



A new *Typhlocharis* (Coleoptera: Carabidae: Anillina) from Spain: combining adult and larval morphological data with DNA information

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Abstract

Adult and larva of *Typhlocharis toletana* **n. sp.** (Coleoptera: Carabidae: Anillini) are described from the province of Toledo, Central Spain. Identical mitochondrial *cox1* gene haplotype suggested that the adults and larvae are conspecific. *Typhlocharis toletana* is the third species of the genus with an unguiform shape of gonocoxite 2 of the ovipositor, a character state that is considered as ancestral. However, *T. toletana* is not closely related to the other two species having a similarly shaped gonocoxite 2, as judged by their morphological characters and geographic distribution. It is concluded that this ancestral state has been kept in different lineages within *Typhlocharis*. Species relationships inferred from a preliminary molecular analysis of five *Typhlocharis* taxa are not congruent with putative morphological lineages. Finally, it was found that the larva of *T. toletana* is morphologically indistinguishable from that of *Typhlocharis* sp. described by Arndt et al. (1999).

Key words: Taxonomy, new species, *Typhlocharis toletana*, Iberian Peninsula, larval description, DNA identification, unguiform gonocoxite, lineages

Resumen

Se describe el adulto y la larva de *Typhlocharis toletana* **n. sp.** (Coleoptera: Carabidae: Anillini), procedente de la cuenca media del río Tajo (provincia de Toledo, España). La identidad conespecífica de ambos estadios se corrobora mediante el análisis de la secuencia del gen mitocondrial *cox1*. *T. toletana* es la tercera especie del género que presenta los gonocoxitos del ovipositor unguiformes, característica que se considera ancestral. Sin embargo, *T. toletana* no es parecida a las otras dos especies en relación a otros caracteres morfológicos y la distribución geográfica. Se concluye que dicha característica ancestral se ha conservado en diferentes linajes de *Typhlocharis*. Las relaciones derivadas de un estudio molecular de cinco especies de *Typhlocharis* no son congruentes con las que se derivan de los supuestos linajes basados en caracteres morfológicos. Finalmente, se halla que la larva de *T. toletana* es indistinguible morfológicamente de la de *Typhlocharis* sp. descrita por Arndt et al. (1999).

Palabras clave. Taxonomía, nueva especie, *Typhlocharis toletana*, Península Ibérica, larva, taxonomía molecular, gonocoxito unguiforme, linajes

Introduction

The genus *Typhlocharis* Dieck, 1869 is a member of the tribe Anillini characterised by a number of setae in the elytral umbilicate series between 4 + 4 and 4+1, instead of the 4+ 5 formula found in the rest of Anillini (Ortuño & Sendra 2007). Likewise, the humeral setal group is evenly distributed in *Typhlocharis*, whereas it is more irregular in the related genera. The genus is also distinct because of the tubular shape of gonocoxite 2 of

the ovipositor found in most species, instead of the sclerotized unguiform shape commonly found within Anillini (Vigna Taglianti 1972).

Members of the genus *Typhlocharis* are endogean, that is, they inhabit the soil layers of B horizon and perhaps the Superficial Underground Compartment (or MSS as described by Juberthie et al. 1980). The majority (46) of the species are known from the Iberian Peninsula, while the remaining two are recorded from North Africa. The number of *Typhlocharis* species has noticeably increased in the last 14 years due to new discoveries: 16 species were recorded in the catalogue of Zaballos and Jeanne (1994), 37 were cited in the catalogue of J. Serrano (2003), and 10 more species have been described since (Andújar et al. 2008; A. Serrano et al. 2005; A. Serrano & Aguiar 2006a, b, 2008; Ortuño 2005). Zaballos and Ruíz Tapiador (1997) and Zaballos and Wrase (1998) have proposed and keyed seven morphologically- and geographically-defined species groups, although these keys were subsequently modified to accommodate new taxa (e.g., A. Serrano & Aguiar 2006b, 2008; Andújar et al. 2008).

Most *Typhlocharis* species are known only from a single or a very few closely adjacent localities and are thought to have low dispersal power. Local populations often become isolated which, in turn, favours allopatric speciation. This is expected to lead to parallel evolution in a number of morphological characters related to adaptation to the particular habitats occupied by these carabids. This hypothesis can be tested, assuming that the morphological groups put forward by Zaballos and Ruíz Tapiador (1997) and Zaballos and Wrase (1998) represent true lineages that may be corroborated with the study of newly described taxa, and by studying other characters such as DNA sequences.

In this paper we describe a new Iberian *Typhlocharis* species using both adult and larval stages, and test whether they belong to the same species by sequencing a mitochondrial DNA *cox1* fragment. A few other *Typhlocharis* species representing different lineages are also sequenced. The results are discussed in relation to the presumed ancestral nature of the sclerotized unguiform shape of gonocoxite 2, and the congruence between the morphology-based lineages of *Typhlocharis* and the newly obtained molecular data.

