IMMUNOHISTOCHEMICAL AND LECTIN-BASED APPROACH TO *ACERENTOMON* SP. ANATOMY (PROTURA: ACERENTOMIDAE)

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ABSTRACT

The present study describes for the first time in *Acerentomon* sp. the distribution of glutamate decarboxylase-like immunoreactivity. The presence of this GABA-synthetizing enzyme was described in: pharynx wall, cephalic muscles, posterior part of the supraesophageal ganglion, abdominal glands, neuropil near to the posterior ganglion, cuticular muscle insertion and in sensilla. Furthermore the use of fluorochrome-conjugated lectins was tested in order to visualize taxonomic features (like pores and setae) on whole mount fixed Protura specimens. The development of a lectin-based technique could render more precise the species determination, allowing a more accurate identification.

KEY WORDS

Lectins, GAD, indirect immunohistochemistry, Protura

INTRODUCTION

Protura is a group of Hexapoda described by Silvestri in 1907. Just two years later, Berlese (1909) provided more detailed data about such taxon, describing their anatomy in his valuable monograph. Since then, only few research were carried out about the anatomy of such small animals (e.g. François, 1969; Dallai 1976, 1978; Dallai et al., 1986) and the vast majority of papers on Protura concern their external morphology (e.g. Rusek et al., 2012; Shrubovych, 2014) and their taxonomy (cf Szeptycki, 2007; Pass and Szucsich 2011).

We here describe the immunoreactivity for the enzyme glutamate decarboxylase (GAD), which is a biosynthetic enzyme of the neurotransmitter γ-aminobutyric acid (GABA), in *Acerentomon* sp. The amino acid sequence of the enzyme GAD is quite conserved throughout animals and the same antiserum anti-GAD 65/67 here used, was also used, in other studies, on arthropod nervous system (e.g. Gallus et al., 2010).

Analysis of some morphological characters, such as porotaxy and chaetotaxy, is needed for the identification of Protura (e.g. Shrubovych, 2014). The difficulty in species determination led to the development of techniques which allow species determination and DNA extraction from the same specimen (Böhm et al., 2011). Thus a second aim of this study was to test the possibility to highlight pores and setae in fixed and preserved whole mount specimens by the use of fluorochrome-conjugated lectins. Lectins are carbohydrate-binding proteins that bind to specific configurations of carbohydrate molecules and can serve to identify cell types or cellular components (Sharon and Lis, 1989). The development of a new technique in order to visualize pores and setae and the possibility to recognize a species-specific lectin-pattern, would be an improvement in the precision of species determination.

MATERIAL AND METHODS

Samples collections and extraction

Soil samples were collected from an oak *Quercus ilex* forest in the San Martino Hospital Park (Genoa, Liguria, NW Italy). Protura were extracted during the following 5 days using Berlese-Tullgren funnels (2.5 mm mesh size). The specimens were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS 0,1M - pH7.4) over night, washed three times in PBS and preserved in 70% ethanol.

Prior to immunohistochemical treatments, specimens belonging to genus *Acerentomon* Silvestri, 1907 were selected, under

the microscope to 100x magnification on the basis of their larger size (body length, nearly 2 mm), their fully sclerotized cuticle and their prominent rostrum.

Indirect immunohistochemistry

Specimens were dehydrated, embedded in paraffin according to standard histological methods and processed into 5 μm sections. Indirect immunohistochemistry was performed using a rabbit polyclonal antiserum raised against mammalian GAD, anti-GAD65/67 (1:200 in PBS - Chemicon International, USA). As the secondary antiserum, a goat anti-rabbit conjugated with Alexa Fluor 488 (1:400 in PBS - Invitrogen, USA) was used. Negative controls were performed by omitting primary antiserum. Nuclei were counterstained using DAPI (1:10000 Invitrogen, USA). Histological sections as well as whole mount specimens stained by fluorochrome-conjugated lectins were examined using Leica DMRB light microscope (Leica, Germany) equipped with epifluorescence; the images were acquired with a Leica CCD camera DFC420C (Leica, Germany).

