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Morphological description and DNA-based association of the last instar larva of *Erotesis schachti* Malicky 1982 (Trichoptera: Leptoceridae), an endemic of the Iberian Peninsula

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Abstract

In this paper we describe the main morphological characteristics that distinguish the full-grown larva of *Erotesis schachti*, an endemic of the Iberian Peninsula. The conspecificity of the larva and adult was confirmed by DNA analysis. Morphological features that easily discriminate it from the similar species *Erotesis baltica* are given.

Key words: caddisfly, molecular sequence, endemic species, mtCOI, South Spain

Introduction

Caddisfly larval stages form an essential portion of macroinvertebrate communities in freshwater habitats, both for their contribution to aquatic trophic networks and for their importance in water quality biomonitoring programs, where the larvae of caddisflies show high value as bioindicators. As highlighted by Graf *et al*. (2018), many of these biomonitoring systems use more-inclusive taxonomic categories, mainly families and genera, which results in a great loss of information on aquatic ecosystems when compared with methodology based on species-level information (e.g., Resh & Unzicker 1975; Usseglio-Polatera *et al*. 2000; Verdonschot 2000; Lenat & Resh 2001; Schmidt-Kloiber & Nijboer 2004). On the other hand, traditional biomonitoring-assessment based on the morphological characteristics of taxa is laborious, expensive, and requioring highly qualified staff. Therefore, new and accurate DNA-based biomonitoring methodologies are currently being developed using species-level DNA barcodes (e.g., Elbrecht *et al*. 2017; Blackman *et al*. 2019; Martins *et al*. 2019). Even phylogenetic (Zhou *et al*. 2016; Thomas *et al*. 2020) and biodiversity conservation studies (e.g., Ruppert *et al*. 2019; Feio *et al.* 2020) can benefit from these DNA sequencing techniques.

The caddisfly family Leptoceridae is distributed throughout the world (Morse 2022) and includes 46 extant genera (Holzenthal & Oliveira-Pes 2004, Morse 2022), of which 12 genera and about 129 species inhabit European aquatic ecosystems (Malicky 2004, 2005; Morse 2022). On the Iberian Peninsula, leptocerids are represented by 10 genera and 42 valid species (Guareschi *et al*. 2017; Coppa *et al*. 2022; González 2022).

The genus *Erotesis* McLachlan 1877 is known from the East and West Palearctic regions (Yang & Morse 2000) and is represented for several amber fossilized species (Wichard & Weitschat 1996) and four living ones, of which three live in Europe: *E. melanella* McLachlan 1884, *E. baltica* McLachlan 1877, and *E. schachti* Malicky 1982. A fourth nominate species, *Erotesis maura* Navás 1922, is a *nomen dubium* (Malicky 2005).

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The taxonomic integrity of the genera *Erotesis* and *Adicella* was discussed by Botosaneanu (1982), transferring *E. melanella* to *Adicella* Mclachlan 1877. Later, this author described the subspecies *A. melanella marocana* Botosaneanu 1989 for specimens from Morocco (Botosaneanu 1989). However, because of their possession of all the *Erotesis* synapomorphies cited by Yang & Morse (1993) and none of the *Adicella* synapomorphies, Yang & Morse (2000) suggested that *Adicella melanella melanella* (McLachlan 1884) and *Adicella melanella marocana* should be transferred back to the original genus *Erotesis*. Finally, Malicky (2005) considered that the differences between the two subspecies are minimal.

Only *E. melanella* and *E. schachti* have been recorded from the Iberian Peninsula (González & Martínez 2011; González 2022); the third species of the genus, *E. baltica*, is distributed over large areas in central and northern Europe (Neu *et al*. 2018).

Regarding knowledge of immature forms, only the European species *E. baltica* has been described (Hickin 1967; Lepneva 1971; Moretti 1983; Wallace *et al*. 2003; Waringer & Graf 2011; Rinne & Wiberg-Larsen 2017), while the larva of the Iberian (*E. schachti*) and the Ibero-Maghrebian (*E. melanella*) endemics are unknown.

This work aims to describe the main morphological characteristics that discriminate the full-grown larva and provide the DNA barcode of three Andalusian specimens of *E. schachti*.

