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Research paper

On the affinities and systematic position of *Pachytychius* Jekel, a genus currently *incertae sedis* in Coleoptera, Curculionidae: Evidence from immature stages and the COI gene support its placement in Smicronychini

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ABSTRACT

The affinities and systematic position of *Pachytychius* Jekel, a genus currently *incertae sedis* in Coleoptera, Curculionidae, are assessed using new evidence from the immature stages and DNA sequences from the COI gene. The taxonomic history of genus *Pachytychius* denotes uncertainty in tribal placements from Erirhinini to various tribes of Curculioninae. In this paper, immature stages of genus *Pachytychius* are described and illustrated for the first time, including the mature larva of the type species, *Pachytychius sparsutus* (Olivier), and the larva and pupa of *Pachytychius haematocephalus* (Gyllenhal). The structural features of the larva and pupa of *Pachytychius*, as well as bionomic data, agree with those commonly found in subfamily Curculioninae and are consistent with a close relationship with *Smicronyx* Schoenherr. In addition, we used sequences from the 5′region of the COI gene, available in genetic databases, to test phylogenetic relationships of *Pachytychius* with other genera with which affinities had been suggested (77 terminal taxa including outgroups). Results from maximum likelihood and parsimony analyses agree: *Pachytychius* is not closely related to the erirhinines with pedo-tectal male genitalia, and belongs among the curculionine weevils, forming a clade with *Smicronyx*. Finally, the systematic position of *Pachytychius* suggested by the immature stages and the COI hypothesis results corroborated when adult morphological characters are re-evaluated, and can be extended to genus *Aubeonymus* Jacquelin du Val for its clear affinity to *Pachytychius*. The New placements are: *Pachytychius* Jekel, 1861 and *Aubeonymus* Jacquelin du Val, 1855 to Smicronychini from Curculioninae *i. sed*. (Curculionidae).

1. Introduction

The genus *Pachytychius* Jekel, 1861 remains with uncertain taxonomic placement and unclear affinities to other genera of Curculionidae, being currently classified as "*incertae sedis*" in Curculioninae (Caldara et al. 2014). Jekel (1861) created *Pachytychius* (type species *Rhynchaenus sparsutus* Olivier, 1807) together with two allied genera *Barytychius* and *Styphlotychius* (currently synonyms of *Pachytychius*) extracting them from *Tychius* Germar, 1817. A possible affinity of *Pachytychius* with *Smicronyx* Schoenherr, 1843 was implied by LeConte & Horn (1876) when LeConte included in his erirhinine group "Desmorhines" (= Smicronychini Seidlitz 1891, see Alonso-Zarazaga & Lyal 1999) the two North American species of *Pachytychius* Jekel known by then, although both were later considered to belong in *Smicronyx* by Casey, (1892), mainly because of their claws connate at base. Since then, *Pachytychius* was always kept separated from Smicronychini by subsequent authors (e.g., Anderson 1962). The genus *Pachytychius* was traditionally placed in the subfamily Erirhininae tribe Erirhinini Schoenherr, 1825 of the family Curculionidae (Hoffmann, 1958; González, 1968; Caldara, 1978; Dieckmann, 1986). However,

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Kuschel (1971) restricted the concept of Erirhininae to include only the genera with a pedo-tectal type of male genitalia. In consequence, the genus *Pachytychius*, as well as many others with male genitalia of the (derived) pedal type, were excluded from Erirhininae, but unfortunately without receiving a novel placement. Morris (1995) suggested *Pachytychius* to be transferred together with the closely related genus *Aubeonymus* Jacquelin du Val, 1855 to Curculioninae: Styphlini Jekel 1861, but subsequently Morris & Booth (1997) proposed a new subfamily, Pachytychiinae, which remained *nomen nudum* for not being accompanied by any description (Alonso-Zarazaga & Lyal 2002). On the other hand, Alonso-Zarazaga & Lyal (1999) included *Pachytychius* and *Aubeonymus* in Curculioninae: Storeini Lacordaire, 1863, a decision rejected by Caldara et al. (2014) placing these genera as Curculioninae *incertae sedis*, while Oberprieler (2014) did not discard the possibility of they belonging in tribe Erirhinini.

In order to resolve these taxonomical uncertainties we thought it would be useful to consider additional sources of evidence, like morphological characters of the immature stages and bionomic data, as well as exploring affinities of *Pachytychius* with other genera by establishing a preliminary hypothesis based on molecular data. Focussing on this, main goals of this paper are: 1) to provide, for the first time, descriptions of the larval and pupal stages of representative species of genus *Pachytychius*, including the type species; 2) perform a first phylogenetic analysis of *Pachytychius* and other weevil genera based on COI "barcode" sequences available in genetic databases; 3) evaluate the new evidence from 1 to 2 with the available information from adult morphology to resolve the systematic position of *Pachytychius*.

2. Material and methods

2.1. Insect collection and laboratory breeding

Immature specimens were collected, generally together with a few adults, and preserved in 70% ethanol. The specimens were deposited in the Group Function of Invertebrate and Plant Biodiversity in Agro-Ecosystems of the Crop Research Institute (Prague, Czech Republic). The collector identified the plants.

A search for the putative host-plants (Fabaceae) was done prior to collecting the weevils. Plants were carefully examined in the field looking for damage on stems, leaves and flowers and for the presence of galls. About 500 seed pods were collected, selected because adult weevils had been detected on the plants in the moment of collection or in previous weeks. The pods were placed in a wide-mouth transparent plastic container covered with netting and examined daily in the laboratory. When full-grown larvae appeared, they were transferred to another similar container but with a mixture of sand and peat moss as substrate into which larvae tunnelled to pupate and complete their development to adult. This procedure was repeated daily with different containers to avoid mixing larvae collected on different days and to be able to calculate, as accurately as possible, the times of the change from larvae to pupae and from pupae to adults. After a few days, some pupal cells were opened daily to establish the first day of appearance of the pupae and then that of the adults.

2.2. Preservation and preparation of larvae and pupae for morphological study

Part of the larval and pupal material was preserved in Pampel fixation liquid (see Trnka et al. 2015) and used for the morphological descriptions. These specimens are deposited in the Group Function of Invertebrate and Plant Biodiversity in Agro-Ecosystems of the Crop Research Institute (Prague, Czech Republic). Larvae were dissected and slide-mounted for study under microscope, basically following May (1994): head and body were separated and cleared in a 10% potassium hydroxide (KOH) solution and then rinsed in distilled water. After clearing, the mouthparts were separated from the head capsule, and mounted in Euparal on permanent microscope slides. The remaining body parts, thorax and abdomen, were mounted on temporary microscope slides in 10% glycerine.

The observations and measurements were conducted using a light compound microscope with calibrated oculars. The following measurements were taken for comparison purposes, for each larva: head width, body length (larvae fixed in a C-shape were measured in segments), and body width at widest place (i.e., abdominal segments I–IV). For the pupa, the length and the width at the widest place were measured.

Drawings were made with the aid of a drawing tube on the microscope and edited by programs (Adobe Photoshop 10, Corel Photo-Paint 11, GIMP 2). The numbers of setae of the bilateral structures are given for one side.

Terminology used for the larva generally follows Marvaldi (1998, 1999), and May (1994), except for the setae on the median parts of epipharynx: two pairs are clearly anteromedian in position, one of which is often very small, but there is a third pair with a rather intermediate position and authors differ in naming it as median (e.g., May 1994;Marvaldi 1999) or, as in the present paper, anteromedian (e. g., Anderson 1947; Skuhrovec et al. 2015). Terminology for the pupa follows Scherf (1964) and May (1994).

2.3. Molecular data and phylogenetic analyses

Phylogenetic analyses were performed on the 5′ (barcoding) region of the COI gene as sequences for this locus were available for representative species of *Pachytychius* ((including type species) and of several genera/tribes with which *Pachytychius* had been considered to be probably related (see Introduction). The COI sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and BOLD (http://www.boldsystems.org/) databases. Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses were conducted on a data matrix of 77 terminal taxa and 658 characters. The taxa sampled (see Appendix) include, besides two species of *Pachytychius*, 71 species in various genera/tribes/subfamilies of Curculionidae, of interest to test affinities of *Pachytychius*, plus four terminals in Brentidae and Caridae as more distant outgroups, using the later to root the trees. The program PAUP (Swofford 2002) was used to edit the original matrix and to export it to nexus and phylip formats, for analyses with TNT and RAxML respectively.

Maximum Likelihood analysis was performed on RAxML version 8.2.8 (Stamatakis 2014) on the CIPRES portal (Miller et al. 2010), using the *-f a* algorithm, which computes a rapid bootstrap analysis and search for best-scoring ML tree in one single run, with 100 bootstrap replications and GTRCAT model, without partitioning. Parsimony analysis was conducted in TNT version 1.5 beta (Goloboff et al. 2008), with an heuristic search consisting in 1000 random addition sequences plus TBR as swapping algorithm, saving 10 trees per replication, under equal weights.

3. Results

3.1. Description of immature stages

3.1.1. Pachytychius haematocephalus (Gyllenhal, 1836)

Material examined. Italy: Lombardia, Pavia province, Varzi, at Penice Mount 1000 m, June 1981, 93 larvae of different instars from seeds of *Astragalus monspessulanus* L. (Fabaceae) (leg. R. Caldara);

18 pupae (9 males, 9 females) obtained from larvae collected as above, determined by association with reared adults.