After observation and photography, coverslips were removed from slides used for immunohistochemistry. Then the sections were stained with hematoxylin-eosin (Bio-Optica, Italy); the staining solution, made from Meyer's Emallume and Eosin Y were purchased ready to use. Sections were examined under the same light microscope and photographed in order to compare immunoreactivity and histology of the same frames.

Lectins

Whole mount specimens were incubated at room temperature in the dark for 45 minutes with different fluorochrome-conjugated lectins (Invitrogen, USA), according to concentrations, specificity and fluorochrome shown in Table 1. Specimens were then washed three times in PBS and mounted with PBS/Glycerol 50/50 on a depression

slide covered with a coverslip.

Table 1. Fluorochrome-conjugated lectins: concentrations, specificity excitement.

	Working Dilution	**Specificity**	**Fluorophore**	**Excitement-Emission**
ConA	100 μg/mL	Mannose	Alexa Fluor 594	594 nm – 617 nm
WGA	10 μg/mL	N-acetyl-glucosamine	Alexa Fluor 488	488 nm – 519 nm
SBA	25 μg/mL	N-acetyl-galattosamine	Alexa Fluor 488	488 nm – 519 nm
UEA	1 μg/mL	Fucose	Atto 594	594 nm – 617 nm

RESULTS AND DISCUSSION

Indirect immunohistochemistry

In *Acerentomon* sp., GAD-like immunoreactivity was observed in the pharynx wall (Fig. 1A), in cephalic dorsoventral muscles (Fig. 1A), posteriorly to the supraesophageal ganglion (Fig. 2), in abdominal glands (Fig. 3A), in the neuropile near to the posterior ganglion (Fig. 3B) and, possibly, in cuticular muscle insertion and in sensilla (Fig. 3B). Omission of the primary antibody abolished all fluorescence. Comparison of immunofluorescence and hematoxylin-eosin stained sections allowed to better observe the morphology of tissues (Figs. 4, 5 and 6).

GABA is a major inhibitory neurotransmitter in insects (Enell et al., 2007) and arthropods in general (Benson, 1989; Gallus et al., 2010). From our observation we could hypothesize a role of this neurotransmitter in the central nervous system of *Acerentomon* sp., due to the presence of GAD-like immunoreactivity in the neuron cell body in the supraesophageal ganglia (head) and in the neuropile near to the terminal ganglion (abdomen). An involvement of GABA on control of

muscle can be inferred from the GAD-like immunoreactivity in pharynx and muscle insertion throughout the body. Moreover an involvement of GABA in the modulation of sensory function is suggested by the presence of GAD-like immunoreactivity in sensilla. A role in sensory function also in pharynx cannot be ruled out. Further immunohistochemical investigations will lead, in future studies, to the mapping of central and peripheral nervous system in this insect group

Lectins

Whole mount fluorochrome-conjugated lectin staining of Protura specimens, here preliminary approached, could be a promising technique as we observed that adult *Acerentomon* sp. specimens are not stained by all the lectins in a specific way. WGA and SBA gave, in fact, negative results. On the other hand, ConA lectin highlighted dorsal points of positivity on metanotum (Fig.7A) and lateral points of positivity on abdomen, thorax, legs II-III and forelegs (Fig.7B). UEA lectin highlighted a setae distribution on Pro- Meso- and Metanotum (Fig. 8A, B) which could maybe correspond to the dorsal chaetotaxy of an *Acerentomon* species such as *A. doderoi* (Silvestri, 1907) (Fig. 8C). These first results, though partial, seem to indicate that a refinement of the technique could be promising for future research.