Material and methods

Material examined. A total of 25 larvae, 7 females, and 5 males were collected from Garganta de la Cierva, Barbate Basin, Los Alcornocales Natural Park (Cádiz-Málaga), 381 m a.s.l., UTM: 30S2640, all leg. A. Ruiz-García. The samples were taken by hand net along a stream section. In addition, the presence of the species in the study area was confirmed by the capture of adults by light trap located on the stream bank. The conspecificity of larvae and adults was confirmed by DNA analysis. Collected material was fixed in 96% ethanol. Morphological characters were studied under stereomicroscopes (Kyowa SDZ-PL and Motic SMZ-168) and photographed with a Moticam® 2300 Digital Camera using Motic® Images 2.0 software, and Motic® Images Multi Focus software. In the description, the chaetotaxy follows those of Williams & Wiggins (1981) and Wallace *et al*. (2003).

DNA analyses. In addition to morphological analysis, DNA was extracted from approximately 0.1 g of each specimen, using a guanidine hydrochloride-silica-based DNA extraction method available at the DNA Barcoding 101.org webpage. Samples were amplified with DNA primers LCO1490 and HCO2198 (Folmer *et al.* 1994) under the following PCR conditions: Initial denaturation at 94°C for one minute, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 45 seconds. PCR products were purified by isopropanol-ammonium acetate precipitation at room temperature and sequenced at an external facility (STAB VIDA, https://www.stabvida.com). Sequences were edited using the program Chromas Lite, and consensus was obtained using SeqTrace software (Stucky 2012). Identification was performed using Boldsystems' resources that use Hidden Markov Model (HMM) profile of the mtCOI protein, followed by a linear search of the reference library (Sujeevan & Hebert 2007). Sequences were deposited in GenBank. Reference sequences were retrieved from GenBank (see Table 1 for accession numbers). A distance matrix was obtained using MEGA X software (Kumar et al. 2018). After a multiple ClustalW alignment, genetic distances were scored using the Maximum Composite Likelihood model (Tamura *et al*. 2004).

TABLE 1. Intra- and interspecific uncorrected pairwise distances (p) in % of nucleotide differences of the mitochondrial cytochrome oxidase I (mtCOI) gene recorded in the sequenced *Erotesis schachti* specimens of the Iberian Peninsula and *Erotesis baltica* from Finland with GenBank accession numbers. * = specimens sequenced in this paper; M = male; L = larva.

	E. schachti (L1)	*E. schachti* (L2)	*E. schachti* (M)	*E. schachti*	Acc. Number	Country
E. schachti (L1)					OP364027	Spain*
E. schachti (L2)	0.15				OP364028	Spain*
E. schachti (M)	0.00	0.15			OP364029	Spain*
E. schachti	0.00	0.15	0.00		HQ959391.1	Portugal
E. baltica	16.53	16.23	16.53	16.53	KX141634.1	Finland

Results

Erotesis schachti Malicky 1982—last instar larva

Body length: 7–8 mm (n = 5).

Head (Figs 1–4). Head width: 0.55–0.58 mm, (n = 5). Head capsule elongate, distally slightly narrower and uniformly dark brown in colour, except pale ring around each eye, with complete set of primary setae; darker muscle attachment spots visible on parietalia and on posterior third of cephalic capsule, including both sides of coronal suture (Fig. 1). As in all final instar larvae of Leptoceridae, subocular ecdysial lines present on parietalia (Fig. 2), but in this case with dorsal branch running sinuously from foramen occipitale on each side of head to lateral section of parietalia under each eye and visible posteriorly in dorsal view (Figs 1 and 2, red arrows). Frontoclypeus dark brown in colour, without distinct constriction at mid-length (Fig. 1). Antennae near distal parietal borders, very long, slightly thicker apically, each with single apical seta (Fig. 1, black arrow). Labrum slightly notched apically with four pairs of setae on dorsal surface. Mandibles stout, not elongate and with two cutting edges, one dorsal and one ventral, and three apical teeth (Fig. 3). Ventral apotome dark brown, subrectangular with rounded corners, slightly narrower in posterior 2/3 (Fig. 4).

