On the verge of below-ground speciation: a new species complex of microendemic endogean carabid beetles, Typhlocharis Dieck, 1869 (Coleoptera: Carabidae: Anillini), from south-west Iberian Peninsula

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Abstract. A new species complex of genus Typhlocharis Dieck, 1869 (Coleoptera: Carabidae: Trechinae: Anillini: Typhlocharina) is described. Six populations from southern Badajoz (Spain), referred as the "coenobita species complex", are the first documented case of an expected situation within Typhlocharina and potentially other lineages of endogean ground beetles: the presence of closely related allopatric populations within a reduced geographical range that, despite certain genetic isolation, show a gradient of morphological differences that challenge taxonomic assignment. Previous phylogenies of Typhlocharina recovered these populations as a monophyletic lineage, represented by three potential new species in need of further examination to validate their status. Here, we test the congruence of this taxonomic hypothesis through direct observation, statistical analyses applied to morphological characters and analysis of COI sequences. Such integrative approach, revealed as a powerful tool to solve situations where phenotypic differences are very subtle, is used for the first time to discriminate Anillini species. The results are coherent with the three species hypothesis, formally described as Typhlocharis coenobita sp.n., T. eremita sp.n. and T. anachoreta sp.n. The implications of the internal variability within this species complex to the systematics of Typhlocharina and their affinities to other Typhlocharis species are discussed. The entity of T. eremita sp.n. as new species is well established within the standards of the genus. However, the populations of T. coenobita sp.n. show high variability and their relationship with T. anachoreta sp.n. is in the verge of what can be considered species-level differentiation, suggestive of an incipient speciation process. The proposed species boundaries maximize the consistence among the different sources of evidence. The intraspecific variability within T. coenobita sp.n. is properly described, contributing to elucidate the ongoing differentiation processes within this endogean lineage. Finally, an identification key for the coenobita species complex is provided.

Key words. Endogean, Coleoptera, Carabidae, Typhlocharis, taxonomy, new species, speciation, systematics, species complex.

1. Introduction

“Given any species in any region, the related species is not likely to be found in the same region” (JORDAN 1905: p. 547).

The evolution of genetic reproductive barriers between geographically separated populations (allopatric speciation, MAYR 1963) has been widely accepted as a prevalent mode of speciation in animals (FUTUYMA 1998; COYNE & ORR 2004). The initial geographical separation may be due to the emergence of an extrinsic barrier, extinction of intervening population, or migration into a separate region (FUTUYMA 1998; COYNE & ORR 2004; LOMOLINO...
et al. 2010). Indeed, dispersion through a heterogeneous geography or landscape has the potential to generate geographically isolated populations that become the source for the speciation process. In species that disperse little or are strongly tied to a particular habitat, spatial scale of speciation can be strongly reduced and barriers to gene flow may isolate populations at a microgeographic scale (Futuyma 1998; Kisel & Barralough 2010).

Both conditions are generally met by species adapted to live in deep soil layers, and particularly by the subtribe Typhlocharina (Coleoptera: Carabidae: Trechinae: Anillini). This endogean lineage of carabid beetles is endemic to some areas of the western Mediterranean region, distributed through the Iberian Peninsula (Spain and Portugal) and the north of Africa (Morocco and Tunisia) (Zaballos 2003). A strong pattern of geographical speciation has been shown in Typhlocharina (Jeannel 1963; Andújar et al. 2016, 2017), becoming the most diversified group of Anillini known up to date. These animals are specialists of the endogean environments, inhabiting the soil horizons A and B (Oortuš 2000). Thus, they are morphologically well suited to the specific conditions below soil: eyeless, wingless, depigmented, with short limbs, narrow, rectangular bodies and tiny sizes (0.9 – 2.9 mm). Within Anillini, they are easily recognizable by the square-shaped pronotum and the unusual female genitalia (Vigna-Taglianti 1972; Zaballos & Wrase 1998; Pérez-González & Zaballos 2012).

Currently, given the unprecedented and increasing rate of new species descriptions, the study of the group is going through one of its most complex moments (Zaballos et al. 2016; Serrano & Aguilar 2017). The first approach to the systematics of Typhlocharina established species groups based on key morphological features, with special emphasis in the umbilicate series of setal insertions (Zaballos & Ruiz-Tapiador 1997; Zaballos & Wrase 1998; Pérez-González & Zaballos 2013c). Notwithstanding, recent efforts to resolve the phylogeny of Typhlocharina based on morphological, molecular and total evidence data (Pérez-González et al. 2017; Andújar et al. 2017) showed that these species groups do not correlate with true clades and concluded in the subdivision of the former genus Typhlocharis Dieck, 1869 in three different genera: Lusotyphlus Pérez-González, Andújar & Zaballos, 2017; Typhlocharis Dieck, 1869 and Microcharidius Coiffait, 1969 (Pérez-González et al. 2017). As a consequence of the sampling efforts towards the phylogeny of the group, many new populations were discovered, which may represent more than 45 potential new species yet to be formally corroborated and described (Pérez-González et al. 2017; Andújar et al. 2017).

Now, the case of six closely related populations of Typhlocharis found in a small area of about 60 × 60 km in south-west Iberian Peninsula is presented. These populations were recovered as a well-supported monophyletic lineage represented by three potential species with clear morphological affinities (named “T. sp. 6”, “T. sp. 7” and “T. sp. 8” in Pérez-González et al. 2017 and Andújar et al. 2017).

However, “species delimitation” is not an easy task and these potential “new species” need further examination to validate their status and provide a formal description. Cases of intraspecific phenotypic variation that challenge the identification criteria for species-level taxa in Typhlocharina have been recently evidenced, as recorded for Typhlocharis singularis Serrano & Aguilar, 2000; T. mixta Pérez-González, Zaballos & Ghannem, 2013 and Microcharidius zaballosi Serrano & Aguilar, 2014 (Serrano & Aguilar 2000, 2002, 2014; Pérez-González et al. 2013). The problem of species discrimination is widely extended in zoology (e.g. De Queiroz 2007; Willis 2017; Andújar et al. 2014) and the frontiers between intra- and interpopulation variability and speciation are diffuse at the scale of microevolutionary changes. Here, we test the congruence of morphological and molecular data with the hypothesis of the six studied populations as three different species. The new species are described and their relationships and limits are discussed, as well as the implications of microevolutionary changes and intrapopulation variability for the systematics of the genus, which would help to understand population dynamics and speciation in endogean environments.

2. Material and methods

2.1. Collecting

The study area occupies a range of about 60 × 60 km, located in the south of Badajoz province (Spain). Soil samples were collected in winters 2012 and 2013 from six localities (Figs. 1, 2): Valverde de Leganés (VL); Almendral, Ribera La Albuer (LA); Higuera de Vargas (HV); Aceuchal, Rio Guadajira (RG); Valverde de Burguillos (VB) and Oliva de la Fratera, Arroyo Zaos (AZ). Samples included superficial and deep soil layers (horizons A and B) up to 30–50 cm deep and were processed in the field using an optimized version of the soil washing technique (Normand 1911). The fauna was extracted from the samples with Berlese apparatus (Berlese 1905). In some sites, additional specimens were collected by hand, under deeply buried boulders of several sizes, using a thin (nº 000) white haird paintbrush. Overall, 1020 specimens were collected and stored in absolute ethanol.