Mature larva (Figs. 1–10). *Dimensions*: body length 4.45–6.70 mm (mean 5.60 mm); maximum body width at mid abdomen, up to 1.76 mm; head width 0.72–0.89 mm (mean 0.80 mm). *General aspect* (Fig. 7): body elongated, slightly curved, rounded in cross section; head brownish, pale to dark, thoracic and abdominal segments white to whitish yellow, body vestiture of setae thin, of various lengths from minute to relatively long. *Head* (Fig. 1): exposed, head capsule suboval in outline; endocarinal line distinct, two-thirds as long as frons; frontal sutures distinct in entire length up to antennae; only anterior stemma (st) present, as small dark pigmented spot with convex cornea. Cranial setae (Fig. 1): *des1*, *des2 des3 and des5* long, *des4 short*, *des3* located on epicranium close to frontal suture; *fs1* minute to very short, *fs2* short, *fs3* minute or very short, *fs4* and *fs5* long, well developed; *les1* and *les2* as long as *des1* or *fs5*; *ves1–2* relatively long; postepicranial area with minute setae and sensilla (together 6–8). Antennae (Figs. 1, 4), with oblique position on each side at anterior margin of head; membranous basal segment convex, bearing subconical, longer than wide, sensorium, and 5 small sensilla different in length (Fig. 4). Clypeus (Fig. 2), approximately 3 X wider than long, *cls1–2* well developed, with sensillum between them; anterior margin slightly concave. Labrum (Fig. 2) approximately 2 X wider than long, anterior margin bisinuate; *lms1–3* well developed, *lms1* and *lms2* longer than *lms3*. Epipharynx (Fig. 3) with 3 blunt *als*, subequal in length; with 3 *ams* as in Fig. 3, inner *ams* of anteromedian margin (*ams 1*) blunt and bigger than other *ams* which are distinctly smaller and setiform; *mes1–2* short and blunt; epipharyngeal sensilla arranged in 2 clusters of 2-sensilla each; labral rods (lr) elongated, more sclerotized at apex and wider towards base. Mandibles (Fig. 5), with 3 apical teeth of unequal height, the inner one subapical and much smaller; *mds1–2* well developed, longitudinally placed, in distinct holes. Maxillae, each (Fig. 6) with

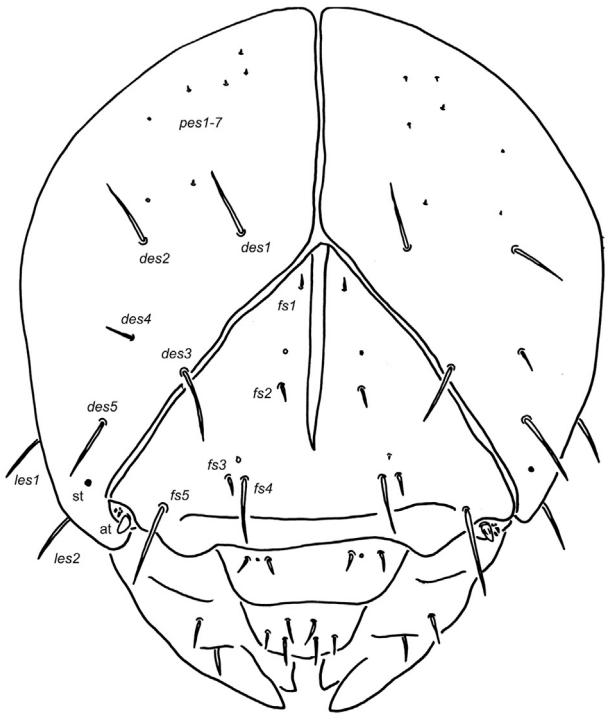

Fig. 1. *Pachytychius haematocephalus* mature larva head, frontal view: *des* – dorsal epicranial s., *fs* – frontal epicranial s., *les* – lateral epicranial s., at – antenna, st – stemma. Scale bar: 0.5 mm.

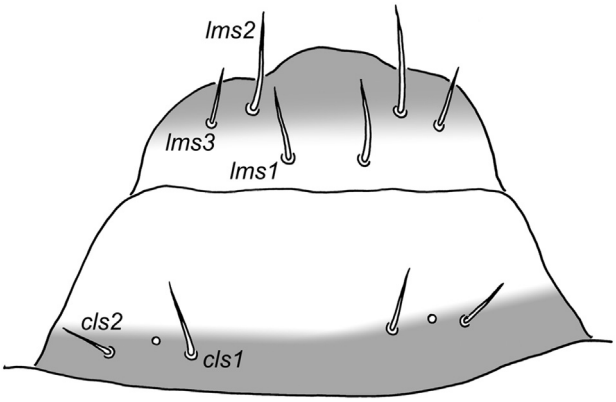

Fig. 2. *Pachytychius haematocephalus* mature larva, mouth parts: labrum and clypeus (*lms* – labral s., *cls* – clypeal s.). Scale bar: 0.2 mm.

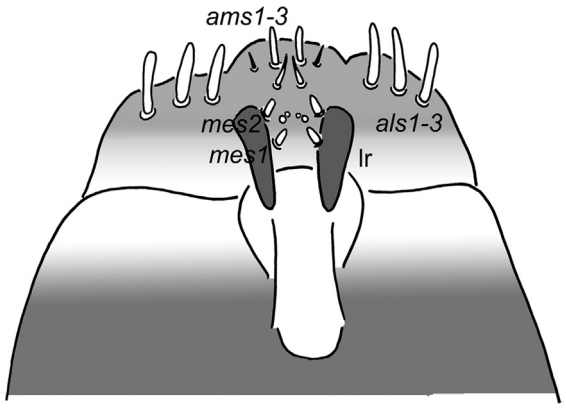

Fig. 3. *Pachytychius haematocephalus* mature larva, mouth parts: epipharynx (*ams* – anteromedian s., *als* – anterolateral s., *mes* – median s., lr – labral rods). Scale bar: 0.2 mm.

Fig. 4. *Pachytychius haematocephalus* mature larva, antenna. Scale bar: 0.1 mm.

stipes bearing 1 *stps*, 2 *pfs* and 1 min *mbs* plus sensillum; mala with row of 6 blunt *dms* and 5 *vms*, one *vms* distinctly bigger and 2 min; maxillary palpi with two palpomeres; basal palpomere with 1 very short *mxps* and 1 sensillum; length ratio of basal and distal palpomeres: 1:0.6; distal palpomere with 1 sensillum and a group of apical sensorial papillae. Labium (Fig. 6) with postmentum wide, membranous, oval shaped, with *pms1–3* located laterally, *pms2* pair slightly more widely separated than *pms1* and *pms3* pairs; *pms1* and *pms2* relatively long, *pms3* distinctly shorter; prementum with trident-shaped premental sclerite; ligula sinuate at margin, *ligs1-2* short; labial palpi with 2 palpomeres; length ratio of basal and distal palpomeres: 1:0.8. *Thorax* (Figs. 7 and 8): Spiracle bicameral, placed on prothorax. Prothorax (Fig. 8) with lightly pigmented pronotal shield, medially subdivided in two subtriangular plates; 8

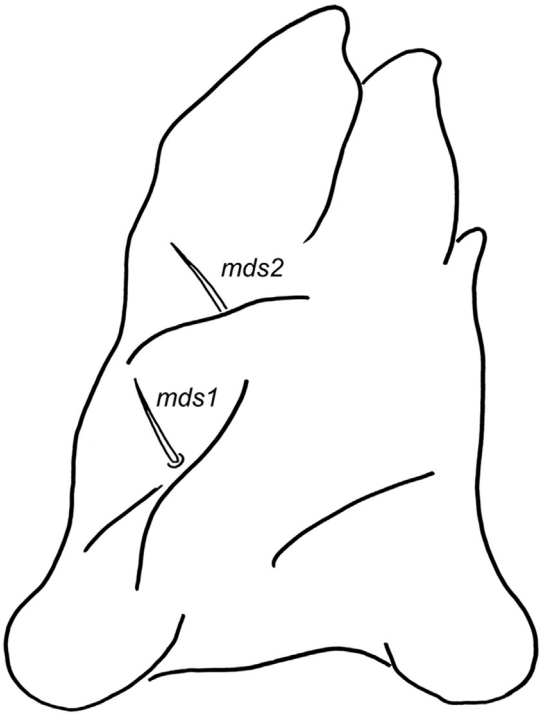

Fig. 5. *Pachytychius haematocephalus* mature larva, mouth parts: mandible (*mds* – mandible dorsal s.). Scale bar: 0.2 mm.