Material and methods

Soil samples were collected from humid slopes on a hillside and processed using the Berlese apparatus. Extracted *Typhlocharis* adults and larvae were preserved in Scheerpeltz fluid or absolute ethanol. Details of the studied individuals (type series) are given below. Morphological study of adults was carried out using either a Zeiss stereomicroscope or a scanning electronic microscope JEOL-6100 at an acceleration speed of 20 KV. Total body length was measured from apex of mandible to apex of elytron. The first visible abdominal sternum of *Typhlocharis* is probably the result of a fusion between sterna II, III and IV commonly found in most carabids (Andújar et al. 2008), although previous authors refer to it as the second abdominal sternum (e.g. Zaballos & Ruiz-Tapiador 1997; A. Serrano & Aguiar 2006b).

Larvae were cleared in a hot 5% water solution of potassium hydroxide (KOH) for about two hours, and then kept overnight in iso-propanol and mounted in Euparal on microscope slides. Photographs of larvae (Figs 1–9) were taken using Nikon DXM1200F digital camera mounted on Nikon SMZ1500 dissecting microscope (Figs 1, 2) or MBI-1 dissecting microscope with oculars removed (Figs 3–9). For figures 4 and 5, a few images were taken at different focal depths and were automatically combined into a single sharp image using CombineZM software (Hadley 2008). Morphological terms and chaetotaxy system for Carabidae larvae follows those proposed by Bousquet & Goulet (1984).

Two adult individuals and one larva of *T. toletana* n. sp. have been sequenced for a fragment of the mitochondrial *cox1* gen. Other *Typhlocharis* species representing three different hypothetical lineages and *Hypotyphlus navaricus* Coiffait, 1958 were also sequenced. Data concerning specimens used in the molecular analysis are shown in Table 1.

DNA was extracted with QIAGEN Dneasy tissue kit (Qiagen, Hilden, Germany). A fragment of 750 bp of *cox1* gen (from the middle of region E3 to the 3' end, Lunt et al. 1996) was amplified using primers Pat (5' TCCAATGCACTAATCTGCCATATTA) and Jerry (5' CAACATTTATTTTGATTTTGG) (Simon et al.

1994). A pairwise divergence analysis of mitochondrial DNA sequences was conducted in PAUP 4.0b10 (Swofford 2000) using PaupUp graphical interface (Calendini & Martin 2005). The best-fit model of nucleotide substitution was calculated with ModelTest 3.7 under the AIC criteria (Posada & Crandall 1998).

TABLE1. Data of *Typhlocharis* and *Hypotyphlus* specimens included in the molecular analysis, species group of *Typhlocharis*, sampling locality and sequence accession numbers (A.N.) to GenBank. All localities are from Spain.

	Species group	Locality	A.N.
<i>T. diecki</i> 43	<i>diecki</i>	Santa Cruz de Moncayo (Zaragoza)	HM009029
<i>T. martini</i> 18	<i>diecki</i>	Fuente del Prado, Liétor (Albacete)	HM009024
<i>T. estrellae</i> 66	<i>outereloi</i>	Arroyo Becea (Ciudad Real)	HM009031
<i>T. armata</i> 33	<i>silvanoides</i>	Tarifa (Cádiz)	HM009028
<i>T. toletana</i> larva 28	-	Villarrubia de Santiago (Toledo)	HM009027
<i>T. toletana</i> adult 26	-	Villarrubia de Santiago (Toledo)	HM009025
<i>T. toletana</i> adult 27	-	Villarrubia de Santiago (Toledo)	HM009026
<i>Hypotyphlus navaricus</i> 48	-	Nagore (Navarra)	HM009030

Typhlocharis toletana Lencina & Andújar new species

(Figs. 1–5)

Type series. Holotype: 1♂, 19–VI–2007, Arroyo de Vitoria, Villarrubia de Santiago (province of Toledo, Spain), 590 m (N 40,02 W –3,30) C. Andújar and J.L. Lencina leg., deposited in the Departamento de Zoología y Antropología Física, Universidad de Murcia (ZAF–UMU).

Paratypes: 52♂ and 21♀, same locality and date, C. Andújar and J.L. Lencina leg. (2♂ and 2♀ gold coated); 79♂ and 69♀, 15–IX–2007 same locality, C. Andújar, J.L. Lencina and J. Sánchez leg.

Two paratypes, one male and one female are deposited in the collection of Museo Nacional de Ciencias Naturales, Madrid. The other paratypes are deposited in the Departamento de Zoología y Antropología Física, Universidad de Murcia (ZAF–UMU), and in J. L. Lencina collection, Museo de Ciencias Naturales de Jumilla (Murcia).

Diagnosis: Anophthalmous. Body long, depressed and parallel (Fig. 1), light brownish. Integument microreticulate with scattered short setae. Antennomeres 3–11 moniliform (Fig. 2c). Clypeus without median tooth. Pronotum (Fig. 2d) relatively slender ($L/W = 1.2$) with minute dentiform projection at hind angle and base arcuate. Each elytron with seven setae in the lateral umbilicate series distributed in two groups of 4+3 (Fig. 2j); no dentiform projection is found at apex or laterally at the end of the slight carina corresponding to seventh stria (Fig. 2h). Female with lateral slight fovea on first ventrite (Fig. 2i). Male genitalia (Fig. 3b) with median lobe slightly arcuate and ventral side almost straight. Female genitalia (Fig. 3a) with gonocoxite 2 of IX gonocoxa unguiform, with one internal seta and two external nematiform setae.