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ANNEX – IMAGES

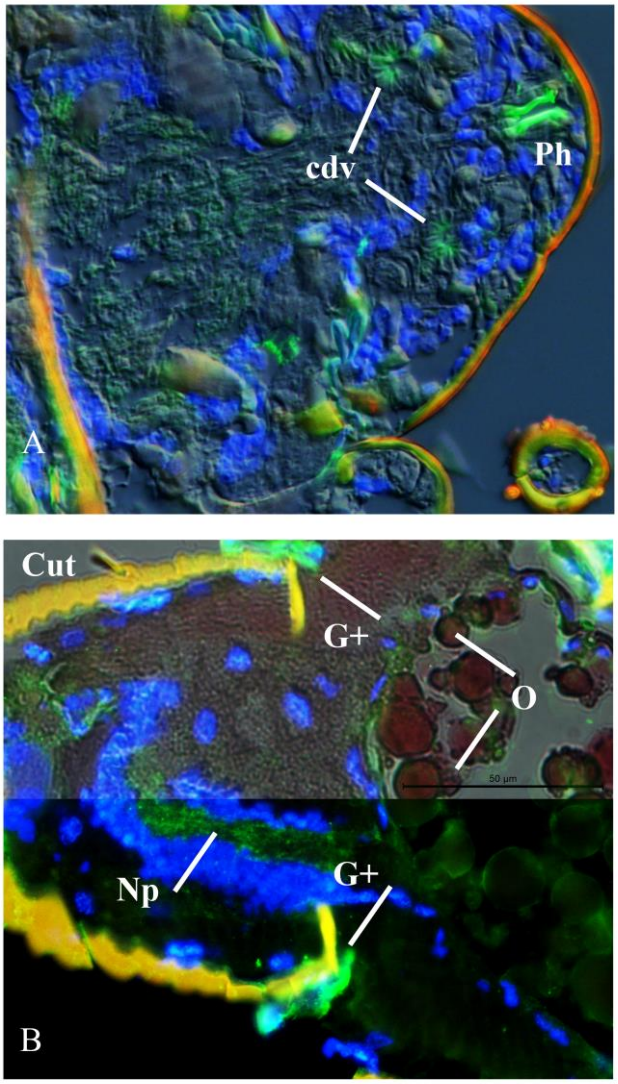

Figure 1. *Acerentomon* sp histological section (A), head detail: positivity to GAD (**Ph** = pharynx, cdv = cephalic dorsoventral muscles); (B) abdomen section (**Ab Mus** = abdominal muscles, **G+** = positivity to GAD, **Np** = neuropil, **O** = oocytes, **TG** = terminal ganglion).