2.2. Morphological study

For the morphological observations, specimens were rinsed in lactic acid to clear the cuticle. A minimum of five males and five females of each population (except for VL population, where only three specimens were available) were dissected by separation of the body parts and extraction of the male genitalia for a detailed observation of the structures. Female genitalia were studied in situ, by transparence, to avoid the damage of delicate
structures during manipulation. There are 44 specimens with a voucher number, selected for DNA extraction (detailed in Pérez-González et al. 2017 and Andújar et al. 2017) that were also observed in detail, but dissection were restricted to the extraction of male genitalia. The remaining specimens of each population were kept intact but observed and compared to dissected specimens to ensure the identification.

All the observations were made using light microscopy. The nomenclature used follows Zabállos (2005) for cephalic chaetotaxy, Pérez-González & Zabállos (2012, 2013c) for the rows of setae and Pérez-González...
& Zaballos (2013b) for antennal features. Terminology for the IX sclerite of males follows Sokolov & Kavanagh (2014). Measurements were made with a Wild Heerbrugg M8 stereomicroscope (Switzerland). Drawings were made from photographs obtained using a Zeiss 474620-9900 microscope (Germany), processed and outlined with Adobe Photoshop CS6 13.0.

After the observations, dissected specimens and extracted genitalia were mounted on entomological cards with glass window using dimethyl hydantoin formaldehyde resin (Bameul 1990). Untreated specimens were mounted on regular entomological cards. Specimens with voucher number (El. Suppl. File 1 Appendix S1: Table S1.1) were fluid-preserved in Eppendorfs with absolute ethanol. The type specimens are deposited in Coll. J.P. Zaballos and Coll. S. Pérez-González, Universidad Complutense de Madrid (UCM, Madrid), Natural History Museum (NHM, London) and Museo Nacional de Ciencias Naturales (MNCN, Madrid).

The morphological matrix used in Pérez-González et al. (2017) was adapted to code those morphological features that showed variation between the studied populations (Table 1) in the 44 voucher specimens (8 from AZ, 8 from HV, 8 from VB, 8 from LA, 2 from VL, 10 from RG). Character states within some traits (e.g. ring sclerite) were re-coded to register additional variations at population level. Characters were coded as binary or multistate if they can be considered part of a transition (e.g. degree of development of lateral denticles of elytra). Multistate characters that could not be considered ordered (ring sclerite) were split as several binary dummy variables, one for each character state, generating a final set of 23 characters (El. Suppl. File 1 Appendix S1: Table S1.2). Two matrixes were produced, one at specimen level (vouchered individuals, 44 terminals) and one at population level (6 terminals) (El. Suppl. File 1 Appendix S1: Tables S1.3, S1.4).

UPGMA analyses were conducted on both matrixes to cluster the terminals by morphological similarity using DendroUPGMA online facility (http://genomes.urv.cat/UPGMA/), assuming Euclidean distances and applying bootstrap with 100 replications (El. Suppl. File 2 Appendix S2).
The hypothesis of three species for these populations (Pérez-González et al. 2017; Andújar et al. 2017) was tested through discriminant analysis using STATGRAPHICS Centurion XVII (StatPoint, Inc., USA, 2014), applied to the individual level matrix excluding non-informative characters (El. Suppl. File 2 Appendix S3). This analysis distinguishes between groups in a given dataset, generating a series of discriminant functions based on the observed variables (the morphological characters). The 44 cases of the matrix were used to develop a model to discriminate among the three proposed groups (“species”: “A” for “T. sp. 8”, “B” for “T. sp. 7” and “C” for “T. sp. 6”), using stepwise regression (backward selection) to determine which variables are significant predictors. The obtained discriminant functions can be used to classify new observations in one of the three groups. To test the performance of the classification function coefficients, 43 specimens were additionally coded and analyzed: 5 males and 5 females from VB, RG, LA and HV respectively, and 3 females of AZ. Due to lack of extra specimens, VL was not included. Data matrix of the 43 additional specimens, table of classification function coefficients, functions used to classify observations and results of the predictions are given in El. Suppl. File 1 Appendix S1: Table S1.5; El. Suppl. File 2 Appendix S3: Table S3.1.

The results are discussed within the phylogenetic framework of Typhlocharina proposed in Pérez-González et al. (2017) and Andújar et al. (2017).

2.3. Molecular study

Genetic differentiation between populations was inferred using sequences of the barcoding region of the Cytochrome Oxidase Subunit I (COI) gene, available for 34 of the 44 vouchered specimens (Genbank accession numbers in El. Suppl. File 1 Appendix S1: Table S1.1; from Andújar et al. 2017). DNA was aligned using MAFFT G-INS-I algorithm (Katoh et al. 2002) and trimmed to a final dataset of 657 bp in Geneious 7.1.9 (Kearse et al. 2012). Phylogenetic maximum likelihood inferences were done with IQTree 1.5.5 (Nguyen et al. 2015). The best fitting model of evolution was estimated with ModelFinder (Kalyaanamoorthy et al. 2017) and nodal support was obtained by 1000 ultrafast bootstrap (UFBoot) replicates (Minh et al. 2013).

The software TCS (Clement et al. 2000) was used to estimate an haplotype network based on Statistical Parsimony (Templeton et al. 1992) using the 34 COI sequences and a dataset trimmed to 523 bp. K2p distances (Kimura 1980) were calculated with MEGA version 5 (Tamura et al. 2011) and were visualized on a principal coordinates plot (PCoA) using NTSYSpc v.2.10q software (Rohlf 2000). To assess structuring within and among the populations, Analyses of Molecular Variance (AMOVA) were carried out in Arlequin v.3.5.1.2 (Excoffier & Lischer 2010) with 1000 permutations (El. Suppl. File 2 Appendix S4).

3. Results

3.1. Morphological variation between populations

The studied specimens from VB, VL, LA, RG, HV and AZ belong to genus Typhlocharis (sensu Pérez-González et al. 2017), defined by the shape of last ventrite, with a smoothly curved posterior margin and presence of abdominal belt. These populations are very akin to each other and are characterized within Typhlocharis by the combination of several morphological features (Table 1). All of them share the presence of two terebral teeth, a row of setae in the anterior margin of pronotum with a trend to alternate lengths, umbilicate series with 4+3 or 4+2 patterns, elytral apex without denticles and a characteristically projected apex of the ring sclerite of the male genitalia, with diverse types of “scoop-like” shapes.

The observed range of intrapopulation variability is similar to that described for other species of Typhlocharis, such as T. mixta or T. mendesi Serrano & Aguilar, 2017 (Pérez-González et al. 2013; Serrano & Aguilar 2017). The labrum, mandibles, pronotum, transverse scutellar organ, elytral buttonholes or the elytral apex shape are prone to minor variations between individuals, as well as body size and degree of sclerotization. Individual variability is also common on the patterns of chaetotaxy in labium, basilar, sensilla coeloconica (se) of the last antennomere, anterior and posterior rows of pronotum or last ventrite.