Description of the adult: Length of holotype: 1.2 mm. Length of paratypes: 1.1–1.3 mm. Body long, depressed and parallel, light brownish. Integument microreticulate, mesh pattern isodiametric, with scattered short setae. Head capsule (Fig. 2a) robust, slightly narrower than pronotum and almost as wide (0.27 mm.) as long (0.29 mm.), slightly depressed in the middle of the frons, with two frontal divergent depressions delimiting it. Dorsal surface reticulate, except stridulatory area without microlines (Fig. 2f). Clypeus without median tooth. Anophthalmous. Appendages: antennomeres 1–2 cylindrical, 3–11 moniliform (Fig. 2c); labial mentum with one tooth and lateral lobes rounded (Fig. 2b); penultimate labial palpomere thickened, ultimate palpomere minute. Setation: clypeus with two setae, the external pair longer than the internal; cephalic capsule with 3–4 frontal setae, two median, and three large periorbital setae (supraorbital plus temporal); cephalic capsule and lateral carina with some other minor setae; labrum with five setae, three short and medial, one long and lateral and one external to the latter; labial mentum with two pairs of long setae, one close to the base of tooth and another on the lateral lobes, prebasally there are two other lateral pairs of setae.

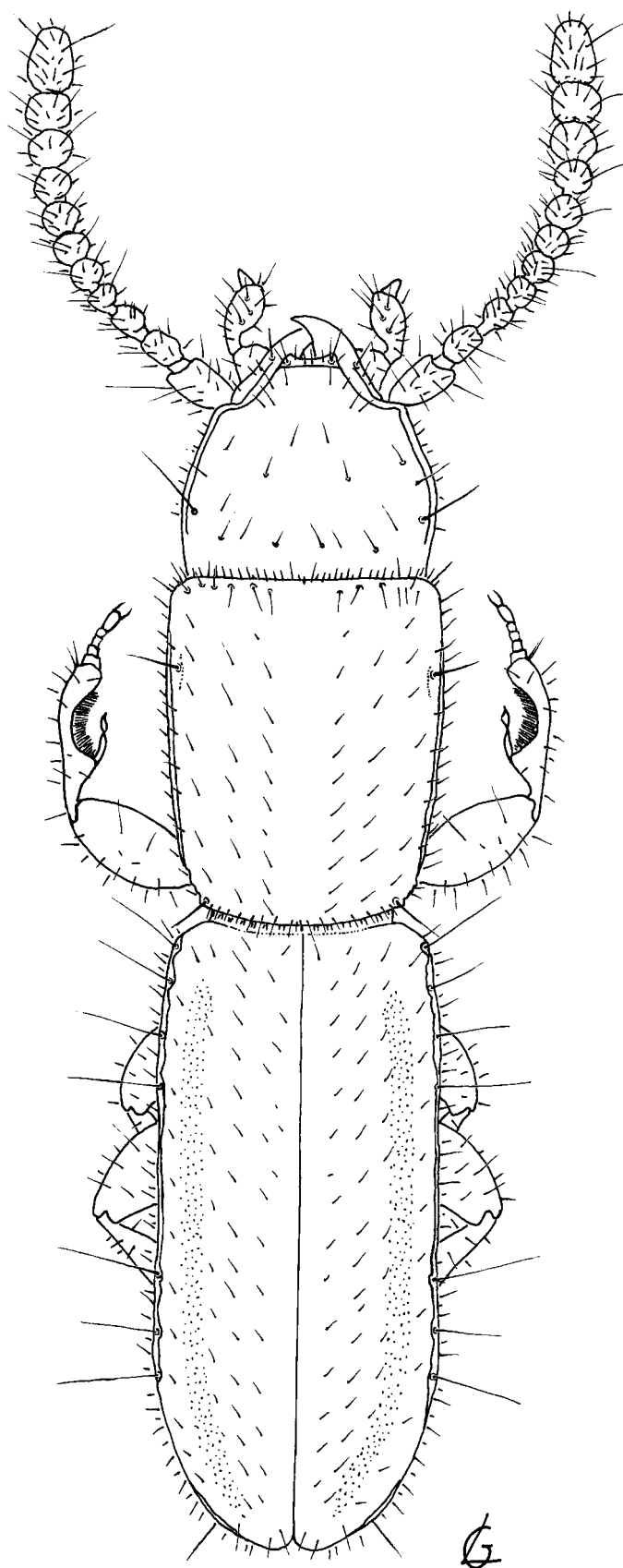


FIGURE 1. *Typhlocharis toletana* n. sp., adult, habitus, dorsal view, total body length 1.2 mm.