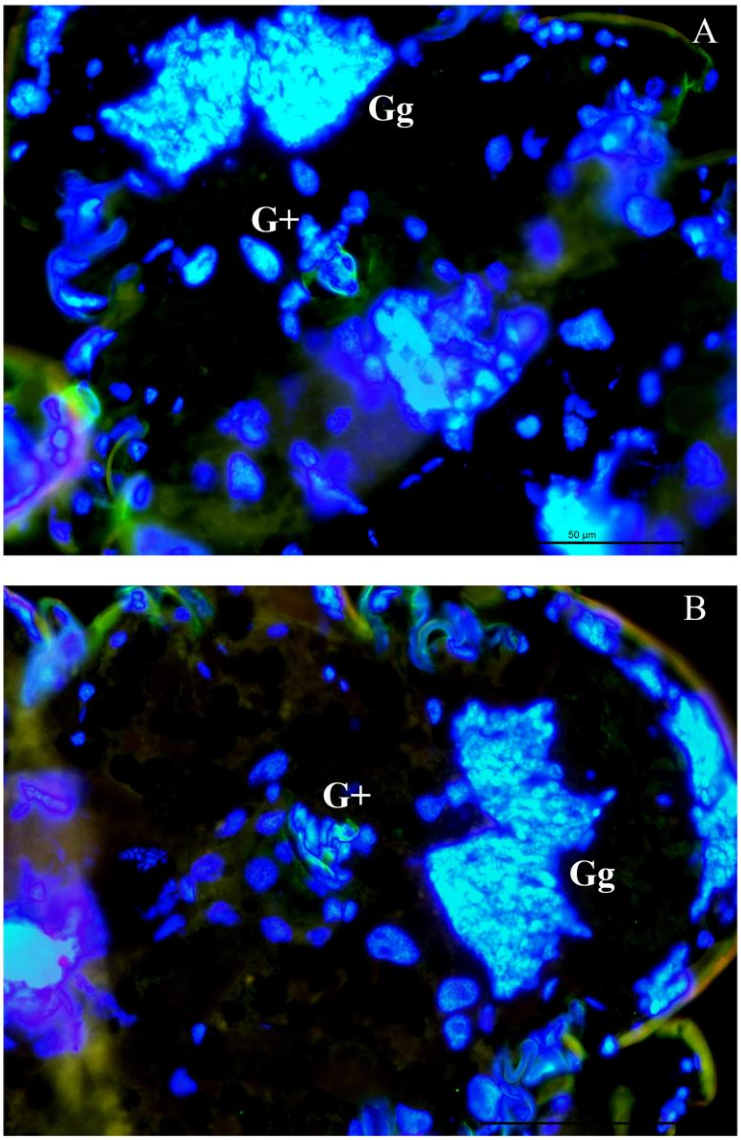

Figure 2. *Acerentomon* sp histological section, head. (A) section showing the bilobed supraesophageal ganglion (**Gg**) and a region positive to GAD (**G+**), probably the end of **Gg**; (B) section superior to the level of A showing the **G+** region posterior to **Gg**.