The main morphological traits that differ among populations are detailed in Table 1. It is noteworthy that the majority of differentiating characters are not discrete, but part of a morphological spectrum and frequently overlaps between populations. For example, the median lobe of the ligula is especially prominent and notorious in the population of HV (Table 1), yet in the other populations, some individuals show very prominent median lobes of ligula that approach to the less developed shapes seen in HV population. For this reason, differentiation between species should not rely on one specific character, but a combination of several features.

UPGMA analyses based on these morphological characters clustered the populations as shown in Fig. 3. The results from the “population level” matrix and the “specimen level” matrix were consistent (El. Suppl. File 2 Appendix S2: Tables S2.1, S2.2 and Figs. S2.1, S2.2), despite general low support. Both analyses recovered a low supported topology with AZ and HV as well differentiated entities and the remaining populations as part of the same cluster. Within this cluster, LA, VL and VB were recovered close to each other and RG more distant. This topology, even if non-supported, is consistent with the proposed hypothesis of three species, corresponding to “species A” - LA+VL+VB+RG, “species B” - HV and “species C” - AZ (Fig. 3).

Results of the discriminant analysis over the 44 voucher specimens also supported the same classifica-
Table 1. Comparison of the morphological features that show differences among the studied populations of *Typhlocharis* from south west Iberian Peninsula. — **Abbreviations:** AZ, Oliva de la Frontera, Arroyo Zaos; HV, Higuera de Vargas; VL, Valverde de Leganés; LA, Almendral, Ribera La Albueña; VB, Valverde de Burguillos, RG, Aceuchal, Río Guadajira.

<table>
<thead>
<tr>
<th>Population</th>
<th>Total length (mm)</th>
<th>Left mandible</th>
<th>Right mandible</th>
<th>Median lobe of ligula</th>
<th>Labrum</th>
<th>Semilunar notch</th>
<th>Shape of pronotum</th>
<th>Medial hiatus</th>
<th>Crenulation of anterior margin</th>
<th>Posterolateral denticles of pronotum</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ</td>
<td>1.26 – 1.43</td>
<td>Inner edge subtly projected (smooth flap)</td>
<td>Two tergal teeth</td>
<td>Curved and prominent (shorter than paraglossae)</td>
<td>Subquadrate or rounded</td>
<td>faint</td>
<td>Subquadrate or subrectangular</td>
<td>2 – 2.5 spaces</td>
<td>Well marked</td>
<td>3 – 5, strong, well defined</td>
</tr>
<tr>
<td>HV</td>
<td>1.17 – 1.45</td>
<td>Inner edge subtly projected (smooth flap)</td>
<td>Two tergal teeth</td>
<td>Very prominent (as long or longer than paraglossae)</td>
<td>Subquadrate or rounded</td>
<td>Well marked</td>
<td>Subquadrate</td>
<td>3 spaces</td>
<td>Modestly marked</td>
<td>3 – 5 well defined, irregular</td>
</tr>
<tr>
<td>VL</td>
<td>1.24 – 1.27</td>
<td>Inner edge subtly projected (smooth flap)</td>
<td>Two tergal teeth</td>
<td>Curved and prominent (shorter than paraglossae)</td>
<td>Rounded</td>
<td>Well marked</td>
<td>Subquadrate</td>
<td>1.5 – 2 spaces</td>
<td>Slightly marked</td>
<td>2 – 3, low and blunt</td>
</tr>
<tr>
<td>LA</td>
<td>1.12 – 1.27</td>
<td>Inner edge subtly projected (smooth flap)</td>
<td>Two tergal teeth, the second with individual variation</td>
<td>Curved and prominent (shorter than paraglossae)</td>
<td>Subquadrate or rounded</td>
<td>Well marked</td>
<td>Subquadrate</td>
<td>1.5 – 2 spaces</td>
<td>Slightly marked</td>
<td>2 – 4, low and blunt</td>
</tr>
<tr>
<td>VB</td>
<td>1.11 – 1.40</td>
<td>Smooth inner edge</td>
<td>Two tergal teeth, the second with individual variation</td>
<td>Curved and prominent (shorter than paraglossae)</td>
<td>Subquadrate or rounded</td>
<td>Well marked</td>
<td>Subquadrate</td>
<td>1.5 – 2 spaces</td>
<td>Slightly marked</td>
<td>2 – 3, low and blunt</td>
</tr>
<tr>
<td>RG</td>
<td>1.13 – 1.27</td>
<td>Inner edge subtly projected (smooth flap)</td>
<td>Two tergal teeth, the second with individual variation</td>
<td>Prominent, but shorter than paraglossae</td>
<td>Subquadrate</td>
<td>Well marked</td>
<td>Subquadrate</td>
<td>2.5 – 3 spaces</td>
<td>Slightly marked</td>
<td>2 – 4, blunt and irregular</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Population</th>
<th>Transverse scutellar organ</th>
<th>Shape of elytral apex</th>
<th>Lateral denticles of elytra</th>
<th>Umbilicate series</th>
<th>Subapical / Apical setae of elytra</th>
<th>Shape of metafemora</th>
<th>Inner margin of femora</th>
<th>Endophallic sclerites</th>
<th>Distal expansion of ring sclerite</th>
<th>Shape of spermatheca</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ</td>
<td>Variable, straight or smoothly curved</td>
<td>“v-shaped” notch</td>
<td>Moderately marked</td>
<td>4 + 2</td>
<td>Row of short setae, apical pair not longer than the rest</td>
<td>Normal</td>
<td>Smooth</td>
<td>Rod-shaped, arranged in a “branched” structure</td>
<td>Subtriangular</td>
<td>Subsphaeric (predominant) or ovoid</td>
</tr>
<tr>
<td>HV</td>
<td>Variable, from straight to strongly subtriangular</td>
<td>Round</td>
<td>Strongly marked, defined in last third</td>
<td>4 + 3</td>
<td>Row of long, thin setae, apical pair slightly longer than the rest</td>
<td>Angular</td>
<td>Smooth</td>
<td>Forked, with a curved lateral projection pointing upwards</td>
<td>“spoon-shaped”, variable, usually more or less square</td>
<td>Subsphaeric (predominant) or ovoid</td>
</tr>
<tr>
<td>VL</td>
<td>Variable, typically smoothly subtriangular</td>
<td>Round</td>
<td>Very faint</td>
<td>4 + 2</td>
<td>Row of long, thin setae, apical pair variable, usually slightly longer than the rest</td>
<td>Normal or slightly angular</td>
<td>Smooth</td>
<td>Forked, with a curved lateral projection pointing upwards</td>
<td>(males unknown)</td>
<td>Ovoid (predominant) or subsphaeric</td>
</tr>
<tr>
<td>LA</td>
<td>Variable, typically smoothly subtriangular</td>
<td>Round</td>
<td>Very faint</td>
<td>4 + 2</td>
<td>Row of long, thin setae, apical pair variable, usually slightly longer than the rest</td>
<td>Normal or slightly angular</td>
<td>Smooth</td>
<td>Forked, with a curved lateral projection pointing upwards</td>
<td>“spoon-shaped”, variable</td>
<td>Ovoid (predominant) or subsphaeric</td>
</tr>
<tr>
<td>VB</td>
<td>Variable, typically curved or smoothly subtriangular</td>
<td>Round or smoothly notched</td>
<td>Faint</td>
<td>4 + 2</td>
<td>Row of long, thin setae, apical pair not longer than the rest</td>
<td>Angular</td>
<td>Coarse or smooth</td>
<td>Forked, with a curved lateral projection pointing upwards</td>
<td>“spoon-shaped”, rounded</td>
<td>Subsphaeric, rarely ovoid</td>
</tr>
<tr>
<td>RG</td>
<td>Subtriangular</td>
<td>Round</td>
<td>Faint</td>
<td>4 + 3</td>
<td>Row of long, thin setae, apical pair variable, usually slightly longer than the rest</td>
<td>Angular</td>
<td>Smooth</td>
<td>Rod-shaped, forked, with a curved lateral projection pointing upwards</td>
<td>“spoon-shaped”, variable, usually more or less square</td>
<td>Subsphaeric</td>
</tr>
</tbody>
</table>
tion (Fig. 4A). Six characters (median lobe of ligula, posterolateral denticles of pronotum, shape of elytral apex, lateral denticles of elytra, pattern of umbilicate series and shape of metafemora) were effective altogether to recognize the proposed three species and correctly classify 100% of the cases (El. Suppl. File 2 Appendix S3).