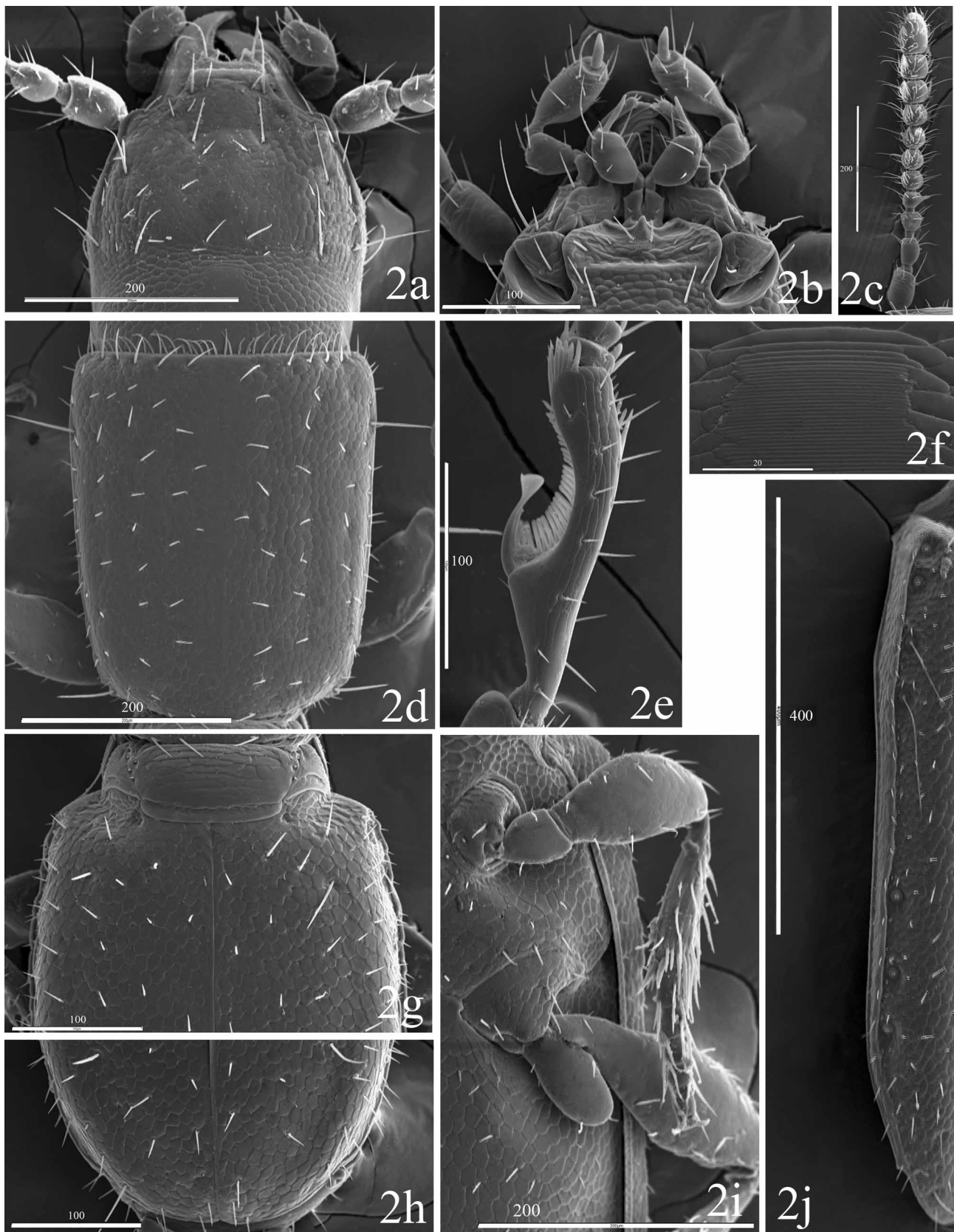


FIGURE 2. *Typhlocharis toletana* n. sp., adult, SEM photographs, (a) details of head, dorsal view; (b) mouth parts, ventral view; (c) antenna; (d) pronotum, dorsal view; (e) right protibia, posterior view; (f) stridulatory area, internal view; (g) basal portion of elytra, dorsal view; (h) apex of elytra, dorsal view; (i) metathorax, ventral view; (j) left elytron, lateral view.