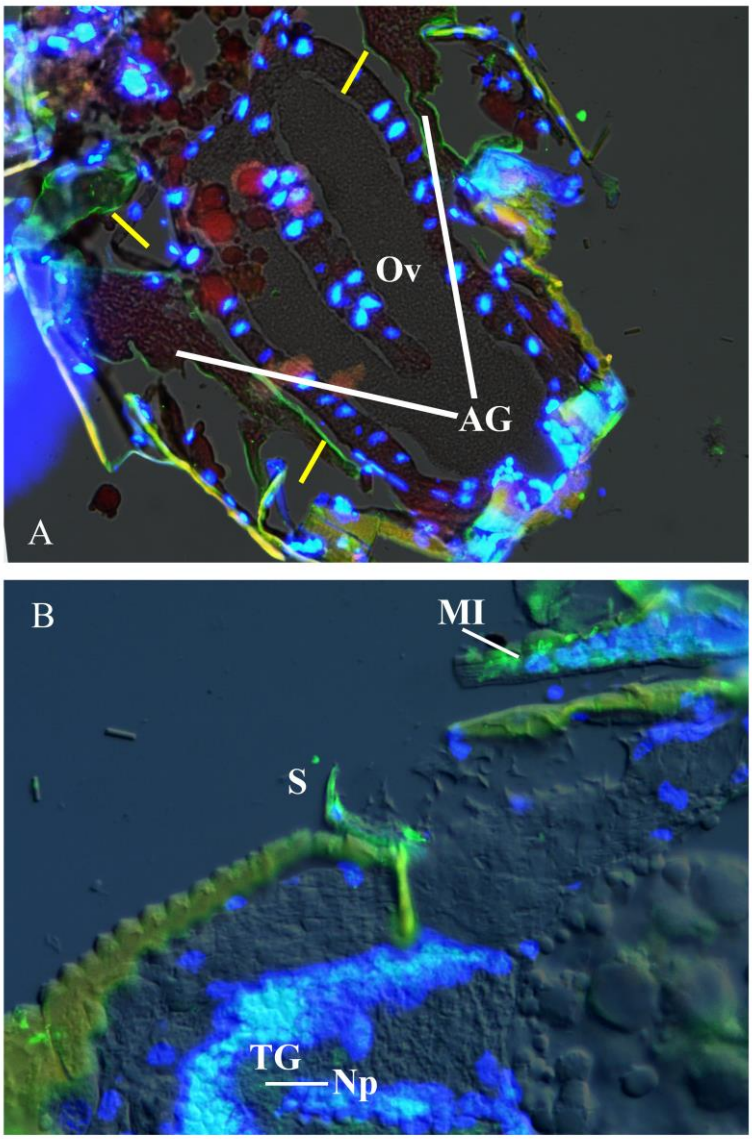

Figure 3. *Acerentomon* sp histological section, abdomen. (A) section showing ovary and probable positivity at the abdominal gland (**AG**) level (yellow bars); (B) section showing terminal ganglion (**TG**) positivity to GAD, neuropil (**Np**), a probable sensillum (**S**) and a probable cuticular muscle insertion point (**MI**).

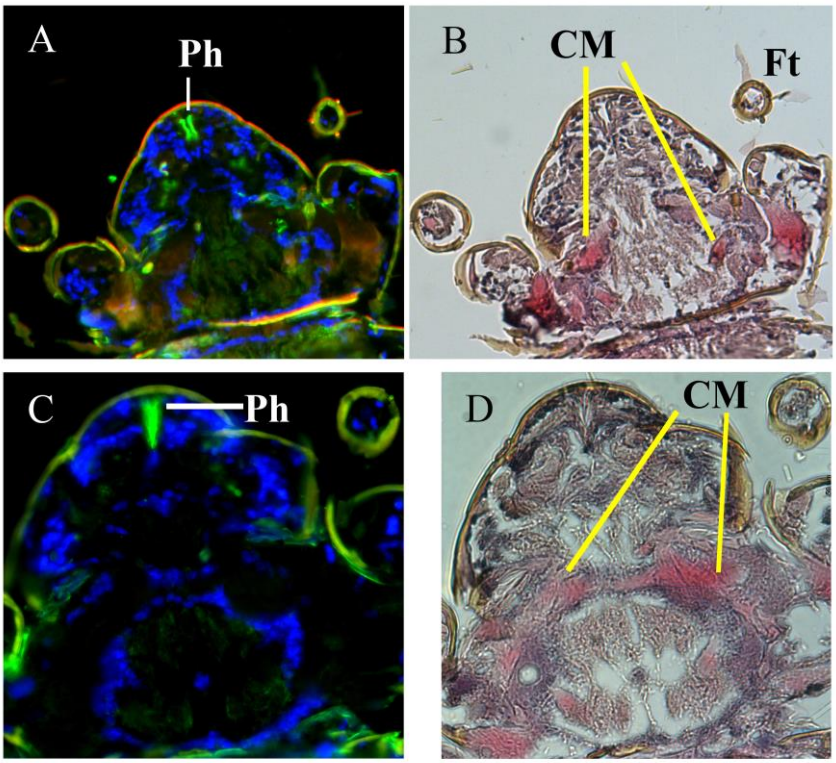

Figure 4. *Acerentomon* sp histological section, head. (A) green fluorescence (GAD), blue (DAPI, cells nuclei); (B) same section hematoxylin-eosin colored (**Ph** = pharynx, **CM** = cephalic muscles, **Ft** = foretarsus); (C) and (D) section cut at a much dorsal level.