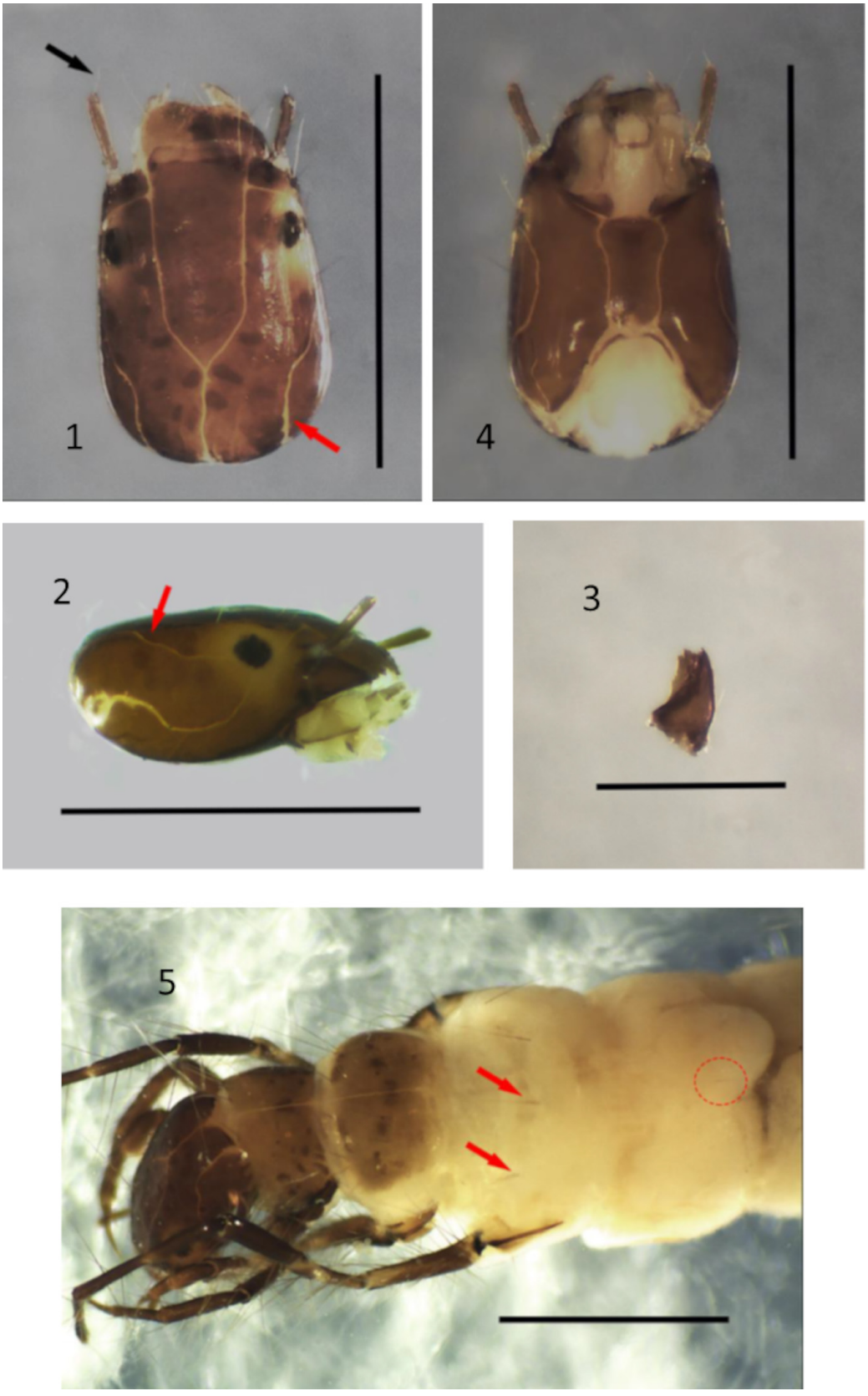
Thorax (Figs 5, 6, 8). Pronotum covered by two light brown sclerites, each with muscle attachment spots in its posterior half, with 27–30 long black setae on each sclerite (Figs 5, 8). Mesonotum covered by two large sclerites light brown in colour with several brown muscle attachment spots arranged in V-shape with 7 or 8 long black setae on each sclerite (Fig. 5). Metanotum completely unsclerotized; metanotal setal areas *sa2* and *sa3* each with single seta only (Fig. 5, red arrows); setal area *sa1* without setae. Prosternum and mesosternum without setae and metasternum with one seta on each side (Fig. 6, red arrows).