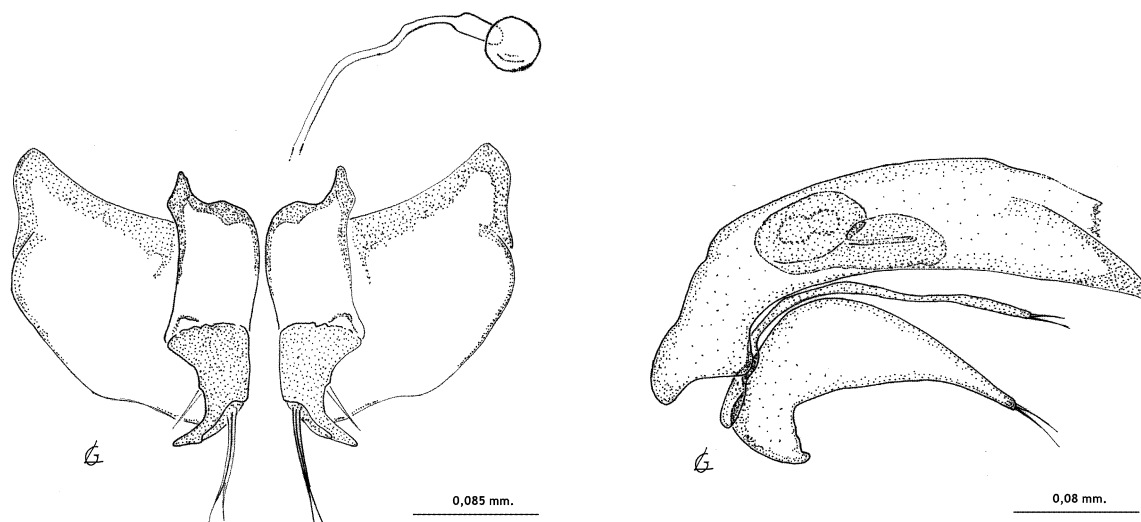


FIGURE 3. *Typhlocharis toletana* n. sp., adult, (a) ovipositor sclerites, ventral view; (b) male genitalia, left lateral view.

Prothorax. Pronotum (Fig. 2d) longer (0.35 mm) than wide (0.29 mm), surface microreticulate, slightly trapezoidal with maximum width close to the apical margin. Disc flattened. Anterior margin slightly arcuate. Sides slightly crenulate only in the posterior quarter. Hind angle obtuse and distinctly marked with a small acute tooth. Posterior margin arcuate. One seta in anterior quarter of lateral margin and another on hind angle. Three longitudinal series of minute setae. Six pairs of strong setae on the anterior margin and two pairs of setae on the posterior margin. Both anterior and posterior margins with a row of tomentose setae. Prosternum with sparse and short setae.

Elytra (Fig. 2g; 2h; 2j) twice longer (0.60 mm) than wide (0.28 mm), totally reticulate, with longitudinal carina at seventh stria slight and short, not extended to the apical margin of elytron (it disappears in the posterior fifth), and thus without forming there a small dentiform projection. Basal and lateral margins perpendicular, humeral region clearly marked and humeral angle smoothly rounded. Sides parallel with lateral margin slightly serrate to posterior quarter. Sutural angle not dentate (Fig. 2h). There are four longitudinal series of setae. Lateral margin with four setae in the anterior umbilicate group and three setae in the posterior group (Fig. 2j).

First ventrite of female with slight lateral fovea (Fig. 2i).

Legs similar in both sexes without special features such as dentiform projections or distally dilated femora. Front tibia with antenna cleaner (toilette organ) long and regularly arcuate, with one securiform clip seta at its proximal border (Fig. 2e), also reported in other species (e.g., *T. gonzaloi*: Ortuño 2005; *T. martini* Andujar et al. 2008). Trochanter long with rounded apex (Fig. 3a), similar in male and female.

Median lobe of male genitalia (Fig. 3b) slightly curved, ventral side almost straight and dorsal side with a membranous appearance to the apex. Apex short and acute. Internal sac with a small sclerotized piece. Parameres with two apical setae.

Gonocoxite 2 of IX gonocoxa of female genitalia unguiform (Fig. 3a), with one internal seta and two external nematiform setae. Spermatheca rounded, sclerotized, spermathecal gland distally dilated.

Description of the larva: Material studied. Twenty apparently older-instar larvae were collected in the type locality, ten in June 19, 2007 and another ten in November 12, 2007. Larvae were labelled “SPAIN Villarrubia de Santiago Toledo 19.VI—2007 Spring on a hillside 590m N 40,02 W -3,30 Andújar and Lencina leg., and “SPAIN Villarrubia de Santiago Toledo 15.IX-2007 Spring on a hillside 590 m N 40,02 W -3,30 Andújar, Lencina and Sánchez leg.. Five larvae of these two dates were mounted on Euparal microscope slides and stored at the Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa, Canada. Larvae are not designated as type specimens.

Diagnosis. Three-segmented antennae is a unique feature readily distinguishing *Typhlocharis* from those of the vast majority of Carabidae genera, including the genus *Geocharidius* Jeannel, 1963, the only other member of Anillina with described larvae (Grebennikov 2002). Larval characters of *T. toletana* are similar to those described for *Typhlocharis* sp. (Arndt et al. 1999).