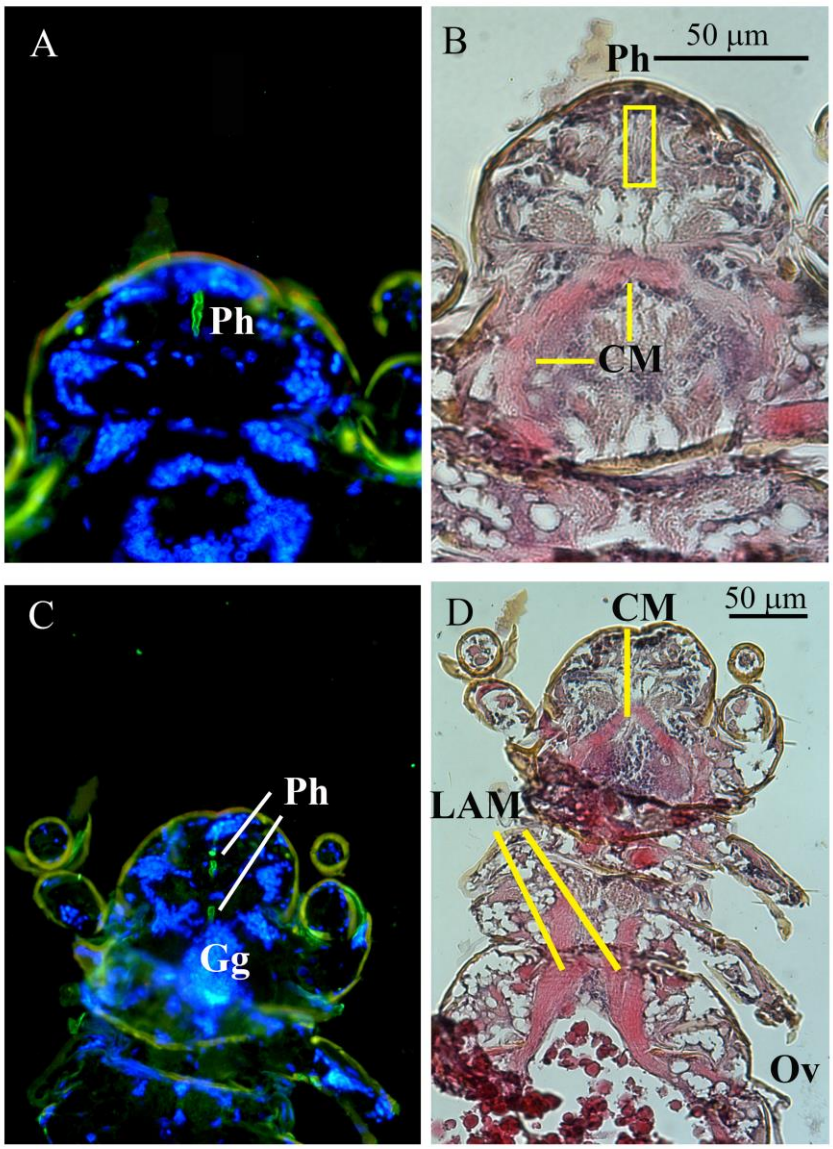

Figure 5. *Acerentomon* sp histological section, head. (A) positivity of the pharynx (**Ph**) longitudinally sectioned; (B) hematoxylin-eosin evidenced cephalic muscles (**CM**). (C, D) pharynx (**P**) lies beneath the supraesophageal ganglion (**Gg**), at the same level of longitudinal abdominal muscles (**LAM**).