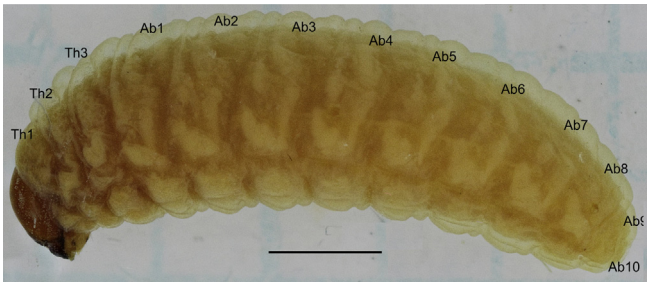

Fig. 7. *Pachytychius haematocephalus* mature larva, habitus, lateral view. Scale bar: 1 mm.

relatively long and 1 short *prns*, 2 long *ps*; and 2 relatively long *eus*. Meso- and metathorax (Fig. 8) with 4 *pds* (order: short, short, long, long); alar area with 1 very short *as*; 1 long and 1 short *ss*; 1 long *eps*; 1 long *ps*; and 2 relatively long *eus*. Pedal areas of thoracic segments well separated, each with 4 relatively long and 1–3 very short to minute *pda*. *Abdomen* (Figs. 7, 9 and 10): Abdominal segments I–VI of similar size, next abdominal segments tapering toward posterior body end. Spiracles bicameral, with airtubes posteriorly directed, the eight abdominal spiracles located laterally, close to the anterior margin of abdominal segments I–VIII. Abdominal segments I–VII (Figs. 9, 10) with 1 short *prs*; 5 *pds* (order: short, short, long, short, long); 2 short *ss*; 1 long and 1 very short to minute *eps*; 1 long and 1 very short to minute *ps*; 1 short *lsts*; and 2 relatively long *eus*. Abdominal segment VIII (Fig. 10) with 1 very short *prs*; 3 *pds* (order: short, long, short); 1 very short *ss*; 1 long and 1 short *eps*; 1 long and 1 short *ps*; 1 short *lsts*; and 2 relatively long *eus*. Abdominal segment IX (Fig. 10) with 2 *ds* (1 long, 1 short); 2 *ps* (1 long, 1 short); and 1 short *sts*; abdominal segment X (Fig. 10) with anus terminal, 4 anal lobes of unequal size, dorsal lobe largest and ventral smallest, each lateral lobe with 1 small seta (*ts*).

Pupa (Figs. 11–15). Body length 3.26–4.66 mm; body width at widest part 1.20–2.33 mm (see Supplementary Table S1). Body

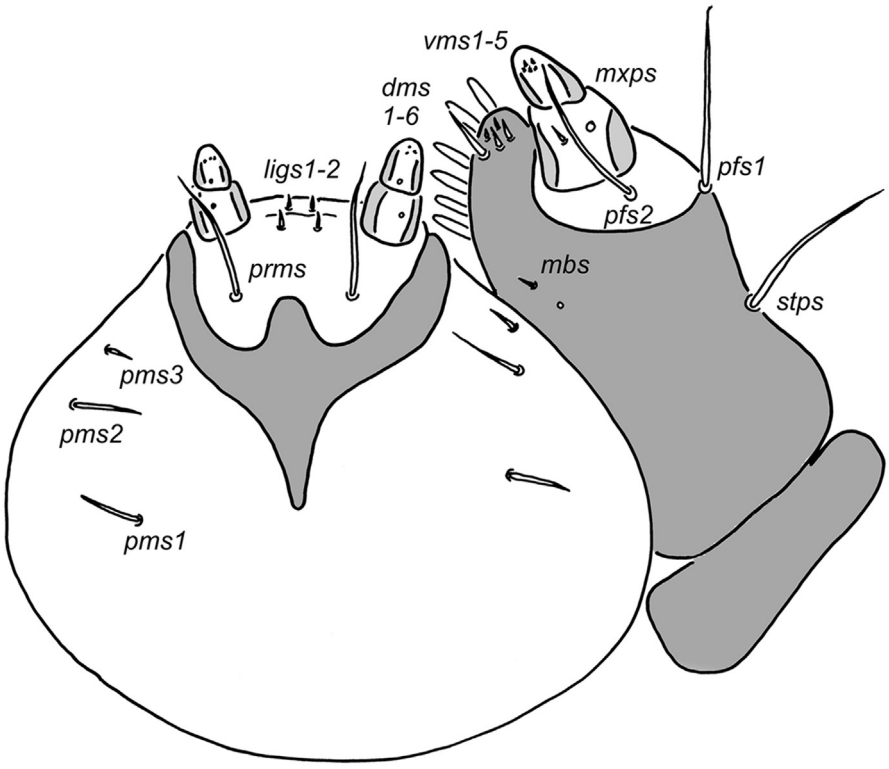

Fig. 6. *Pachytychius haematocephalus* larval mouthparts, maxillolabial complex in ventral view: maxilla (*dms* – dorsal malar s., *vms* – ventral malar s., *mpxs* – maxillary palp s., *mbs* – basioventral s., *pfs* – palpiferal s., *stps* – stipital s.), prementum and postmentum, ventral view (*prms* – premental s., *pms* – postmental s., *ligs* – ligular s.). Scale bar: 0.5 mm.

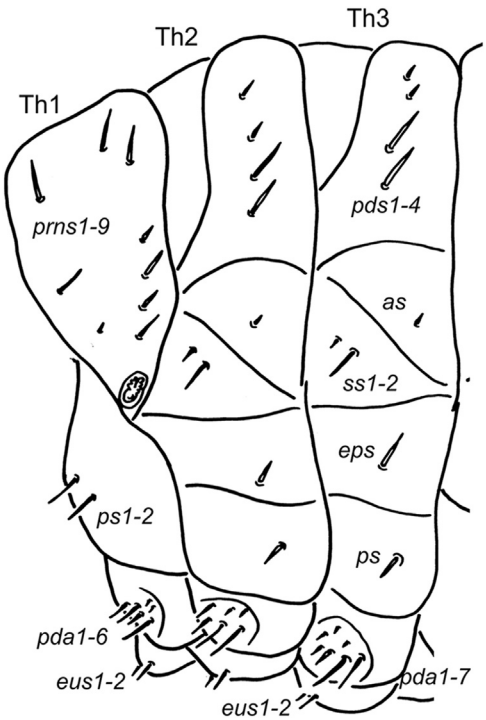

Fig. 8. *Pachytychius haematocephalus* mature larva: lateral view of thoracic segments. (*prns* – pronotal s., *pds* – postdorsal s., *as* – alar s., *ss* – spiracular s., *eps* – epipleural s., *ps* – pleural s., *pda* – pedal s., *eus* – eusternal s.). Scale bar: 1 mm.

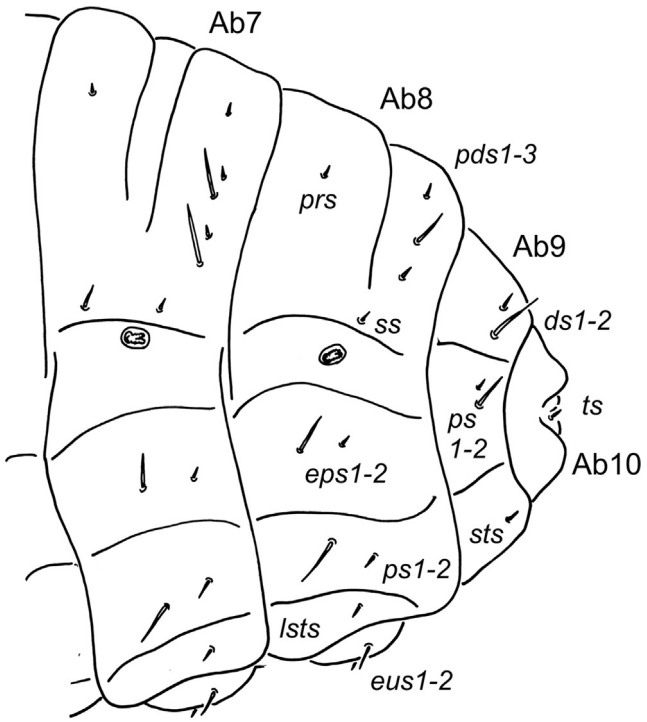

Fig. 10. *Pachytychius haematocephalus* mature larva: lateral view of abdominal segments VII-X (*pds* – postdorsal s., *ss* – spiracular s., *eps* – epipleural s., *ps* – pleural s., *lsts* – laterosternal s., *eus* – eusternal s., *ds* – dorsal s., *sts* – sternal s.). Scale bar: 1 mm.