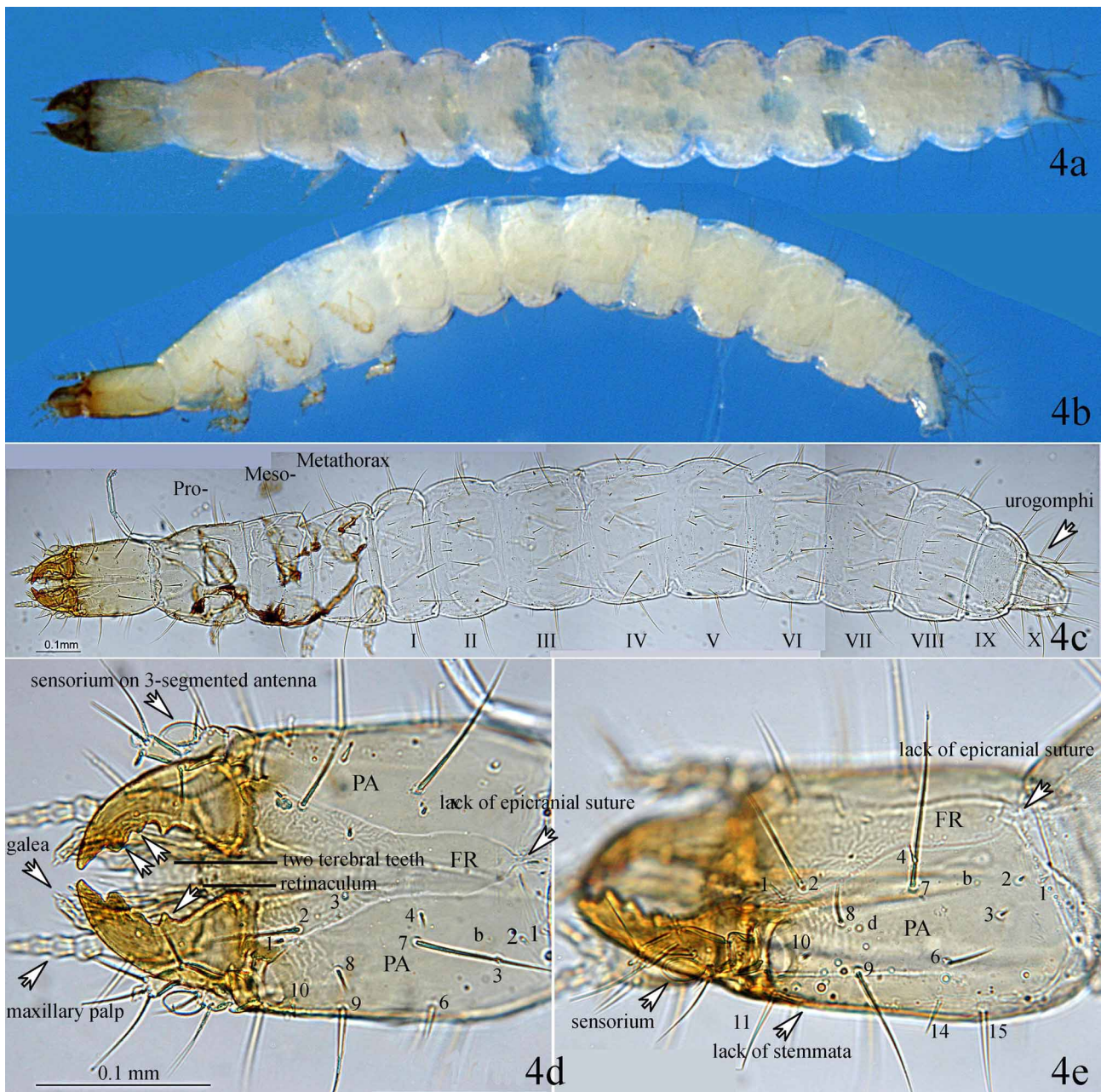


FIGURE 4. *Typhlocharis toletana* n. sp., older-instar larva, habitus, dorsal (a, c) and lateral (b) views, head, dorsal (d) and left dorso-lateral (e) views. Chaetotaxy nomenclature follows Bousquet and Goulet (1984).

Description of the larva. Body length about 2.2 mm (Figs. 4–5); maximal head width 0.173 mm (0.172–0.175 mm, $n = 5$). Cephalic capsule almost parallel-sided and slightly elongated (Figs. 4, 5a), without epicranial suture (Fig. 4d) and stemmata (Fig. 4e), mandible with a wide base, terebrum apically of retinaculum with one apical and large tooth, and one medial and small. All other characters of these larvae, including details of the chaetotaxy, are identical with a *Typhlocharis* larva previously described by Arndt et al. (1999).

Molecular analysis: ModelTest showed that GTR+G was the best-fit substitution model, with a γ value of 0.1603. A pairwise divergence analysis conducted in PAUP with this parameter showed that divergences

between the adults and the putative larva of *T. toletana* are low, between 0 and 0.013%, while divergences among different *Typhlocharis* species vary from 15% to 26%. Divergences between *Typhlocharis* and *Hypotyphlus* are around 30% (Table 2).