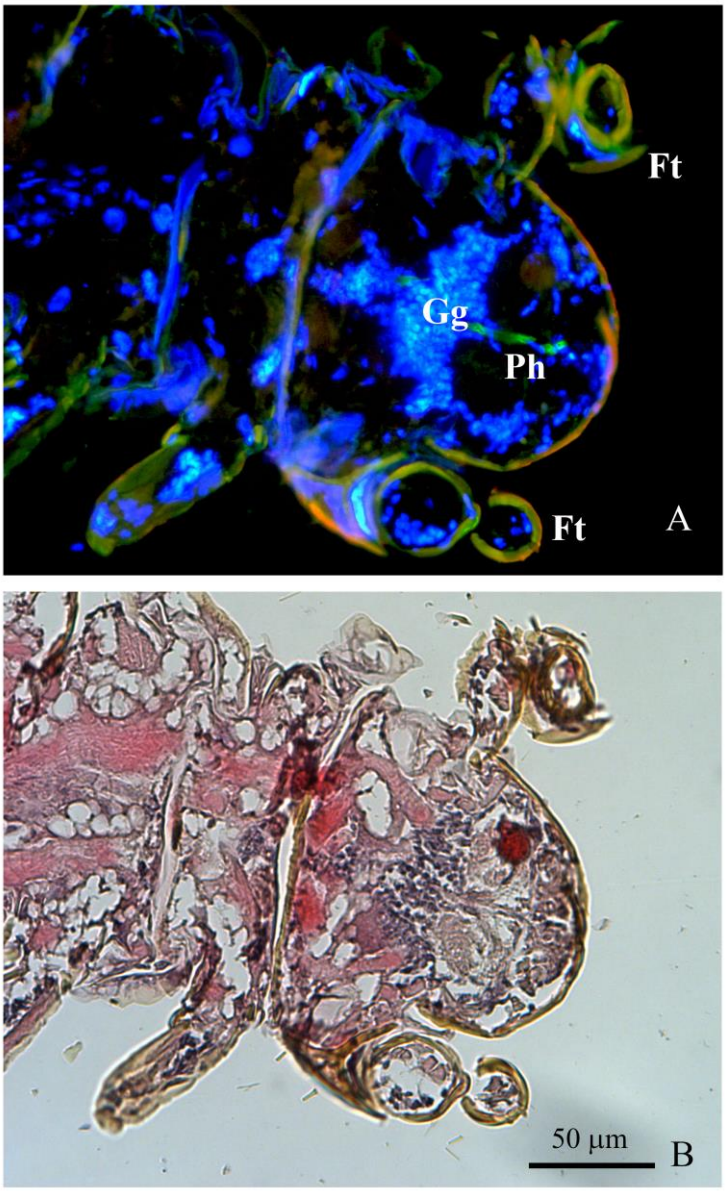

Figure 6. *Acerentomon* sp histological section, head. Much dorsal section. (A) The supraesophageal ganglion (**Gg**) occupies nearly the whole head, laying over the pharynx (**Ph**); (B) same section hematoxylin-eosin colored.

