate (either to a greater or lesser degree), or the apex is indented. Larvae of *Gyretes* bear one additional ventral seta both on the stipes and on the distal portion of last maxillary palpomere, which is not observed in other Neotropical genera. The mandible has several additional setae on the dorsal surface in all Neotropical genera except *Gyrinus*. The coxal seta CO12 is inserted proximally in *Gyretes* and in the remaining genera, with the exception of *Gyrinus* in which it is inserted distally. The coxa lacks additional setae in *Gyretes*, *Gyrinus* and *Dineutus*. Additional coxal setae are present in *Enhydrus* and *Macrogyrus* (*Andogyrus*). The femoral setae FE2 and FE3 and the protibial seta TI3 are bifid in *Gyretes*. Finally, the seta TR2 is present in *Gyretes* and *Gyrinus* and absent in the Dineutini genera.

Finally, it is important to note that in this study we obtained positive results in the association between adults and immatures of *G. nubilus* and *G. minax* using molecular tools (mitochondrial gene COI). Indicating the utility of this marker to increase the taxonomic knowledge on the Gyrinidae immatures, a family with scarce number of immatures described so far.

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