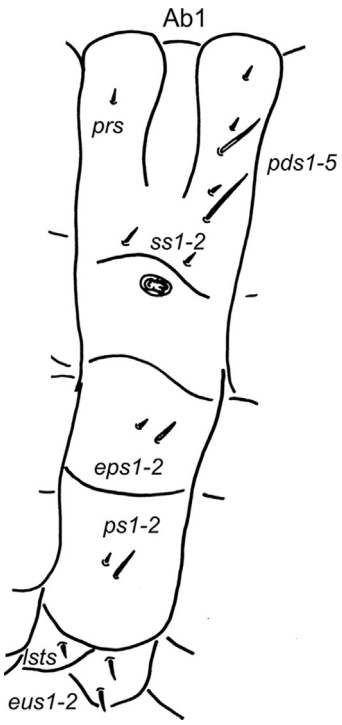

Fig. 9. *Pachytychius haematocephalus* mature larva: lateral view of abdominal segment I. (*prs* – prodorsal s., *pds* – postdorsal s., *ss* – spiracular s., *eps* – epipleural s., *ps* – pleural s., *lsts* – laterosternal s., *eus* – eusternal s.). Scale bar: 1 mm.

yellowish, cuticle smooth. Rostrum reaching mesocoxae. Pronotum 2.36 X wider than long, rounded laterally, tapering anteriorly. Mesonotum as long as metanotum. Abdominal segments I–IV of equal length; segments V–VIII tapered gradually toward end of body; IX terminal, with "pupal urogomphi" (ur) small, pointed at sclerotized apex. Spiracles placed dorso-laterally on abdominal segments I–VI, those on I–V functional, VI vestigial. *Chaetotaxy* (Figs. 11–15) [setal numbers given for one side of body]: setae rather short, placed on small protuberances. Head with 1 *vs* and 1 *os*, without *sos*; rostrum with 1 *pas* and 1 *rs* placed medially (Fig. 14). Setae on head and rostrum of similar size than those on prothorax. Pronotum with 1 *as*, 2 *sls*, 2 *ds*, 1 *pls* and 2 *ls* (Figs. 12, 15). Meso- and metathorax with 2 short *ds* placed medially on dorsum. Abdominal segments I–VIII with 2 min *ls* and 3 dorsal setae (*d*), *d1–2* close and placed medially and *d3* situated more laterally. Abdominal segment IX with 2 *ds*, 1 min seta on urogomphi. Femora with 2 setae at apex (*fes1–2*) (Figs. 11–13).

Biological notes. Caldara (1984) already reported some observations on the collection of the specimens herein described. One to three larvae were found feeding seeds in a single pod, which however did not show external signs of infection. When fully grown, larvae left the pods after making a small hole and reached the ground, where they soon began to burrow tunnelling through the soil for several hours. Then they began to form a pupal cell with viscid anal secretions and small grains of soil. Pupation was observed 7–10 days after the larvae entered the soil. Adults were found in the cells 10 days later and free in the soil after other 10 days.

Remarks. *P. haematocephalus* is a common species distributed in the Mediterranean area at north from Iberian Peninsula to the Balkans and at south in Morocco and Algeria. Moreover, it was accidentally introduced, although still rare, in U.S.A (eastern coast: New York, Massachussets). This species was placed by Caldara (1978) in an informal group composed of about 15 species characterized by rectangular long elytra, hind femora toothed and penis completely sclerotized. Before the record of *P. haematocephalus* feeding on *A. monspessulanus* by Caldara, (1984), this species was quoted as probably living on other species of Fabaceae, such as *Lotus corniculatus* L (Hoffmann, 1958; Perris, 1873; Dieckmann, 1986), and *L. hispidus* Desf (Dieckmann, 1986).

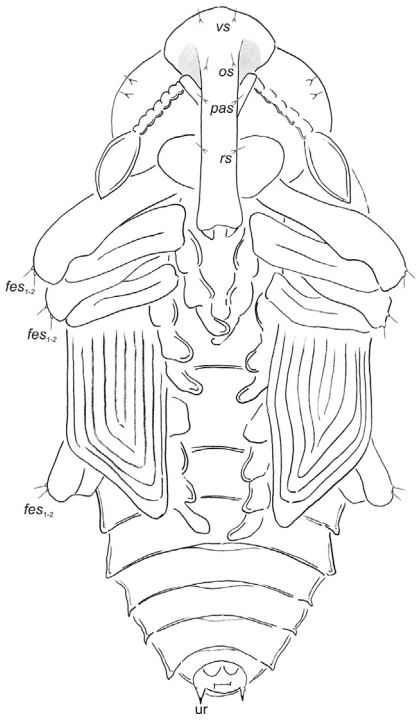

Fig. 11. *Pachytychius haematocephalus* pupa: habitus, ventral view (setae; *fes* – femoral s., *os* – orbital s., *pas* – postantennal s., *rs* – rostral s., *vs* – vertical s.; ur – urogomphi).

Fig. 13. *Pachytychius haematocephalus* pupa: habitus, lateral view (setae; *as* – apical s., *d* – dorsal s., *ds* – discal s., *fes* – femoral s., *l* – lateral, *ls* – lateral s., *pls* – posterolateral s.; ur – urogomphi).

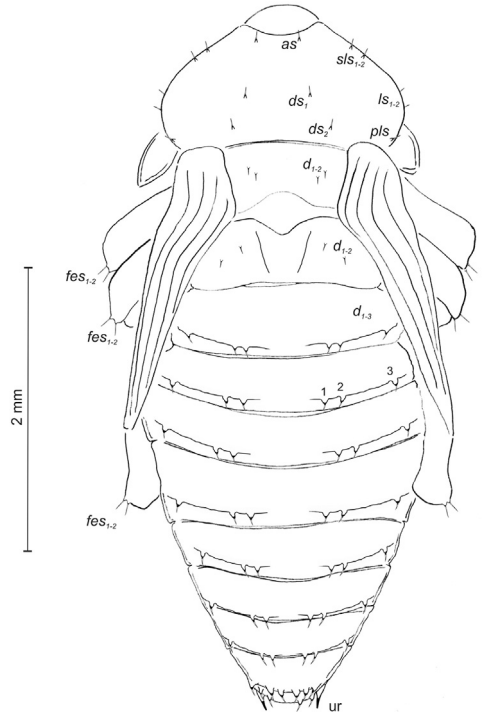

Fig. 12. *Pachytychius haematocephalus* pupa: habitus, dorsal view (setae; *as* – apical s., *d* – dorsal s., *ds* – discal s., *fes* – femoral s., *ls* – lateral s., *pls* – posterolateral s.; ur – urogomphi).

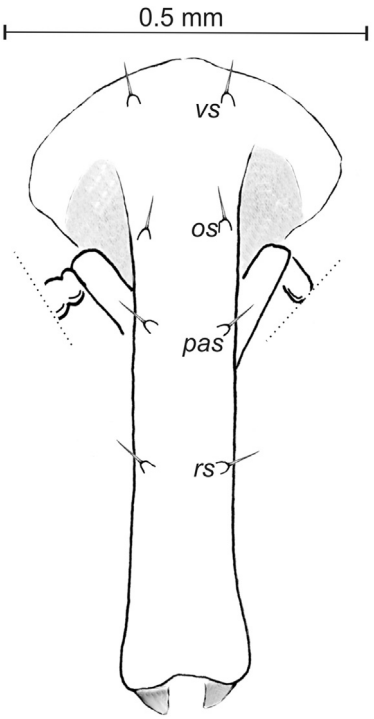

Fig. 14. *Pachytychius haematocephalus* pupa: magnification of head (setae; *os* – orbital s., *pas* – postantennal s., *rs* – rostral s., *vs* – vertical s.).

3.1.2. Pachytychius sparsutus (Olivier, 1807)

Material examined. **Italy**: Piemonte, Alessandria province, Dernice 600 m, July 2015, 74 larvae of different instars from seeds of *Genista pilosa* L. (Fabaceae) (leg. R. Caldara). Determined by association with adults collected on the same plants a month before.

Mature larva (Figs. 16–25). *Dimensions*: body length 5.75–7.39 mm (mean 6.65 mm); maximum body width at mid abdomen, up to 2.01 mm; head width 0.80–1.00 mm (mean 0.93 mm). *G. aspect* (Fig. 22): body elongated, slightly curved,

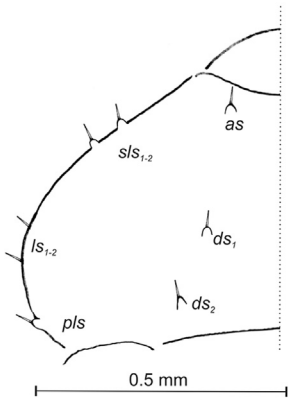

Fig. 15. *Pachytychius haematocephalus* pupa: magnification of pronotum (setae; *as* – apical s., *ds* – discal s., *ls* – lateral s., *pls* – posterolateral s.).

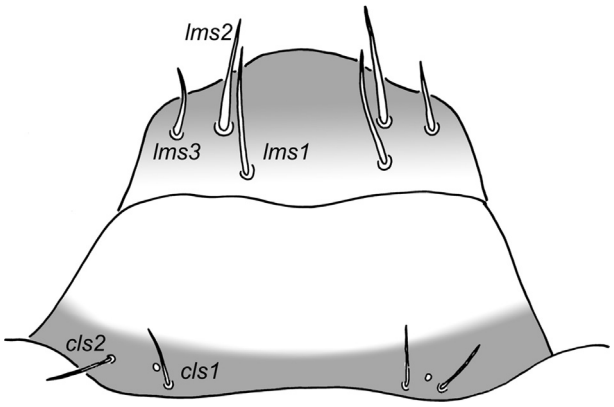

Fig. 17. *Pachytychius sparsutus* mature larva, mouth parts: labrum and clypeus (*lms* – labral s., *cls* – clypeal s.). Scale bar: 0.2 mm.