Legs (Figs 7, 8) brown, with numerous setae. Foreleg femora short and wide (Fig. 7); foretrochantins elongate, subrectangular and each with single seta (Fig. 8, red arrow). Tarsal claws of midlegs and hind legs evenly curved and each with prominent basal seta (Fig. 7). Hind legs much longer than others, each tibia and tarsus having constriction and pale band at midlength (Fig. 7, red arrows), without fringes of long setae.

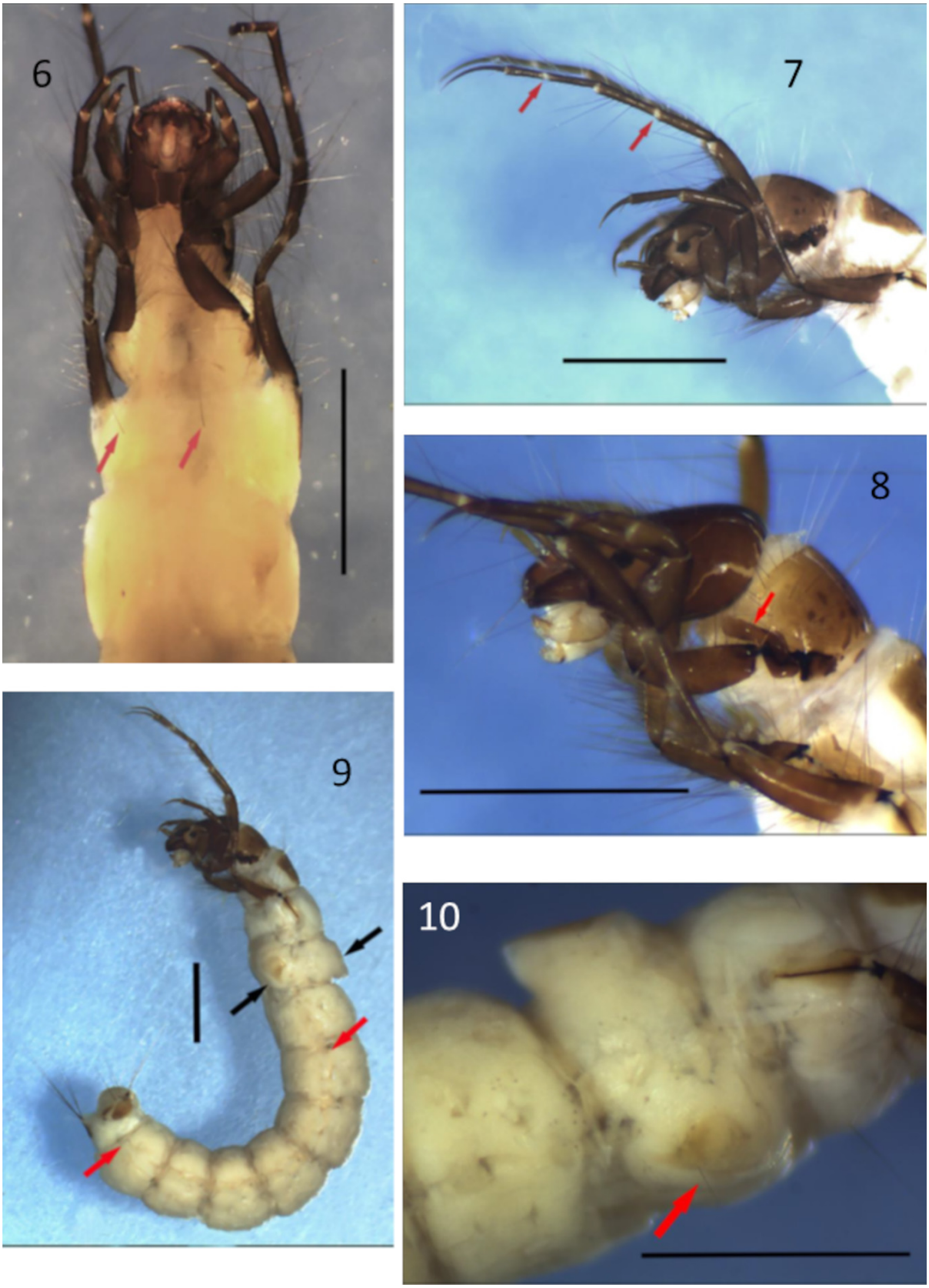
Abdomen (Figs 9–13) white in colour and cylindrical. Gills absent; lateral fringes extending on each side from beginning of abdominal segment III to end of segment VIII (Fig. 9, red arrows). Abdominal segment I with one dorsal and two lateral protuberances (Fig. 9, black arrows); dorsal setal areas *sa1* and *sa3* not developed and without setae, dorsal setal areas *sa2* with single seta on each side (Fig. 5, red circle); lateral sclerite on each side without strongly sclerotized dark posterior process; sclerite may range from pale tan to grey in colour; each lateral sclerite with one seta (Fig. 10, red arrow). Abdominal tergite IX well sclerotized, brown, bearing 6 long and 4 short terminal setae; only one dorsolateral seta on each side (Fig. 11, red arrows). Anal prolegs each with large lateral sclerite and strongly sclerotized anal claw with two small accessory hooks (Fig. 12); each proleg with five strong black setae apically (Figs 11, 13). Anal pads each with areas of dense fine and soft long setae laterally and rows of spinules medially, without tooth-edged plates or rows of strong, posteriorly directed spines (Fig. 13).