Fig. 3. Hypothesis of three species within the six studied populations contrasted to morphological and molecular evidences (species A, B and C equivalent to “T. sp. 8”; “T. sp. 7” and “T. sp. 6” respectively, from Pérez-González et al. 2017). A: “Specimen-level” UPGMA dendrogram clustering the 44 vouchered specimens by morphological similarity according to 23 characters. B: ML tree from the COI sequences of 34 specimens. Numbers at each node represent bootstrap values (over 100).

Fig. 4. A: Discriminant Analysis based on morphological characters. B: Principal Component Analysis based on the k2p distances.
3.2. Analyses of genetic differentiation

As a preliminary approach trying to figure out if the morphological data were supported by the available molecular data, the 34 DNA sequences from the studied populations were used to assess the genetic structure within this *Typhlocharis* complex.

The maximum likelihood tree obtained in IQTree for the COI dataset shows clades well supported as monophyletic grouping specimens from individual localities (with VL and LA together) and is fully congruent with the hypothesis of three species (Fig. 3). Specimens from “species C” - AZ are supported as monophyletic (Bootstrap support = bs 100) and are sister to the lineage with the remaining specimens. This lineage is divided in two main clades, one including all specimens from “species B” - HV (bs 96) and the other, “species A” - LA+VL+VB+RG (bs 91), including all the remaining populations. Within the latter, there are three clades corresponding to specimens from RG (bs 96), VB (bs 78) and LA+VL (bs 93) respectively, with RG and VB (bs 78) well supported as monophyletic (Fig. 3).

The 34 COI sequences, after trimming to 523 bp, defined 14 haplotypes, none of them shared among populations. The parsimony haplotype network from TCS (Fig. 2) showed how haplotypes from HV (named H2, H3 and H4) diverge from haplotype H1 (characteristic from AZ) by 40 mutational steps supporting the differentiation of these two groups. HV is separated by at least 17 mutational steps from the closest haplotype (H6) within the third group, which included haplotypes from VB, RG, LA and VL. Among the latter populations, the distance between VB and VL is 11 mutational steps, between RG and VL is 8 and between VB and RG is 17.

AMOVA results grouping populations according to the three species hypothesis revealed that 2/3 of the genetic variance were due to the differences among groups. Also, these three genetic groups seemed to be heterogeneous given that 27.8% of the genetic variance was caused by differences among population within groups (El. Suppl. File 2 Appendix S4). This genetic structure was supported by K2P distances among the 14 haplotypes detected in the sample. These distances were visualized on a PCoA (Fig. 4B) where the x-axis split the haplotypes in the same three groups defined by the morphological characters.

These results must be taken with caution, since there are very few individuals from each population with available molecular data. However, this preliminary approach is largely congruent with the observed morphological differences and both approaches are consistent with the initial hypothesis. Hence, we propose that the six studied populations are part of a complex within genus *Typhlocharis* represented by three different species.

3.3. Description of species

*Typhlocharis coenobita* sp.n.

**Locus typicus.** Valverde de Burguillos, Badajoz, España.


**Diagnosis.** Small endogeon Anillini, anophtalmous, with subparallel body covered by microreticulated integument and scattered pubescence, recognizable by the following combination of characters: Vertex with *pars stridens*. Right mandible with two teeth. Subsquare pronotum, with two or three low posterolateral denticles. Elytra with smoothly rounded apical region, lacking denticles. Transverse scutellar organ variable: curved or subtriangular. Variable pattern of umbilicate series: 4+2 or 4+3. Last ventrite with belt, posterior margin smooth and continuous, without lateral notches, pattern of chaetotaxy l-(s)-s-s-l-s-s/m-s-l-s-s-(s)-l. Male genitalia: Falciform aedeagus, “rod-shaped” endophallic sclerites, with a curved lateral projection pointing upwards. Ring sclerite with a characteristic “spoon-shaped” distal projection. Female genitalia: stout tubular gonocoxites, without lateral setae; ovoid or subsphaeric spermatheca (Figs. 5, 8A,B).