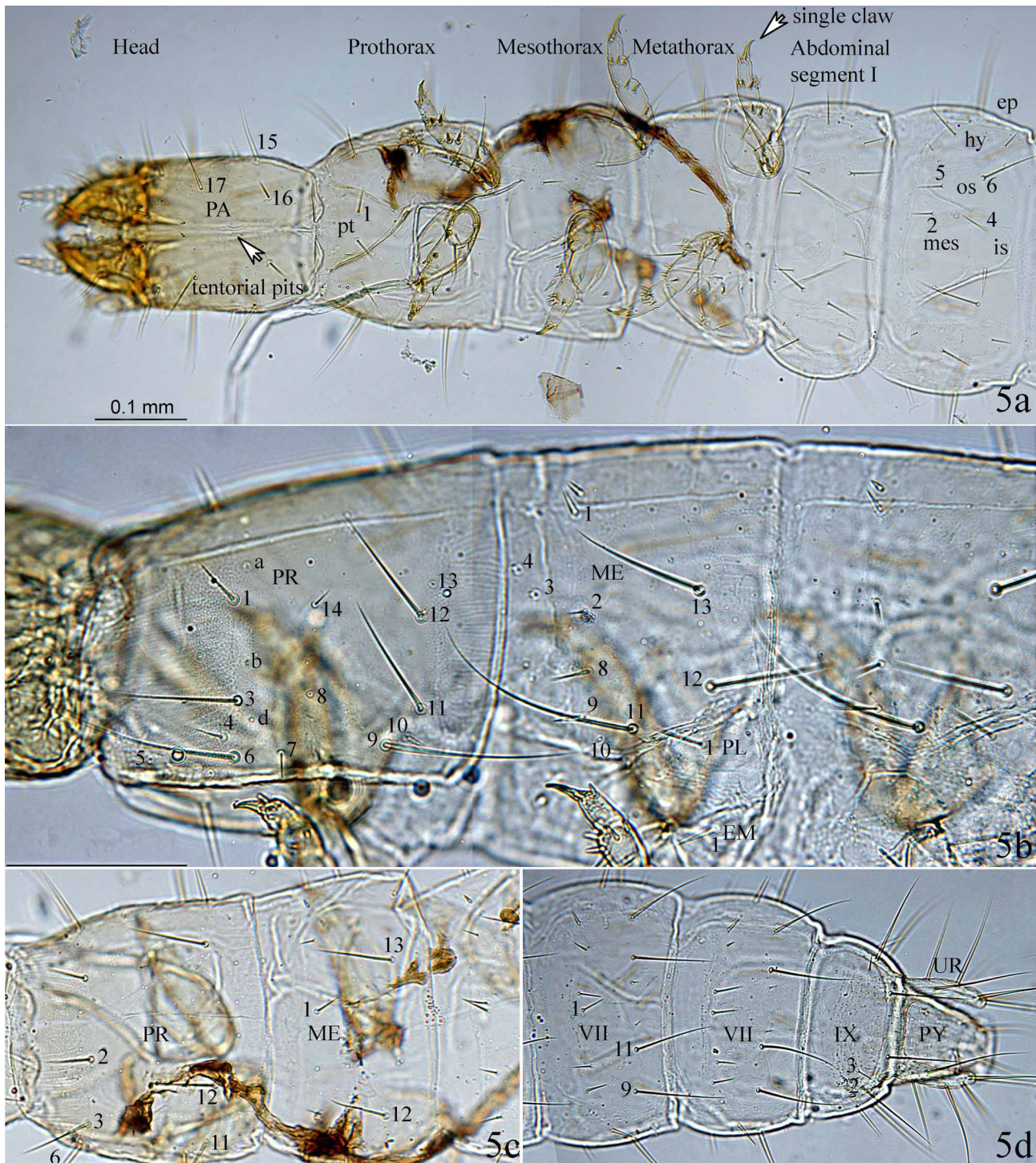


FIGURE 5. *Typhlocharis toletana* n. sp., older-instar larva, (a) head, thorax and abdominal segments I–II, ventral view; (b) pro-, meso- and metathorax, left dorso-lateral and (c) dorsal view; (d) abdominal segments VII–X and urogomphi, dorsal view. Chaetotaxy nomenclature follows Bousquet & Goulet (1984).

Habitat: The type locality of *Typhlocharis toletana* n. sp. is a spring on a lateral hillside facing Arroyo de Vitoria, a stream with permanent water in white loamy soil area close to Villarrubia de Santiago (Toledo, Spain). Type locality is 1.5 km apart from the main course of river Tajo. There was only herbaceous vegetation, mainly composed of grass, like *Brachypodium* sp.

TABLE 2. Pairwise distances between cox1 sequences of 5 species of *Typhlocharis* and one *Hypotyphlus*. Analyses were conducted in PaupUp using the nucleotide substitution model GTR+G ($\gamma = 0.1602$), calculated with ModelTest 3.7.

	<i>T. toletana</i> larva	<i>T. toletana</i> adult 1	<i>T. toletana</i> adult 2	<i>T. diecki</i>	<i>T. estrellae</i>	<i>T. armata</i>	<i>T. martini</i>
<i>T. toletana</i> larva	-						
<i>T. toletana</i> adult 1	0.000	-					
<i>T. toletana</i> adult 2	0.003	0.001	-				
<i>T. diecki</i>	0.205	0.201	0.210	-			
<i>T. estrellae</i>	0.209	0.209	0.209	0.200	-		
<i>T. armata</i>	0.265	0.265	0.265	0.216	0.201	-	
<i>T. martini</i>	0.257	0.257	0.256	0.232	0.156	0.169	-
<i>Hypotyphlus navaricus</i>	0.319	0.320	0.331	0.302	0.289	0.288	0.248

Etymology: The specific epithet is a noun in genitive case deriving from Toletum (=Toledo), the province in central Spain where the new species has been found.