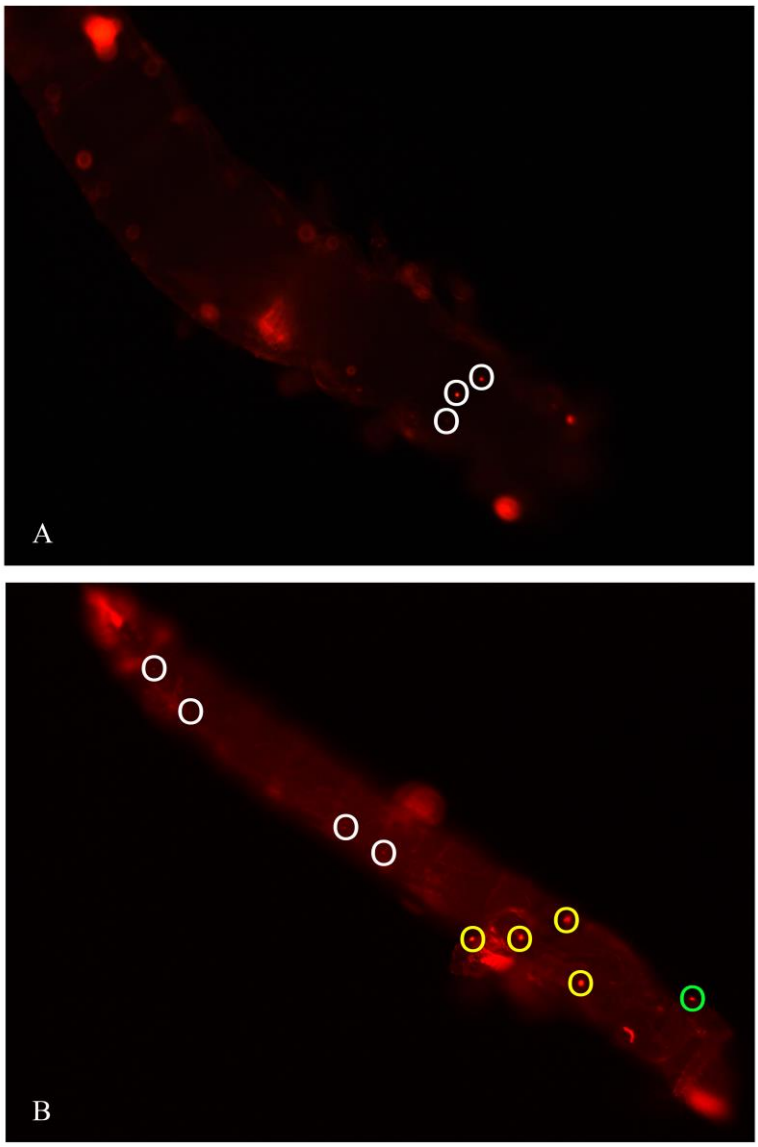

Figure 7. Whole mount specimen of *Acerentomon* sp. stained with lectin ConA. (A) dorsal view: points of positivity on metanotum (white circles); (B) side view: points of positivity on abdomen (white circles), thorax and legs II, III (yellow), and leg I (green).

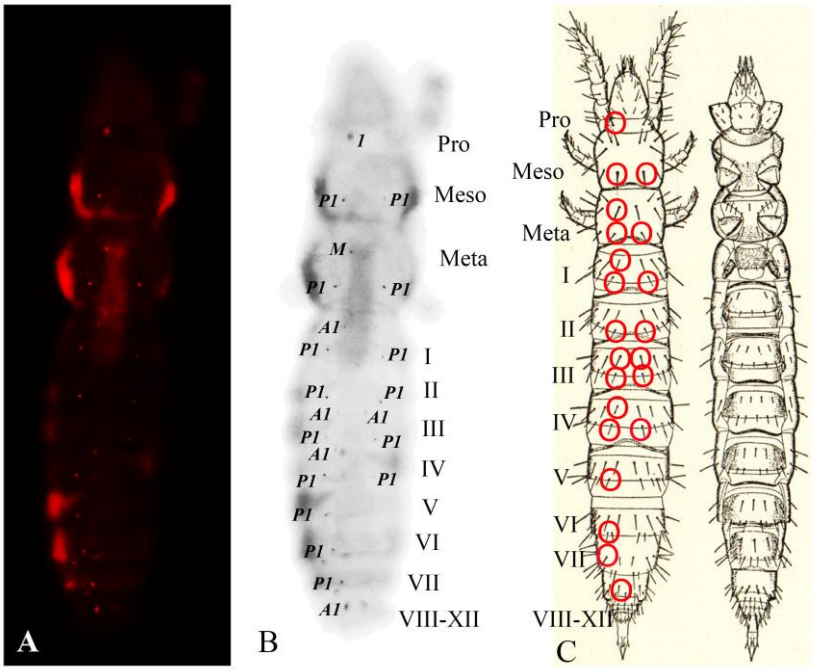

Figure 8. (A) Whole mount specimen of *Acerentomon* sp. stained with the lectin UEA; (B) negative image (**A** = anterior setae, **P** = posterior setae, **M** = median setae, **Pro**-, **Meso**-, **Meta** = Pro-, Meso- and Metanotum); (C) presumed correspondence of the positive points with dorsal chaetotaxy (from the original description of *Acerentomon doderoi* Silvestri, 1907); (**Ab Mus** = abdominal muscles**, O** = oocytes, **Ov** = ovary, **TG** = terminal ganglion).