**Description.** Length 1.11 – 1.32 mm (males), 1.25 – 1.40 mm (females). Anophtalmous, depigmented, with pubescent and microreticulated integument, ranging from yellowish to brown (Fig. 5). Head (Fig. 5): approximately as wide (0.23 – 0.30 mm) as long (0.23 – 0.30 mm), covered by subhexagonal microreticulation. Stridulatory organ (*pars stridens*) present in vertex region in both sexes. Posteralateral semilunar notch at both sides of cephalic capsule. Labrum subquadrate or slightly rounded, with thicker cuticle in a triangular region with a middle button. Clypeus with straight anterior margin. Moniliform antennae with 11 antennomeres, progressively more square-shaped towards distal **(morph 1)**, the last one pyriform. Stem of 3rd antennomere not elongated. Sensilla coeloconica (**sc**) on last antennomere arranged in a pattern of 3 anterodorsal and 1 posterodorsal. 1 ventral **sc** on antennomeres 5 and 6. Right mandible with two terebral teeth, left mandible without teeth, but with a smooth edge. Labium without special features for the genus, with a blunt middle tooth. Long paraglossae, middle lobe of ligula curved and prominent (shorter than paraglossae). Wide gula, approximately twice as long as
wide. *Cephalic chaetotaxy*: 6 pairs of labral setae (s-s-l-m-s-m/m-s-m-l-s-s), 2 pairs of clypeal setae (l-s/s-l), 1 pair of frontal setae, 2 supraocular pairs (anterior and posterior), 1 supraantennal pair, 2 pairs of occipital setae and 1 pair of genal setae, as well as scattered pubescence. Labium with 1 pair of setae near base of middle tooth, 1 pair of long setae near base of epilobes, 1 pair of very short setae near apex of epilobes and 1 or 2 pairs of very short setae near posterior suture. Prebasilar with 1 pair of lateral setae near anterior margin, 1 pair of very short lateral setae in middle region and 2 pairs (the lateral one much longer) in posterior region, irregularly distributed among specimens. **Thorax** (Fig. 5): pronotum subquadrate, barely longer (0.28 – 0.36 mm) than wide (0.28 – 0.34 mm), slightly narrowed posteriorly. Anterior margin straight or smoothly curved inwards, slightly crenulated, with medial hiatus (approximately as wide as 2 adjacent intersetal spaces). Posterior margin smoothly sinuated. Lateral margins with 2 or 3 posterior denticles, low, blunt and irregular. Surface covered by subhexagonal microreticulation. Disc flattened, with a median line and a pair of faint lateral sulci. Chaetotaxy: 1 pair of long setae in anterior third of lateral margins, 1 pair of long setae in posterior angles, a row of 6 – 8 pairs of setae [l-(l)-(l)-(l)-(m)-(m)-(l)-(l)] parallel to anterior margin (in general, 2 or 3 of them are notably shorter than the rest, alternated with long setae and highly variable among specimens), 3 pairs of setae parallel to posterior margin [s-s-l-l-l-s], a row of small, thin setae, regularly placed along anterior and posterior margins, a row of short setae along lateral margins and 5 pairs of irregular longitudinal rows on disc. Propisternal suture visible. Prosternal apophysis rounded. Anterior margin of prosternum with a row of long and thin setae and 6 – 8 pairs of short setae parallel to them. Prosternum covered by scattered pubescence, absent in proepisterna. Mesoepisterna covered by scales, with 1 pair of lateral setae near anterior margin, 1 pair of long setae near base of epilobes, 1 pair of very short setae near apex of epilobes and 1 or 2 pairs of very short setae near posterior suture. Prebasilar with 1 pair of lateral setae near anterior margin, 1 pair of very short lateral setae in middle region and 2 pairs (the lateral one much longer) in posterior region, irregularly distributed among specimens. **Male genitalia** (Fig. 8A): Aedeagus with falciform median lobe (length 0.21 mm), slightly bent to the right in dorsal view (anatomically oriented). Subtriangular, blunt apex. Endophallus with rod-shaped, forked sclerites, with a curved lateral projection pointing upwards. Subtriangular parameres, with 2 mid-sized apical setae. Ring sclerite (IXth abdominal sternum) subtriangular-arcuate, projected apically in a rounded, broad, “spoon-shaped” expansion. **Female genitalia** (Fig. 8B): Stout tubular gonoxites, with 2 apical setae, according to the general model described by Vigna-Taglianti (1972), without lateral setae but scattered pores. Gonosuboxites smoothly rounded. Spermathecal duct short to medium width, with two well-differentiated regions: proximal, thinner (diameter 0.003 mm) and distal, thicker (diameter 0.011 mm). Subsphaeric to slightly ovoid spermatheca (length 0.025 mm). Spermathecal gland conical (length 0.021 mm), distally sclerotized. Genital armature (abdominal segment/tergite VIII) with margin smooth and round, covered in a row of thin setae; lateral projections long and thin.

**Derivatio nominis.** The specific epithet refers to the apparent isolation from the outside world where these animals spent their lives, in homage to the spiritual retirement of the cenobitic monks (from latin *coenobitas* as member of a *coenobium*, a group of monks living in isolated communities).

**Habitat.** This species was collected in four localities within a range of approximately 1000 km² (Fig. 2). In the type locality, Valverde de Burguillos (Fig. 1C), *Tiphlocharis coenobita* sp.n. was captured in soil samples from a small meadow surrounded by cultures and open holm oak forest (*Quercus ilex* L.), where it coexists with *T. mixta*. In Valverde de Leganés (Fig. 1D), the sample was taken in reddish to dark brown soil with small embedded boulders from an olive tree culture (*Olea europaea* L.), with dense patches of brooms (*Retama* sp. Raf.) over grasses and thistles, with some holm oaks dispersed (*Quercus ilex* L.). The population of La Albuera (Fig. 1E) was found in a land used for pasture, on a low slope under holm oaks (*Quercus ilex* L.), near a small river surrounded by ash trees (*Fraxinus* sp. Tourn. ex. L.). The locality of Río Guadajira (Fig. 1F) was an open area with reddish, clayish soil and abundant scattered boulders and stones of diverse sizes. Vegetation was composed mainly by...
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Variability. The description above is based on the specimens from Valverde de Burguillos, but the other populations show a notable range of variation (Table 1).

grasses, thistles and small bushes. The sample was taken from the soil under a large deeply buried boulder, in the unaltered boundaries of a recently ploughed culture.

Fig. 9. Comparative detail photographs of the six main discriminant characters in the recognition of the three new Typhlocharis species. A: pattern of umbilicate series (position of setae indicated by “*”) and lateral denticles of elytra. B: shape of elytral apex; note the “v-shaped” notch of T. eremita sp.n. C: shape of metafemora. D: posterolateral denticles of pronotum (indicated by black arrows). E: median lobe of ligula; note the specially prominent lobe of T. anachoreta sp.n.
Within Valverde de Burguillos (372 specimens), the shape of labrum, development of the terebral teeth (the second tooth can be very blunt in some specimens), shape of the pronotum (specially the anterior margin), width of the hiatus, number and shape of the postero lateral denticles in pronotum, shape of the transverse scutellar organ, shape of the elytral buttonholes, shape of the apex of the elytra or texture of the inner margin of femora are all structures that can express minor differences among individuals. In three specimens the umbilicate series is asymmetric, with a 3+2/4+2 pattern.

The specimens from La Albuera (38 specimens) and Valverde de Leganés (3 specimens) are nearly identical to each other and fall within the range of variation observed in the T. coenobita sp.n. from Valverde de Burguillos. However, in average, these populations vary from the specimens in Valverde de Burguillos in a more prominent median lobe of ligula, fainter lateral denticles of elytra, less angular metafemora and smooth inner margin of femora in all limbs (Table 1). The “spoon-shaped” projection in the ring sclerite of males is more heterogeneous. The rest of cephalic features, pronotum, elytra, abdomen and genitalia are coincident.

The specimens from Rio Guadajira (56 specimens) are the most morphologically divergent to the other populations (Table 1), with differences that mainly affect pronotum and elytra. In average, they show a wider medial hiatus and generally more sinuous anterior margin (instead of straight) as well as more pronounced postero lateral denticles. The transverse scutellar organ is subtriangular in the majority of specimens (this is the population with stronger development and less variation in this trait). The most conspicuous difference is the pattern of the umbilicate series, 4+3 instead of 4+2 as in the other populations. Ligula, lateral denticles of elytra, inner margin of femora and male genitalia vary in the same degree as the populations of La Albuera and Valverde de Leganés, but the metafemora are angular (as in the specimens from Valverde de Burguillos). Intrapopulation variability is in the same degree and affects the same structures as described for the other populations.