Case (Fig. 14). Length: 8–10 mm. Larval case composed of plant fragments such as roots and pieces of leaves arranged in two helices at least in some sections of case, one on right and one on left, tracing a zig-zag line on ventral side and another on dorsal side. In other sections of case, fragments aligned parallel or randomly. Some cases with few small mineral particles. Posterior end open, without membrane.

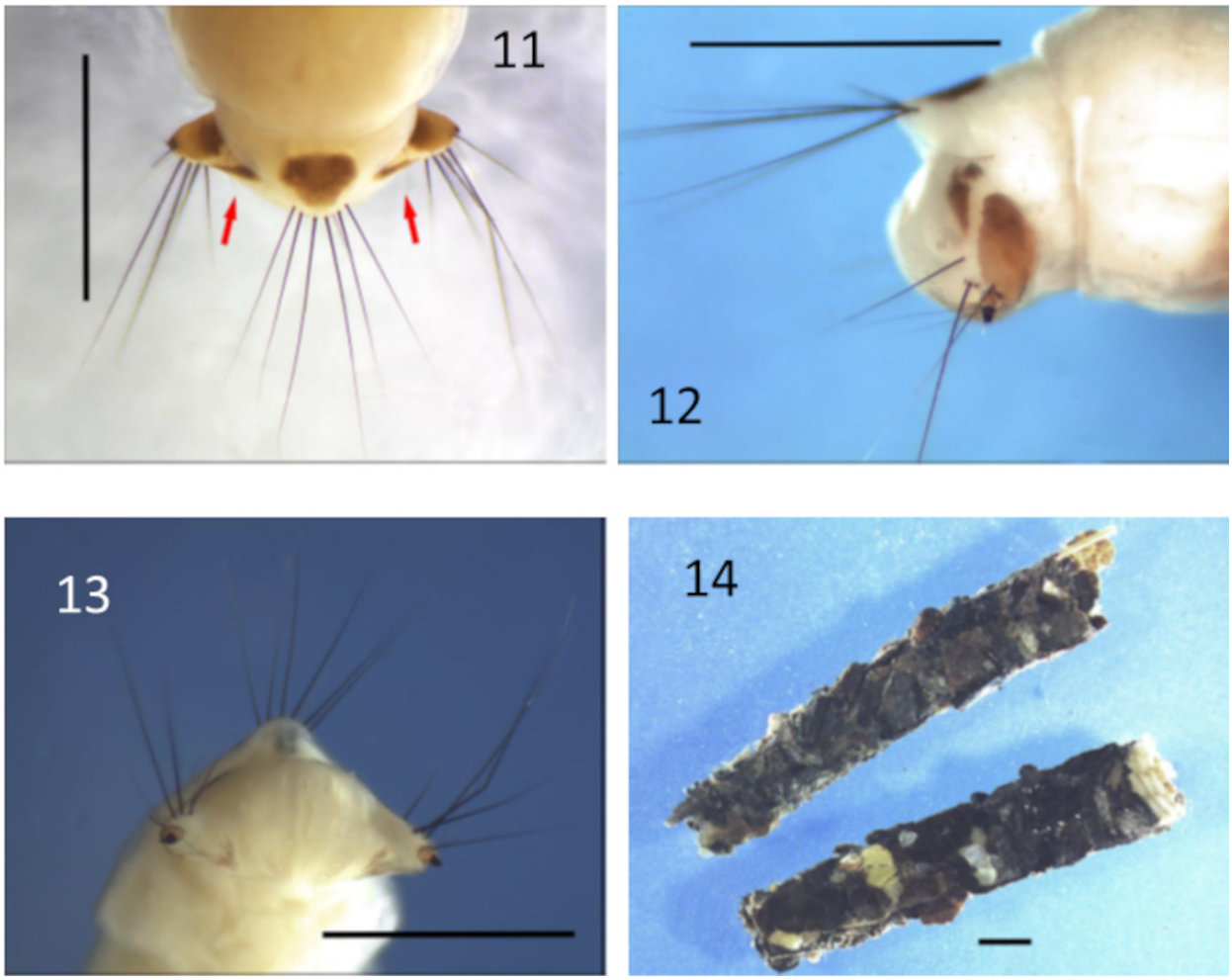
DNA analysis. The analysis of the barcode region of an adult of *E. schachti* from Garganta de la Cierva (Spain) (GenBank accession number: OP364029) and two previously unknown larvae collected in the same locality showed a genetic distance of 0.00% and 0.15% (Table 1). These values fit well within the intraspecific variability of mtCOI usually observed in caddisflies (Pauls *et al*. 2009, 2010; Previšić *et al*. 2009, 2014; Graf *et al*. 2015). Moreover, the uncorrected p-distances based on the mtCOI gene of these three individuals, a specimen of *E. schachti* from Portugal, and an *E. baltica* specimen from Finland (Table 1) are in line with interspecific distances commonly reported in Leptoceridae (Kučinić *et al.* 2020). Thus, the data enable confident association of the larva and the adult of *E. schachti*. Furthermore, the association of larvae and adults is based not only on comparisons of sequences from these specimens as Zhou (2007) and Zhou *et al*. (2007) recommended, but also by their co-occurrence at the same locality.