rounded in cross section; head brownish, pale to dark, thoracic and abdominal segments white to whitish yellow, body vestiture of setae thin, of various lengths from minute to relatively long. *Head* (Fig. 16): exposed, head capsule suboval in outline; endocarinal line distinct, two-thirds as long as frons; frontal sutures distinct in entire length up to antennae; only anterior stemma (st) present, as small dark pigmented spot with convex cornea. Cranial setae (Fig. 16): *des1*, *des2*, *des3 and des5* long, *des4 short*, *des3* located on epicranium close to frontal suture; *fs1* minute to very short, *fs2* short, *fs3* minute or very short, *fs4* and *fs5* long, well developed; *les1* and *les2* as long as *des1* or *fs5*; *ves1–2* relatively long; postepicranial area with minute setae and sensilla (together 6–8). Antennae (Figs. 16, 19), with oblique position on each side at anterior margin of head; membranous basal segment convex, bearing subconical, longer than wide, sensorium, and 5 small sensilla different in length (Fig. 19). Clypeus (Fig. 17), approximately 3 X wider than

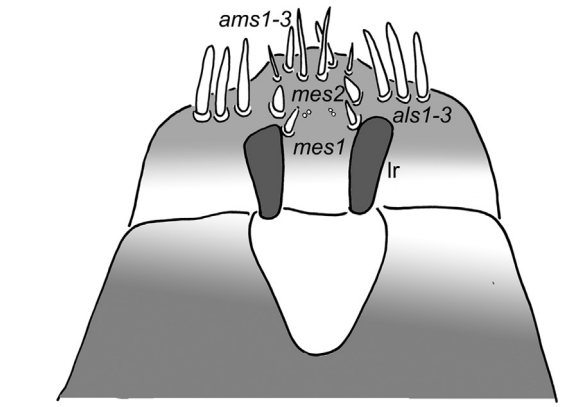

Fig. 18. *Pachytychius sparsutus* mature larva, mouth parts: epipharynx (*ams* – anteromedian s., *als* – anterolateral s., *mes* – median s., lr – labral rods). Scale bar: 0.2 mm.

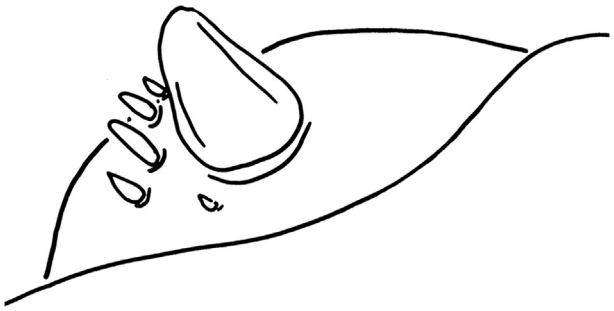

Fig. 19. *Pachytychius sparsutus* mature larva, antenna. Scale bar: 0.1 mm.

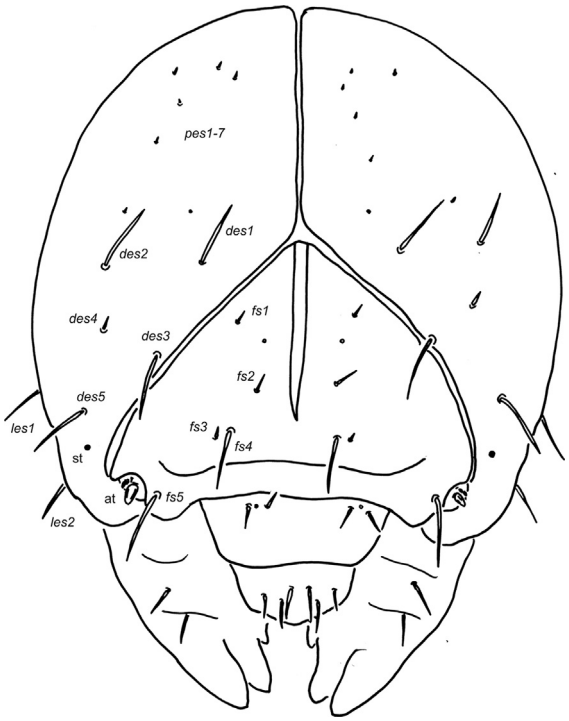

Fig. 16. *Pachytychius sparsutus* mature larva head, frontal view: *des* – dorsal epicranial s., *fs* – frontal epicranial s., *les* – lateral epicranial s., at – antenna, st – stemma. Scale bar: 0.5 mm.

long, *cls1–2* well developed, with sensillum between them; anterior margin slightly concave. Labrum (Fig. 17) approximately 2 X wider than long, anterior margin bisinuate; *lms1–3* well developed, *lms1* and *lms2* longer than *lms3*. Epipharynx (Fig. 18) with 3 blunt *als*, subequal in length; with 3 *ams* as in Fig. 18, pair of "intermediate" *ams* (*ams 3*) elongated, rather blunt and distinctly longer than *ams1* and *ams2*; *mes1–2* short; epipharyngeal sensilla arranged in 2 clusters of 2-sensilla each; labral rods (lr) elongated, more sclerotized at apex and wider towards base. Mandibles (Fig. 20), with 3 apical teeth of unequal height, the inner one subapical and much smaller; *mds1–2* well developed, longitudinally placed, in distinct holes. Maxillae, each (Fig. 21) with stipes bearing 1 *stps*, 2 *pfs* and 1 min *mbs* plus sensillum; mala with row of 6 blunt *dms* and 5 *vms*, one *vms* bigger and 2 min; maxillary palpi with two

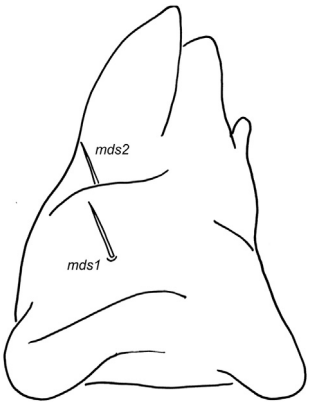

Fig. 20. *Pachytychius sparsutus* mature larva, mouth parts: mandible (*mds* – mandible dorsal s.). Scale bar: 0.2 mm.

palpomeres; basal palpomere with 1 very short *mxps* and 1 sensillum; length ratio of basal and distal palpomeres: 1:0.7; distal palpomere with 1 sensillum and a group of apical sensorial papillae. Labium (Fig. 21) with postmentum wide, membranous, oval shaped, with *pms1–3* located laterally, *pms2* pair slightly more widely separated than *pms1* and *pms3* pairs; *pms2* relatively long, *pms1* and *pms3* distinctly shorter; prementum with trident-shaped premental sclerite; ligula sinuate at margin, *ligs1–2* short; labial palpi with 2 palpomeres; length ratio of basal and distal palpomeres: 1:0.8. *Thorax* (Figs. 22, 23): Spiracle bicameral, placed on prothorax. Prothorax (Fig. 23) with lightly pigmented pronotal shield, medially subdivided in two subtriangular plates; 9 relatively long and 1–2 short *prns*; 2 long *ps*; and 2 relatively long *eus*. Meso- and metathorax (Fig. 23) with 4 *pds* (order: short, short, long, long); alar area with 1 very short *as*; 1 long and 1 short *ss*; 1 long *eps*; 1 long *ps*; and 2 relatively long *eus*. Pedal areas of thoracic segments well separated, each with 5 relatively long and 1–2 very short to minute *pda*.

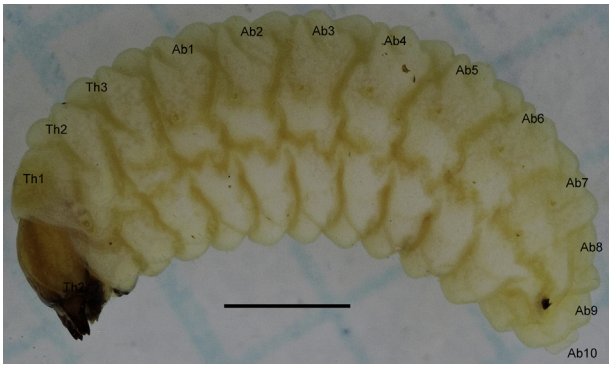

Fig. 22. *Pachytychius sparsutus* mature larva, habitus, lateral view. Scale bar: 1 mm.

Abdomen (Figs. 22, 24 and 25): Abdominal segments I–VI of similar size, next abdominal segments tapering toward posterior body end. Spiracles bicameral, with airtubes posteriorly directed, the eight abdominal spiracles located laterally, close to the anterior margin of abdominal segments I–VIII. Abdominal segments I–VII (Figs. 24 and 25) with 1 short *prs*; 5 *pds* (order: short, short, long, short, long); 2 short *ss*; 1 long and 1 very short to minute *eps*; 1 long and 1 very short to minute *ps*; 1 short *lsts*; and 2 relatively long *eus*. Abdominal segment VIII (Fig. 25) with 1 very short *prs*; 3 *pds* (order: short, long, short); 1 very short *ss*; 1 long and 1 short *eps*; 1 long and 1 short *ps*; 1 short *lsts*; and 2 relatively long *eus*. Abdominal segment IX (Fig. 25) with 1 long *ds*; 2 *ps* (1 long, 1 short); and 1 short *sts*; abdominal segment X (Fig. 25) with anus terminal, 4 anal lobes of unequal size, dorsal lobe largest and ventral smallest, each lateral lobe with 1 small seta (*ts*).