Typhlocharis eremita sp.n.

Locus typicus. Oliva de la Frontera, Badajoz, España.


Diagnosis. Small endoge an Anillini, anophthalmous, with subparallel body covered by microreticulated integument and scattered pubescence, recognizable by the following combination of characters: Vertex with pars stridens. Right mandible with two teeth. Subquadrangular pronotum, with three to five strong postero lateral denticles. Elytra with smoothly rounded apical region, lacking denticles but with a clear “v-shaped” notch. Transverse scutellar organ variable: straight or slightly subtriangular. Umbilicate series with six setae (4+2). Last ventricle with belt, posterior margin smooth and continuous, without lateral notches, pattern of chaetotaxy l(l-s)-s-l-s/m-s-l-s-s-(s)-l. Male genitalia: Falciform aedeagus, “rod-shaped” endophalic sclerites, arranged in a branched structure. Ring sclerite with a broad subtriangular distal projection. Female genitalia: tubular gonoxites, without lateral setae; subsphaeric spermatheca (Figs. 6, 8C, D).

Description. Length 1.26–1.29 mm (males), 1.25–1.43 mm (females). Anophthalmous, depigmented, with pubescent and microreticulated integument, ranging from yellowish to brown (Fig. 6). Head (Fig. 6): almost as long (0.25–0.30 mm) as wide (0.26–0.30 mm). Cephalic features as described for T. coenobita sp.n., with exception of semilunar notches, much less marked, and a generally rounder, smoother labrum. Middle lobe of ligula moderately prominent, curved. Cephalic chaetotaxy: follows the same pattern as in T. coenobita sp.n. Thorax (Fig. 6): pronotum: subquadrature to subrectangular, trend to longer (0.31–0.40 mm) than wide (0.30–0.34 mm) shapes, slightly narrowed in posterior region. Anterior margin straight, crenulated, with medial hiatus as wide as 2–3 adjacent intersexual spaces. 3–5 posterolateral denticles, strong and well defined, in form of undulating edge. Other pronotal features and chaetotaxy as described for T. coenobita sp.n. Proepisternal suture visible and prosternal apophysis rounded. Prosternum, mesosopisterna and metaepisterna as in T. coenobita sp.n. Elytra (Fig. 6): approximately 2 × longer (0.66–0.74 mm) than wide (0.33–0.35 mm), subparallel. Lateral margins with 17–27 subtriangular denticles, progressively less marked towards posterior, but still defined near end. Apical margin without denticles, but with two blunt and rounded angles separated by a clear “v-shaped” notch in the end of suture. Transverse scutellar organ substraight or very smoothly subtriangular. Chaetotaxy: umbilicate series with anterior group of 4 setae and posterior group of 2 setae (4+2). Discal pubescence very short, even in apical region, distributed in 5 pairs of longitudinal rows. The rest of features do not differ from those described in T. coenobita sp.n. Legs (Fig. 6): As described for T. coenobita sp.n., but smooth inner margins in all femora, metafemora less angular and distal end of metatibiae strongly dilated. Abdomen (Fig. 6B): Abdominal features and chaetotaxy as described for T. coenobita sp.n. Male genitalia (Fig. 8C): Aedeagus with falconmidian lobe (length 0.19 mm), slightly bent to the right in dorsal view (anatomically oriented). Subtriangular, smoothly rounded apex. Endophallus formed by several “rod-shaped” sclerites, arranged in a “branched” structure. Subtriangular parameres, with 2 mid-sized apical setae. Ring sclerite (IXth abdominal sternum) with a broad subtriangular
distal expansion. **Female genitalia** (Fig. 8D): adjusts to the model of *Vigna-taglianti* (1972). Tubular gonocoxites with 2 apical setae. Lateral setae absent, but scattered pores. Gonosubcoxites rounded. Short spermathecal duct, with two differentiated regions: thinner, proximal (diameter: 0.003 mm) and thicker, distal (diameter: 0.010 mm). Subsphaeric to slightly ovoid spermatheca, “bulb-shaped” (length: 0.021 mm). Conical spermathecal gland (length: 0.020 mm), distally sclerotized. Genital armature (abdominal segment/tergite VIII) with margin smooth and round, covered in a row of thin setae; lateral projections long and slender.

**Derivatio nominis.** This species is dedicated to the hermit way of life (from latin *eremita*), voluntarily retired from the society, as allusion to the evolutionary history of the lineage, isolated from the external world to live in the endogean environment.

**Habitat.** The species is only known from the type locality, Zaos stream, near Oliva de la Frontera (Fig. 1A). It was captured in a small slope of a temporary watercourse in an alluvial plain between low hills covered with open cork oak (*Quercus suber*) and reeds (*Juncus sp.*), also from the underside of a deeply buried stone. The soil was humid and dark brown, rich in organic matter.

**Variability.** The range of variability in the single population known of *T. eremita* sp.n. (15 specimens) affects mainly the labrum, pronotum, transverse scutellar organ, lateral denticles of elytra, elytra buttonholes and chaetotaxy. This variation occurs in a similar fashion to that observed in *T. coenobita* sp.n., showing subtle variations within the observed specimens in shape, development and number of denticles, or position of setae.

**Typhlocharis anchoretae sp.n.**

**Locus typicus.** Higuera de Vargas, Badajoz, España.


**Diagnosis.** Small endogean Anillini, anophtalmous, with subparallel body covered by micoreticulated integument and scattered pubescence, recognizable by the following combination of characters: Vertex with *pars stridens*. Right mandible with two teeth. Subquadrate pronotum, with three to five blunt posterolateral denticles. Elytra with smoothly rounded apical region, lacking denticles. Transverse scutellar organ highly variable: straight to strongly subtriangular. Strongly marked lateral denticles. Umbilicate series with seven setae (4+3). Last ventrite with belt, posterior margin smooth and continuous, without lateral notches, pattern of chaetotaxy l-(s) s l ss/m s l l-(s)-s l. Male genitalia: Falciform aedeagus, “rod-shaped” endophalic sclerites, with a curved lateral projection pointing upwards. Ring sclerite with a broad and variable “spoon-shaped” distal projection. Female genitalia: robust tubular gonocoxites, without lateral setae; slightly ovoid or subsphaeric spermatheca (Figs. 7, 8E,F).