Affinities: Adults of *T. toletana* n. sp. share with those of *T. monastica* Zaballos and Wrase 1998 and *T. peregrina* Zaballos and Wrase 1998 the unguiform type genitalia. However, *T. toletana* can be easily distinguished from them by the absence of tooth at the posterior margin of elytra and the umbilicate series of 4+3 type (as compared to 4+2 type found in the cited species). These and other characters suggest the inclusion of *T. toletana* in the *T. diecki* species group, whereas the lack of teeth in the posterior half of the lateral margin plus the sutural angle of elytra is characteristic to the *T. silvanoides* species group. Presently described *T. toletana* larva cannot be reliably distinguished from the congeneric larvae described by Arndt et al. (1999) and, therefore, are not informative in clarifying species relationships. Affinities of the new species are, therefore, ambiguous.

Discussion

The majority of *Typhlocharis* species are characterised by having the ovipositor's gonocoxite 2 of a tubular shape, instead of sclerotized and unguiform commonly found within Anillini (Vigna Taglianti 1972; Zaballos & Wrase 1998). Within the tribe Anillini the tubular gonocoxite 2 is only found in *Typhlocharis* and *Anillotarsus*, the latter being a monotypic genus from Peru (Mateu 1980). *Typhlocharis* is characterized by having five to eight setae in the umbilicate series as compared to nine found in the rest of Anillini (Jeannel 1963; Ortuño & Sendra 2007). Vigna Taglianti (1972) considered the sclerotized unguiform gonocoxite 2 of Anillini as a primitive character state. Likewise, Zaballos and Wrase (1998) postulated that this is the ancestral character state for *Typhlocharis* after discovering it in *T. monastica* and *T. peregrina* and established a species group to include these two species (Zaballos & Wrase 1998). The *T. monastica* species group is characterised by having the unguiform female genitalia, noticeable sexual dimorphism, posteriad margin of elytra with two teeth and umbilicate series of 4+2. However, some of these character states are not found in *T. toletana*, thus suggesting that this species belongs to a different lineage. Furthermore, *T. monastica* and *T. peregrina* were described from north Spain (province of Navarra), 400 km distant from the type locality of *T. toletana*. It can be hypothesized that the ancestral unguiform gonocoxite 2 has been either preserved in at least two unrelated *Typhlocharis* lineages, or that these three species are closely related and this is obscured by subsequent morphological changes.

On the other hand, the combination of diagnostic traits shown by *T. toletana* (unguiform female genitalia, umbilicate series of 4+3, lack of teeth in the lateral and apical margin of elytron) does not allow it to be included in any of the species groups indicated by Zaballos and Wrase (1998). Instead of proposing a new lineage (or species group) within *Typhlocharis* to include *T. toletana*, we prefer to reconsider the phylogenetic value of traits currently used in the systematics of the genus. As shown by the preliminary molecular data

(Table 2), *T. martini* is not closely related to *T. diecki* although both species were included in the *T. diecki* species group based on morphological characters (Andújar et al. 2008). Instead, *T. martini* is relatively close to *T. estrellae* (*T. outerelei* species group of Zaballos and Ruíz Tapiador 1997) and to *T. armata* (*T. silvanoides* species group). Likewise, *T. toletana* n. sp. is equally related to *T. diecki* and to *T. estrellae*. These and other results shown in Table 2 indicate that the morphological definition of species groups within the genus *Typhlocharis* is not in agreement with the DNA data and, therefore, a holistic approach to assess the evolutionary history of *Typhlocharis* should be applied.

The molecular analysis strongly suggests that the larva we describe belongs to the same species as the two *T. toletana* adults studied, by having the same haplotype. Molecular analysis was not performed when the first *Typhlocharis* larva was described (Arndt et al., 1999); this made the species identification impossible because three *Typhlocharis* species were known to co-occur in the area. Thus, a simple molecular study should be routinely performed to assess the larval identity of these tiny carabids.

Remarks on larval morphology

The remarkable similarity of both known *Typhlocharis* larvae (present description and that by Arndt et al. 1999) contrasts sharply with the numerous differences distinguishing them from a *Geocharidius* larva, the only other Anillini genus known in its immature stages (Grebennikov 2002). Both genera were recently included in a phylogenetic analysis using larval morphology of 32 Trechitae genera (Grebennikov & Maddison 2005; Grebennikov 2008) and formed a well defined clade supported by the following five synapomorphies: (1) basal antennomere with two pores; (2) antennomere 2 markedly reduced in length or absent; (3) antennal fossa separated from pleurosoma by a wide strip of sclerotized cuticle; (4) terebra with two teeth, each not smaller than the retinaculum, and (5) retinaculum markedly reduced. Monophyly of the whole subtribe Anillina with about sixty genera, however, still remains doubtful, as the larvae of only three species of two genera are known.

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