Biological notes. Only one living larva was found feeding on seeds in a single pod, which had no evident external signs of infection. In the field, mature larvae left the pods reaching the soil only when the two parts of the pods, still on the trees, opened

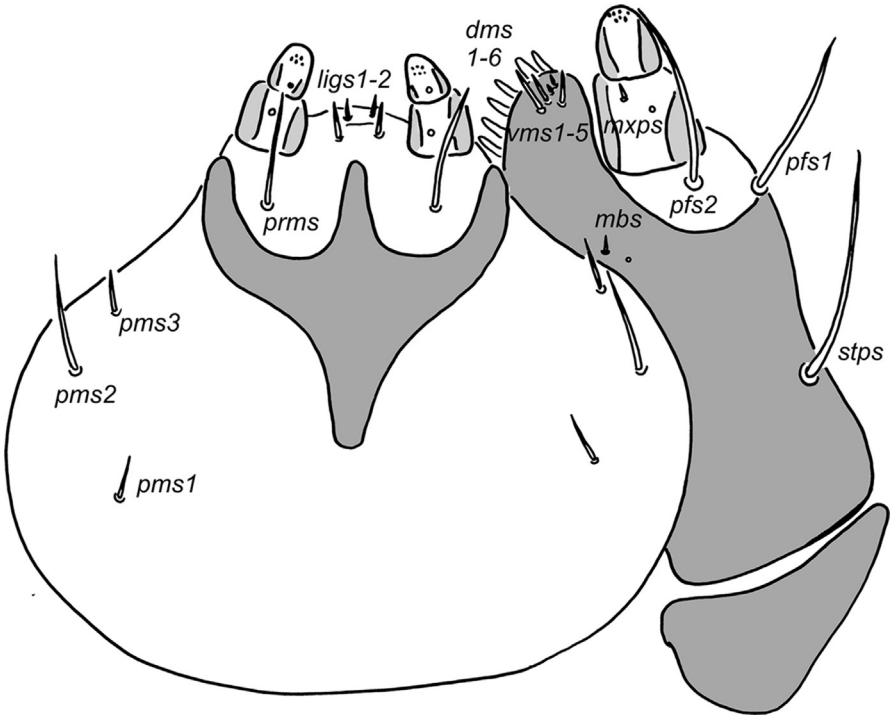

Fig. 21. *Pachytychius sparsutus* larval mouthparts, maxillolabial complex in ventral view: maxilla (*dms* – dorsal malar s., *vms* – ventral malar s., *mpxs* – maxillary palp s., *mbs* – basioventral s., *pfs* – palpiferal s., *stps* – stipital s.), prementum and postmentum, ventral view (*prms* – premental s., *pms* – postmental s., *ligs* – ligular s.). Scale bar: 0.5 mm.

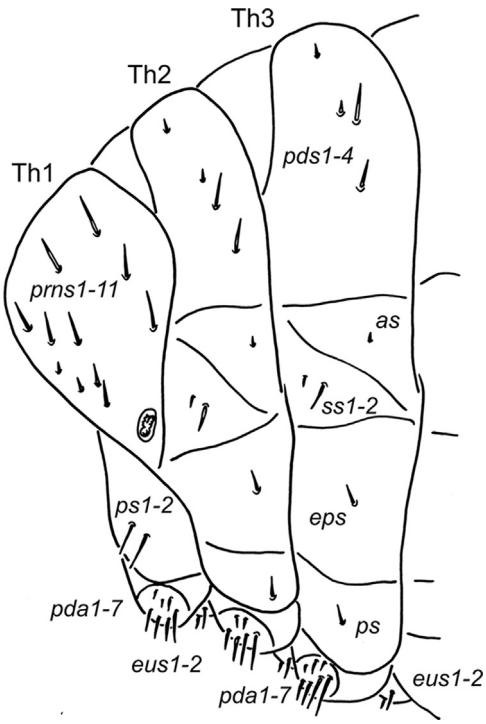

Fig. 23. *Pachytychius sparsutus* mature larva, habitus, lateral view of thoracic segments. (*prns* – pronotal s., *pds* – postdorsal s., *as* – alar s., *ss* – spiracular s., *eps* – epipleural s., *ps* – pleural s., *pda* – pedal s., *eus* – eusternal s.). Scale bar: 1 mm.

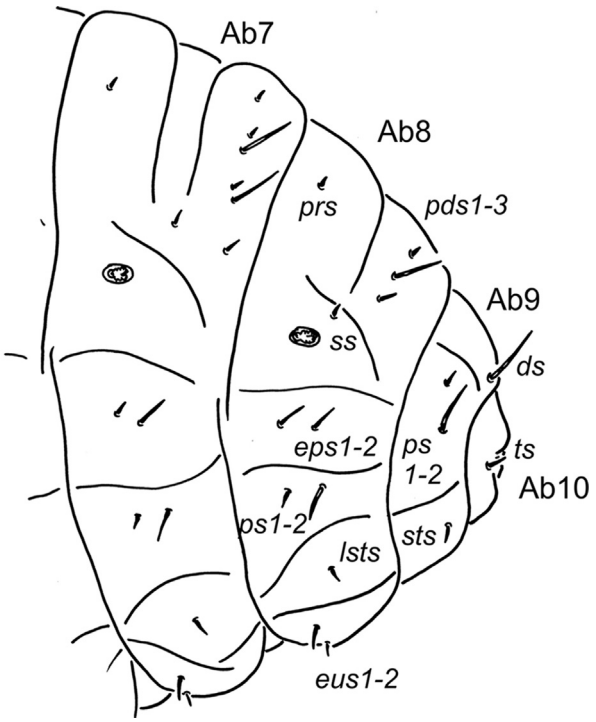

Fig. 25. *Pachytychius sparsutus* mature larva, habitus, lateral view of abdominal segments VII-X (*pds* – postdorsal s., *ss* – spiracular s., *eps* – epipleural s., *ps* – pleural s., *lsts* – laterosternal s., *eus* – eusternal s., *ds* – dorsal s., *sts* – sternal s.). Scale bar: 1 mm.

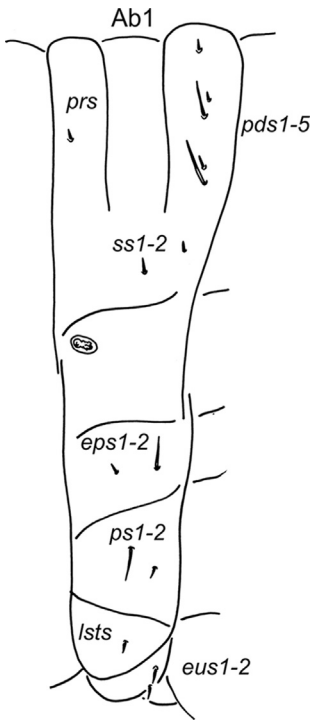

Fig. 24. *Pachytychius sparsutus* mature larva, habitus, lateral view of abdominal segment I. (*prs* – prodorsal s., *pds* – postdorsal s., *ss* – spiracular s., *eps* – epipleural s., *ps* – pleural s., *lsts* – laterosternal s., *eus* – eusternal s.). Scale bar: 1 mm.

spontaneously and forcefully. They did not exit by making a hole in the pods. The same happened in laboratory when closed pods were collected. Once on the soil, larvae began immediately to burrow under the surface tunnelling through the soil for several hours. Then they stopped but did not build a cocoon and did not pupate. Most of them remained alive during the winter until the beginning of the spring and moved rhytmically only when disturbed by a pin. Unfortunately in February and March all of them died. A similar behaviour was observed by Gosik et al. (2017) when rearing larvae of *Lignyodes enucleator* (Panzer), which also survived as larvae during the winter but died before pupation in spring. It seems that in captivity, the artificial conditions negatively affected the metamorphosis in both circumstances. It is noteworthy to mention, however, that Hoffmann (1958) reported that larvae of *Pachytychius sparsutus* pupated in soil and adults emerged about 12 days later.

Remarks. *P. sparsutus* is distributed in nearly all the Mediterranean states, except for Eastern North Africa, and in Central Europe (Caldara 2013). This is the type species of the genus, being clearly different from all the other species of the genus, especially because of the shape of the rostrum and that of the penis. Due to some similarities in the shape of the penis, it was placed near *Pachytychius sobrinus* Tournier, 1874, presently synonymous with *Pachytychius sinaiticus* (Crotch, 1872), from North Africa and Middle East (Caldara 1978). Hoffmann (1958) quoted different host plants, all belonging in the Fabaceae: *Cytisus sessilifolius* L., *Sarothamnus scoparius* L., *Ulex europaeus* L., *Genista tinctoria* L. and *G. pilosa* L., on the last of which our specimens were collected.