**Description.** Length 1.17–1.35 mm (males), 1.30–1.45 mm (females). Anophtalmous, depigmented, with pubescent and micoreticulated integument, ranging from yellowish to brown (Fig. 7). **Head** (Fig. 7): slightly wider (0.26–0.29 mm) than long (0.23–0.26 mm). Cephalic features as described for *T. coenobita* sp.n., but inner edge of left mandible more prominent and middle lobe of ligula highly projected, as long or longer than para-glossae. **Cephalic chaetotaxy**: coincident with the pattern described in *T. coenobita* sp.n. **Thorax** (Fig. 7): prono- subtrigale, slightly longer (0.32–0.40 mm) than wide (0.29–0.36 mm) narrowed posteriorly. Anterior margin straight or smoothly curved inwards with medial hiatus as wide as 2 or 3 adjacent intersepal spaces. 3–5 posterolateral denticles, blunt and irregular but well defined. Rest of pronotal features and chaetotaxy as described for *T. coenobita* sp.n. **Elytra** (Fig. 7): approximately 2 × longer (0.62–0.79 mm) than wide (0.32–0.40 mm), subparallel. Lateral margins serrated with 17–27 strongly marked denticles, progressively smoother towards posterior, but still defined near end. Apical margin without denticles. Transverse scutellar organ highly variable, margin substraight to strongly subtriangular. Chaetotaxy: umbilicate series formed by anterior group of four setae and posterior group of three setae (4+3). Apical row of thin setae, apical pair longer than rest. Other features as described for *T. coenobita* sp.n. **Legs** (Fig. 7): As described for *T. coenobita* sp.n., but smooth inner margins in all femora. **Abdomen** (Fig. 7B): abdominal features and chaetotaxy as described for *T. coenobita* sp.n. **Male genitalia** (Fig. 8E): Aedeagus with falciform median lobe (length: 0.19 mm), slightly bent to the right in dorsal view (anatomically oriented). Subtriangular, smoothly rounded apex. Endophallus with rod-shaped, forked sclerites and a curved lateral projection pointing upwards. Subtriangular parameres, with 2 apical setae. Ring sclerite (IXth abdominal sternum) with a broad “spoon-shaped” distal expansion, irregular and highly variable between individuals, more or less square edges are common. **Female genitalia** (Fig. 8F): adjusts to the model of *Vigna-taglianti* (1972). Robust tubular gonocoxites with 2 apical setae. Lateral setae absent, but scattered pores. Gonosubcoxites rounded.
Short spermatic duct, with two differentiated regions: thinner, proximal (diameter 0.004 mm) and thicker, distal (diameter 0.008 mm). Slightly ovoid or subphaeric spermatic gland (length 0.022 mm). Conical spermatic gland (length 0.029 mm), distally sclerotized (Fig. 8F). Genital armature (abdominal segment/tergite VIII) with margin smooth and round, covered in a row of thin setae; lateral projections long and slender.

Derivatio nominis. Like the cenobitic monks or the hermits, the anchorites (from Latin anachoreta) were people retired from the society to a life of isolation. The new species is named after them by the parallel lifestyle, retired from the outside to a life inside the soil.

Habitat. *T. anachoreta* sp.n. is currently known only from the type locality, near Higuera de Vargas (Fig. 1B), where it was found in an open grassland and thistle pasture field with scattered broom bushes (*Retama* sp. Raf.). Abundant small boulders and stones were scattered all over the place, embedded in the soil at general shallow depths (5–20 cm). The sample was taken from humid soil under the stones.

Variability. The morphological traits that are observed to vary between individuals of *T. anachoreta* sp.n. are the same as commented before in *T. coenobita* sp.n. and *T. eremita* sp.n. Apart from that, the population of Higuera de Vargas is quite diverse in size and degree of sclerotization, from soft, yellowish small specimens around 1.15 mm to tougher, chestnut brown large specimens of more than 1.40 mm.

3.4. Identification key to the “coenobita species complex”

1 Presence of ventral foveae on 1st ventrite, more developed in females. Inner margin of profemora markedly angular .... *T. mendesi* Serrano & Aguiar 2017

1’ Absence of ventral foveae. Inner margin of profemora not or smoothly angular ........................................ 2

2 Faint semilunar notch. Posteralateral denticles of pronotum strong and well defined, 3–5 (Fig. 9D). “v-shaped” notch in the elytral apex (Fig. 9A). Subtriangular distal expansion of ring sclerite (Fig. 8C) .................................................. *T. eremita* sp.n.

2’ Well marked semilunar notch. Moderately marked or low posteralateral denticles of pronotum (Fig. 9D). Elytral apex rounded, without any clear “v-shaped” notch (Fig. 9B). “Spoon-shaped” distal expansion of ring sclerite (Fig. 8A,E) ............................................... 3

3 Very prominent middle lobe of ligula (as long as or longer than paraglossae, Fig. 9E). Posteralateral denticles of pronotum moderately marked, 3–5 (Fig. 9D). Lateral denticles of elytra strongly marked, serrated (Fig. 9A) ............................... *T. anachoreta* sp.n.

3’ Curved middle lobe of ligula, sometimes prominent, always shorter than paraglossae. Posteralateral denticles of pronotum low and blunt, 2–4. Lateral denticles of elytra faint or very faint ... *T. coenobita* sp.n.

4. Discussion

4.1. Affinities

Typhlocharis coenobita, *T. eremita* and *T. anachoreta*, hence referred as the “coenobita complex”, are very close to each other and represent a lineage previously unknown for the genus (Pérez-González et al. 2017). This lineage was shown related to species of the former “baetica” group (except *T. mixta* and *T. besucheti*, *T. martini*, *T. singularis* and *T. gomesai*).

*T. eremita* is easily distinguished within the complex by a fainter semilunar notch, pronotum with well marked crenulation in the anterior margin and 3–5 strong, well defined posteralateral denticles, elytra with moderately marked lateral denticles and a characteristic “v-shaped” notch in the apex (Fig. 9, Table 1). Also, it is recognizable by some features of male genitalia, like the shape of endopalial sclerites and the distal expansion of the ring sclerite.

*T. coenobita* shows high internal variability between populations and it is the second described species of Typhlocharina with a polymorphic pattern of umbilicate series (Serrano & Aguiar 2000, 2002). Morphological features of the RG population are beyond the average variation observed for the other populations of *T. coenobita*. Some characteristics of the RG specimens (like the 4+3 umbilicate series, the prominent middle lobe of ligula, the width of the medial hiatus among others, see Table 1) are close to that of *T. anachoreta*. This fills a morphological gradient that challenges the differentiation between *T. coenobita* and *T. anachoreta*. However, *T. anachoreta* can be identified by the extremely prominent middle lobe of the ligula, the posteralateral denticles of pronotum (more abundant and notorious than in *T. coenobita*) and the strongly marked lateral denticles of elytra (Fig. 9), much more developed than in any population of *T. coenobita*.