FIGURES 1–5. Larva of *Erotesis schachti* Malicky 1982: (1) head capsule, dorsal; antennal apical seta, black arrow; dorsal branch of ecdysial line, red arrow; (2) head capsule, right lateral; dorsal branch of ecdysial line, red arrow; (3) left mandible, ventral; (4) head capsule, ventral; (5) head, thorax, abdominal segment I, dorsal; single seta on each of metanotal setal areas *sa2* and *sa3*, red arrows; single seta on each dorsal setal area *sa2* of abdominal segment I, red circle). All figures scale: 1 mm, except (3) scale: 0.5 mm.



FIGURES 6–10. Larva of *Erotesis schachti* Malicky 1982: (6) thorax, ventral; metanotal setae, red arrows; (7) left legs, posterior/lateral face, left lateral; pale band on tibia and tarsus of hind leg, red arrows; (8) head, prothorax, legs, left lateral; foretrochantin, red arrow; (9) habitus, left lateral; start and end of left lateral fringe, red arrows; dorsal and left lateral protuberances of abdominal segment I, black arrows; (10) metathorax, abdominal segments I and II, right lateral; right lateral protuberance with sclerite and one seta, red arrow. All figures scale: 1 mm.



FIGURES 11–14. Larva of *Erotesis schachti* Malicky 1982: (11) last abdominal segments, dorsal; dorsolateral setae on abdominal segment IX, red arrows; (12) last abdominal segments, right lateral; (13) tip of abdomen, ventral; (14) larval cases. All figures scale: 1 mm.