3.2. Molecular analysis

The phylogenetic tree resulting from maximum likelihood is shown in Fig. 26. Those clades that in ML tree are well supported (80% or higher ML Bootstrap) are also present in the strict consensus of 8 MPT (not shown) recovered with Parsimony analysis. The phylogenetic hypothesis based on COI show that

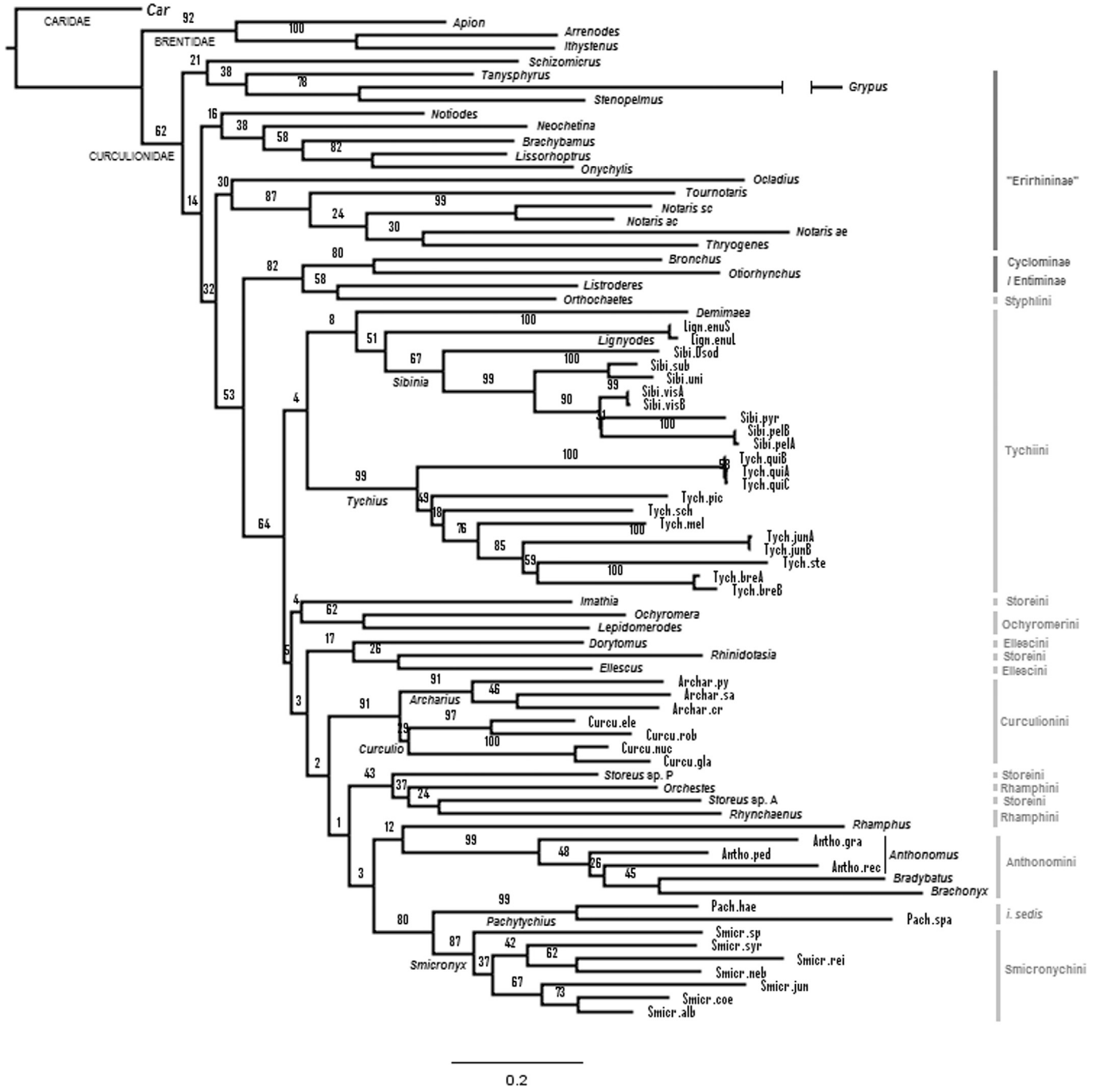

Fig. 26. Phylogenetic tree of COI sequences of selected species of *Pachytychius* and other genera in Curculionidae plus outgroups, based on maximum likelihood. Numbers above branches are ML bootstrap values. See Appendix for complete taxon names, classification, and access codes of sequences.

Pachytychius is nested within a clade of curculionine weevils, being most closely related to genus *Smicronyx*.

4. Discussion

Larvae of *P. haematocephalus* and *P. sparsutus* are morphologically very similar, and most of the characters (except dimensions) provided in both descriptions above may prove helpful in defining the genus *Pachytychius*, with few characters informative to distinguish species. Among the later, they are worth to mention: the shape and size of the "intermediate" pair of *ams* on epipharynx (conspicuous, elongated, blunt at apex in *P. sparsutus*; small, pointed at apex in *P. haematocephalus*) and the number of dorsal setae on abdominal segment IX (one in *P. sparsutus*; two in *P. haematocephalus*).

The larvae of *Pachytychius* have *des3* on epicranium and a conspicuous endocarinal line, features expected to occur in Curculioninae (e. g., Marvaldi, 2003), but not to be found in erirhinine weevils (Brachycerinae Erirhinini sensu Oberprieler, 2014) which are characterised, besides some characters that could be adaptive to aquatic habits, by having *des3* in the frontal line or on frons (also in the clade Cyclominae/Entiminae) and by lacking endocarinal line

(Marvaldi 1997). Similarly, larval characters of *Pachytychius* do not support a close relationship with Styphlini, a tribe near which *Pachytychius* was placed by Morris, (1995). In fact, and besides the Styphlini are in Curculioninae in current weevil classifications, larvae of this tribe studied by Nazarenko (2011), in genus *Paraphilernus* Desbrochers des Loges, 1892, have *des3* on frons and vestigial endocarinal line, suggesting that such an assignment could be erroneous, more so after considering that several aspects of their biology are also atypical for Curculioninae (Caldara et al., 2014). Our molecular results from COI (shown in Fig. 26) are in agreement with larval evidence discussed above, recovering *Pachytychius* within the curculionine weevils and not in close position with any of the erirhinine taxa or with the Styphlini (as represented by *Orthochaetes*), being the latter most likely members of a clade "Cyclominae-Entiminae", again in accordance with larval and biological evidence.

Several characters observed in the larvae of *Pachytychius* herein described are held in common with larvae of tribes in Curculioninae (particularly those sampled in this study, see Appendix), most of which are listed in the fundamental comparative study by May (1993) and herein revised after consulting other sources like Scherf (1964), Anderson (1991) and many others focused on particular taxa, like Anthonomini (e. g., Ahmad & Burke 1972), Ochyromerini (Oberprieler 1993), Smicronychini (Frilli 1966; Oseto & Braness 1979), Tychiini (e. g., Skuhrovec et al. 2014, 2015; Gosik et al. 2017). Such characters could be considered diagnostic of the larvae of curculionine weevils: *fs1,2,3* minute to absent; endocarinal line present; mandibular setae aligned longitudinally; labral rods subparallel to slightly convergent; postmentum with setae aligned laterally, with proximal pairs (*pms1* and *pms2*) separated by equal or similar distance; meso- and metathorax with *pds* arranged short, short, long, long; abdominal segment VIII with 3 *pds*; all spiracles bicameral; abdominal spiracles with airtubes posteriorly directed. In addition, we found that the larvae of *Pachytychius* have 2-sensilla per cluster in the epipharynx (Figs. 3, 18) like in those of other genera in Curculioninae (e. g., Anthonomini, Tychiini, Curculionini, Smicronychini, etc, but not some "Storeini" of May (1993), while other Curculionidae typically have 3-sensilla per cluster. Also, only the anterior pair of stemmata is present.

Larvae of *Pachytychius* differ from those known in Storeini *s. l* (e. g., May 1993, 1994), which show important setal reductions on frons and epicranium, usually just *fs4* and two *des* distinct in storeine larvae, whereas *Pachytychius* has *fs4 and fs5* and four *des* rather well developed. The non-monophyly of Storeini herein established with the COI sequences (Fig. 26) certainly requires further testing with more data, but for the aim of the present study it may suffice to note that *Pachytychius* did not group with any Storeini. Then, evidence from larvae and COI sequences add support for the decision by Caldara et al. (2014), who rejected the placement of *Pachytychius* in this tribe proposed by Alonso-Zarazaga & Lyal (1999) on the basis of adult morphology. On the other hand, the close relationship between *Pachytychius* and *Smicronyx* herein found based on COI evidence (Fig. 26) promoted a shift of focus towards comparing immature stages of these taxa. We note that larvae of *Pachytychius* and *Smicronyx* have similar tridentate mandibles, with two teeth robust at apex and a third smaller placed subapically at inner side. They are both also similar to Anthonomini, having *fs4* and *fs5* both well developed and subequal in length and all abdominal spiracles placed laterally, while larvae of other curculionines often have *fs5* reduced or distinctly shorter than *fs4* and the spiracle of Abdominal segment VIII placed on dorsum. The known pupae of *Pachytychius* and *Smicronyx* also seem to share some diagnostic characters in chaetotaxy (that distinguish them from Tychiini and some other curculionines): on pronotum they have only 1 apical seta (*as*), on abdominal segments I–VIII they have 3 dorsal setae (*d*); on abdominal segment IX they have 2 lateral setae (*l*) and 1 small seta on each urogomphi.