Discussion

Erotesis schachti keys with *E. baltica* in the main available keys (Wallace *et al*. 2003; Waringer & Graf 2011), except in Rinne & Wiberg-Larsen (2017) where it keys with *Mystacides*. Nevertheless, in both cases the larva of *E. schachti* is easily distinguishable by the following characteristics:

- Head capsule uniformly dark brown in colour, except pale ring around each eye in *E. schachti*; whereas in *E. baltica* yellowish longitudinal band present along central axis of frontoclypeus, continuing on cephalic capsule on both sides of coronal suture (e.g., Wallace *et al*. 2003; Rinne & Wiberg-Larsen 2017).
- Tarsus of each hind leg of *E. schachti* with constriction and pale band at midlength, absent in *E. baltica* (Lepneva 1971; Rinne & Wiberg-Larsen 2017).
- Abdominal tergite IX well sclerotized, brown in *E. schachti;* whereas in *E. baltica* this tergite pale, indistinct, with small, pinkish brown dots (Lepneva 1971).
- *Erotesis schachti* and *Mystacides* species with hind tibiae and tarsi each with constriction and pale band at midlength. However, *Mystacides* head pattern of European *Mystacides* species consisting of dots, dark stripes, or characteristic H-shaped black pattern formed by dorsal stripes and band in posterior part of frontoclypeus (Lepneva 1971) easily discriminating these taxa. In addition, *Mystacides* species with dark curved bar on posterior projection of each lateral sclerite of abdominal segment I, absent in *E. schachti*.
- Dorsal branch of subocular ecdysial line present in *E. schachti*, absent in *Mystacides*.

FIGURE 15. Los Alcornocales Natural Park. Garganta de la Cierva: *Erotesis schachti* Malicky 1982 habitat.

From the morphological study of the immature stages of the two species of *Erotesis* described to date, we can summarize the main discriminatory characters of the last instar larva of this genus as follows:

- Head capsule uniformly dark brown except pale ring around each eye or with distinct dark parietal bands;
- Subocular ecdysial lines each with dorsal branch running sinuously from foramen occipital on each side of head to lateral section of parietalia under eye and visible posteriorly in dorsal view;
- Tarsal claws of legs long and curved but never hooked;
- Mandibles short and with two cutting edges;
- Labrum with few setae on dorsal surface;
- Mesonotum without dark posterolateral marks;
- Gills absent;
- Anal region without tooth-edged plates or rows of strong posteriorly-directed spines;
- Anal region with rows of spinules on either side of anal opening.
- Dark curved bar on posterior projection of each lateral sclerite of abdominal segment I absent;
- Tibiae of hind legs, and in some species also hind tarsi, each with constriction and pale band at mid-length;
- Hind legs without fringes of long swimming setae.
- Larval case with vegetal fragments arranged in two opposing spirals, at least partially.

Distributional and Ecological Remarks

In Los Alcornocales Natural Park, *E. schachti* inhabits a permanent headwater stream flowing over the Aljibe sandstones geological unit. The riparian canopy forest is composed mainly of *Salix* sp., *Alnus glutinosa*, and *Nerium oleander*, surrounded by a pristine forest composed almost exclusively by *Quercus suber*, and in the most humid areas by *Q. canariensis*. Herbaceous riparian vegetation is represented mostly by *Oenanthe crocata* (Fig. 15). The larvae are found mainly in shallow pools on the roots of riparian vegetation but we did not observe them to swim during collection work. *Erotesis schachti* shares this stream section with a characteristic larval community of caddisflies including *Rhyacophila fonticola* Giudicelli & Dakki 1984, *Micrasema moestum* (Hagen 1868), *Allogamus kampos* Oláh & Ruiz Garcia 2014 (in Oláh *et al*. 2014), *Allogamus gibraltaricus* Gonzalez & Ruiz 2001, *Schizopelex festiva* (Rambur 1842), *Lepidostoma hirtum* (Fabricius 1775), *Diplectrona felix* McLachlan 1878, and *Calamoceras marsupus* Brauer 1865, among others.

Erotesis schachti is found in slightly buffered acidic waters with alkalinity values close to 0.5 meq/L. The water temperature fluctuates between 7–12 ºC and the concentration of dissolved oxygen is always high (6.5–8 mg/L).

On the Iberian Peninsula, *E. schachti* is distributed in the central and southern parts of Portugal and the Spanish provinces of Huelva, Sevilla, and Cádiz.

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