In summary, the morphological evidence from the immature stages of *Pachytychius*, as well as the bionomic data (i.e., larval development in plant reproductive structures and pupation in soil), agree with a placement in subfamily Curculioninae and are particularly consistent with a close relationship to *Smicronyx*. Regarding molecular evidence from the 5′region of the COI, phylogenetic relationships reconstructed via maximum likelihood and parsimony analyses, indicate that *Pachytychius* is not closely related to the erirhinines with pedotectal type of male genitalia, and belongs instead in the group of "higher weevils" with the derived type of male genitalia, among genera currently classified in Curculioninae and forming a clade with *Smicronyx*. Finally, the adult characters traditionally used to define the weevil tribe Smicronychini (Anderson 1962; Casey 1892) need to be re-evaluated. We conclude that the degree of connation of the tarsal claws is not consistent enough to characterize the tribe, as predicted early on by LeConte & Horn (1876), being instead of better use in diagnosing individual genera or species within it. Other characters seem to be more robust in defining the tribe, allowing inclusion of *Pachytychius* within Smicronychini: mandibles toothed on outer edge; antennal scrobes obliquely directed and almost confluent in ventral side of rostrum; prosternum emarginate anteriorly; ventrites with sutures very slightly curved at sides.

5. Conclusions

In this paper we made an assessment of the systematic position of genus *Pachytychius* considering new information on immature stages, phylogenetic analysis of COI sequences, and a re-evaluation of adult characters. The structural features of the larva and pupa of *Pachytychius*, as well as bionomic data, agree with those commonly found in subfamily Curculioninae and are consistent with a close relationship with *Smicronyx*. In addition, molecular evidence from COI also support the phylogenetic placement of *Pachytychius* among the curculionine weevils and close to *Smicronyx*. These findings lead to consider several adult morphological characters which seem to corroborate the proposed systematic position of genus *Pachytychius*, and of its allied genus *Aubeonymus*, in tribe Smicronychini.

Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to submit the article for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcz.2018.08.007.

Appendix. Taxon sampling and sequences used in this study on *Pachytychius*. Classification follows the *Handbook of Zoology,Coleoptera, Beetles*, vol. 3. (Leschen & Beutel, 2014). In this system subfamily Brachycerinae *s. l.* includes the former subfamily Erirhininae (*sensu* Kuschel 1971).
CURC = Curculionidae.

FAMILY Subfamily	Tribe	Species	Accession code
1 CARIDAE Carinae		*Car condensatus*	LN889437
2 BRENTIDAE Brentinae	Brentini	*Arrenodes minutus*	KR483412
3 BRENTIDAE Brentinae	Trachelizini	*Ithystenus* sp.	LN889508
4 BRENTIDAE Apioninae	Apionini	*Apion frumentarium*	KM451581
5 CURC Brachycerinae	Erirhinini	*Ocladius* sp.	FJ867815
6 CURC Brachycerinae	Tanysphyrini	*Brachybamus electus*	KJ672293
7 CURC Brachycerinae	Tanysphyrini	*Lissorhoptrus oryzophilus*	KJ672285
8 CURC Brachycerinae	Erirhinini	*Notaris acridulus*	KJ966613
9 CURC Brachycerinae	Erirhinini	*Notaris scirpi*	KJ963431
10 CURC Brachycerinae	Erirhinini	*Notaris aethiops*	KJ672289
11 CURC Brachycerinae	Tanysphyrini	*Neochetina eichhorniae*	BBCCA1435-12
12 CURC Brachycerinae	Erirhinini	*Tournotaris bimaculatus*	BBCCN244-10
13 CURC Brachycerinae	Erirhinini	*Grypus equiseti*	COLFE1160-13
14 CURC Brachycerinae	Raymondionymini	*Schizomicrus caecus*	FJ867824
15 CURC Brachycerinae	Tanysphyrini	*Stenopelmus rufinasus*	KM440642
16 CURC Brachycerinae	Tanysphyrini	*Tanysphyrus lemnae*	KM443047
17 CURC Brachycerinae	Erirhinini	*Thryogenes festucae*	COLFF672-13
18 CURC Brachycerinae	Tanysphyrini	*Notiodes celatus*	KJ672283
19 CURC Brachycerinae	Tanysphyrini	*Onychylis longulus*	KJ672284
20 CURC Cyclominae	Hipporhinini	*Bronchus* sp.	FJ867830
21 CURC Cyclominae	Listroderini	*Listroderes costirostris*	JWBP085-15
22 CURC Entiminae	Otiorhynchini	*Otiorhynchus sulcatus*	KJ962464
23 CURC Curculioninae	Smicronychini	*Smicronyx* sp.	FJ867825
24 CURC Curculioninae	Smicronychini	*Smicronyx reichi*	GCOL13226-16
25 CURC Curculioninae	Smicronychini	*Smicronyx jungermanniae*	KM439701
26 CURC Curculioninae	Smicronychini	*Smicronyx coecus*	FBCOQ197-13
27 CURC Curculioninae	Smicronychini	*Smicronyx albosquamosus*	KU942332
28 CURC Curculioninae	Smicronychini	*Smicronyx syriacus*	KU942326
29 CURC Curculioninae	Smicronychini	*Smicronyx nebulosus*	KU942319
30 CURC Curculioninae	Anthonomini	*Anthonomus grandis*	JQ941952
31 CURC Curculioninae	Anthonomini	*Anthonomus pedicularis*	KM450808
32 CURC Curculioninae	Anthonomini	*Anthonomus rectirostris*	KM450830
33 CURC Curculioninae	Anthonomini	*Bradybatus kellneri*	KM452488
34 CURC Curculioninae	Anthonomini	*Brachonyx pineti*	KJ963383
35 CURC Curculioninae	Curculionini	*Curculio glandium*	KM452053
36 CURC Curculioninae	Curculionini	*Curculio nucum*	KJ963139
37 CURC Curculioninae	Curculionini	*Curculio elephas*	KM442041
38 CURC Curculioninae	Curculionini	*Curculio robustus*	CJWBP072-15
39 CURC Curculioninae	Curculionini	*Archarius salicivorus*	KJ966576
40 CURC Curculioninae	Curculionini	*Archarius crux*	KJ962421
41 CURC Curculioninae	Curculionini	*Archarius pyrrhoceras*	KJ962452
42 CURC Curculioninae	Storeini	*Storeus* sp. - Papua	LN889496
43 CURC Curculioninae	Storeini	*Storeus* sp. - Australia	LN889490
44 CURC Curculioninae	Storeini	*Rhinidotasia edentata*	LN889529
45 CURC Curculioninae	Storeini	*Imathia* sp.	LN889497
46 CURC Curculioninae	Ellescini	*Ellescus scanicus*	KM452266
47 CURC Curculioninae	Ellescini	*Dorytomus longimanus*	KM439107
48 CURC Curculioninae	Ochyromerini	*Ochyromera cf. sericea*	LN889498
49 CURC Curculioninae	Ochyromerini	*Lepidomerodes* sp.	LN889500
50 CURC Curculioninae	Rhamphini	*Rhamphus pulicarius*	KJ962003
51 CURC Curculioninae	Rhamphini	*Orchestes avellanae*	KM441716
52 CURC Curculioninae	Rhamphini	*Rhynchaenus lonicerae*	KJ966424
53 CURC Curculioninae	Styphlini	*Orthochaetes setiger*	KM359705
54 CURC Curculioninae	Tychiini	*Demimaea cf. strumosa*	LN889492
55 CURC Curculioninae	Tychiini	*Lignyodes enucleator* - Leipzig	KM444895
56 CURC Curculioninae	Tychiini	*Lignyodes enucleator* - Slovenia	KM450723
57 CURC Curculioninae	Tychiini	*Tychius quinquepunctatus* - A	KM450723
58 CURC Curculioninae	Tychiini	*Tychius quinquepunctatus* - B	GCOL11398-16
59 CURC Curculioninae	Tychiini	*Tychius quinquepunctatus* - C	GCOL3700-16
60 CURC Curculioninae	Tychiini	*Tychius junceus* - A	KM440677
61 CURC Curculioninae	Tychiini	*Tychius junceus* - B	KJ967239
62 CURC Curculioninae	Tychiini	*Tychius meliloti*	BBCCA3788-12
63 CURC Curculioninae	Tychiini	*Tychius breviusculus* - A	KJ965422
64 CURC Curculioninae	Tychiini	*Tychius breviusculus* - B	KM442623
65 CURC Curculioninae	Tychiini	*Tychius schneideri*	KM442126
66 CURC Curculioninae	Tychiini	*Tychius picirostris*	KJ963992

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FAMILY Subfamily	Tribe	Species	Accession code
67 CURC Curculioninae	Tychiini	*Tychius stephensi*	RRMPE175-15
68 CURC Curculioninae	Tychiini	*Sibinia viscariae* - A	KM444698
69 CURC Curculioninae	Tychiini	*Sibinia viscariae* - B	KJ961796
70 CURC Curculioninae	Tychiini	*Sibinia pellucens* - A	KM440637
71 CURC Curculioninae	Tychiini	*Sibinia pellucens* - B	KM441327
72 CURC Curculioninae	Tychiini	*Sibinia subelliptica*	GCOL11928-16
73 CURC Curculioninae	Tychiini	*Sibinia pyrrhodactyla*	GCOL12885-16
74 CURC Curculioninae	Tychiini	*Sibinia unicolour*	GCOL5653-16
75 CURC Curculioninae	Tychiini	*Sibinia (Dichotychius) sodalis*	GCOL11425-16
76 CURC Curculioninae	Incertae sedis	*Pachytychius sparsutus*	KM439725
77 CURC Curculioninae	Incertae sedis	*Pachytychius haematocephalus*	KM445855

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