The combination of a reduced pattern in the umbilicate series (4+2, 4+3) and total lack of apical denticles in elytra is unusual within Typhlocharina. So far, this condition was only known in *T. armata* Coiffait, 1969; *T. deferreri* Zaballos & Pérez-González, 2011 and the recently described *T. mendesi* (Zaballos & Pérez-González 2011a,b; Serrano & Aguiar 2017). *T. armata* and *T. deferreri* are distantly related to the “coenobita complex” (Pérez-González et al. 2017) and, while the overall morphology is similar, they do not share characteristic traits of the complex, such as the alternate length in the setae of anterior margin of pronotum, the shape of the ring sclerite of males or the short spermatic duct and rounded spermaticae of females. By contrast, *T. mendesi* show all these features, suggesting that it is a member of the same species complex. We had the oppor-
tunity of studying four paratypes of *T. mendesi* (2 males, 2 females) that indicates a particularly close relationship to *T. eremita*. Both species share a trend to rectangular pronotums with well marked crenulation in the anterior margin and moderately marked lateral denticles of elytra, as well as a near-identical shape of the ring sclerite projection. *T. mendesi* differs from the three new species in the presence of well developed ventral foveae in the first ventrites (deeper in females) and very angular pro femora. The fact that *T. mendesi* is only known from the surroundings of Bucelas region (Estremadura, Portugal), about 200 km away from the area where the new species were found (southern Badajoz, Spain) points to a wider distribution of the *coenobita* species complex.

The subtriangular shape of the transverse scutellar organ, the pattern of the endophallic sclerites and the shape of the spermatheca resemble those of *T. martinii* Andújar, Lencina & Serrano, 2008; *T. besucheti* Vigna-Taglianti, 1972; *T. gomesalvesi* Serrano & Aguiar, 2002 and *T. singularis* Serrano & Aguiar, 2000, which are characterized by the presence of parasutural denticles in the elytral apex. *T. gomesalvesi* and *T. singularis* are the closest geographically, and also show reduced umbilicate series (4+2), polymorphic in the case of *T. singularis* (Serrano & Aguiar 2000, 2002).

The pattern of the endophallic sclerites, with bifurcated rod-shaped pieces and a lateral branch pointing upwards, is also typical of the *baetica* group (Pérez-González & Zaballos 2013a). This is suggestive of a relationship between all the mentioned species that is supported in the current phylogenies of Typhlocharina (Pérez-González et al. 2017; Andújar et al. 2017). In fact, the main difference between “coenobita complex”, *T. martini*, *T. besucheti*, *T. gomesalvesi*, *T. singularis* and the species of *baetica* group is the different development of apical denticles in the elytra (absent, presence of microdenticles and sutural denticles and multiple fully developed denticles, respectively), which have been shown to be plastic characters with low phylogenetic signal (Pérez-González et al. 2017).

### 4.2. Lumping or splitting? Taxonomic implications of the new populations

While the reality of the species concept as discrete entities and the boundaries of speciation processes has been thoroughly discussed (e.g. De Queiroz 2007; Willis 2017; Sites & Marshall 2003), the designation of evolutionary lineages is still a need that should be attained to register and describe biological diversity (Davyat 2005; Valdecasas 2008).

The three species proposed in this work are supported by morphological and molecular data and all of them differ from any other *Typhlocharis* species. The entity of *T. eremita* as a new species is well established within the standards of the genus. However, the high internal variability observed within the populations of *T. coenobita* and their relationship with *T. anachoreta* are in the verge of what can be considered species-level differentiation. In particular, the population of Rio Guadajira implies an interesting problem. Morphologically, this population shows some features that are intermediate between other populations of *T. coenobita* and *T. anachoreta*, blurring the otherwise clear differences between the two taxa (Table 1). In addition, all populations within *T. coenobita* do not share haplotypes and show certain geographic structure (Fig. 2). This situation led to discuss different taxonomic interpretations:

**Why not consider RG as a different species?** According to the mitochondrial sequences studied, the populations described as *T. coenobita* show heterogeneity, pointing to a certain level of isolation between three large clusters (VL+LA, VB and RG respectively). The current molecular phylogeny suggests that RG population is nested within the same clade as the remaining populations of *T. coenobita*, with a sister relationship with specimens from VB (Fig. 3B). Thus, describing the RG population as a different species would imply that the other two lineages should be at the same taxonomic level, i.e. *T. coenobita* would be split into three different taxa (RG, VB and VL+LA). The subtle morphological differences between the populations of VB and VL+LA do not support their division in two species, while describing specimens from VB and VL+LA as a single species, excluding RG, would generate an artificial, paraphyletic taxon.

**Does the RG population fall within the internal variability of *T. coenobita*?** According to the statistical analyses, yes. It is possible to accurately discriminate between the three new species with a 100% rate of success based on six morphological key characters in agreement with the results of the discriminant analyses.

This classification was tested with the additionally coded specimens and the RG specimens were recognized as *T. coenobita* in all cases. This implies that the RG population is, overall, closer to the rest of *T. coenobita* in spite of the aforementioned affinities with *T. anachoreta*. The use of statistical analysis in taxonomy and integrative studies with molecular and morphological data is becoming more and more common (e.g. Silva et al. 2017), yet this is the first time that such techniques are applied to discriminate species in Anillini endogeans beetles. They suppose a powerful tool that could help taxonomic decisions when phenotypic differences are difficult to assess.

**Why not consider *T. anachoreta* as part of *T. coenobita***? In this case, the molecular data (Fig. 3B) recovered a well supported relationship of the population from HV, described as *T. anachoreta*, as the sister taxon to the whole clade of *T. coenobita* populations. Molecular data indicates a higher separation between both clades than within any of the populations of *T. coenobita*.

Also, the morphological differences between both clades go beyond the usual limits of internal variability known in *Typhlocharis* (Pérez-González et al. 2013).
terborne dispersal mechanisms as well as short-distance It has been stated that these animals use passive wa-

According to the calibrated phylogeny by

Overall, although different taxonomic decisions are possible, we consider that the species boundaries here adopted maximize the consistence among different sourc-
es of evidence data, while the intraspecific variability within T. coenobita is properly described and discussed, thus contributing to elucidate the ongoing differentiation processes within the coenobita species complex.

4.3. Evolutionary remarks

The “coenobita species complex” is the first documented case of an expected situation within Typhlocharina and other lineages of Anillini: the presence of several closely related allopatric populations within a very reduced geograph-
ical range that, despite certain genetic isolation, show a gradient of morphological differences that chal-

It has been stated that these animals use passive wa-
terborne dispersal mechanisms as well as short-distance active displacement of the populations (ORTUNO & GIL-
gado 2011; ANDUJR et al. 2017). The studied populations show a geographic structure (Fig. 2) that could be consistent with two alternative scenarios. In the first one, sporadic dispersal events (active or passive) will allow colonization of new suitable patches of habitat in the sur-
roundings where, due to i) founder effects and ii) sub-
sequent low connectivity between the ancestral and the new populations, differentiation can be achieved in short evolutionary time. In this scenario, the different degrees of variation between the populations of the “coenobita species complex” would be due to the different times since the split of populations.

According to the calibrated phylogeny by ANDUJR et al. (2017), the split of the “coenobita species complex” from other Typhlocharis could be dated back to about 20 Mya. This provides enough time to make probable infre-
quent events of dispersal through unsuitable habitats, but also it is enough time for strong environmental changes to happen. Thus, as a second alternative scenario, the data can be coherent with the fragmentation of an ance-
stral species with a wider distribution and the subsequent isolation and differentiation of resultant populations.

In both scenarios, the differences between the popu-
lations of T. coenobita are suggestive of incipient spe-
ciation processes, where they are in different stages of isolation and show polymorphic characters fixed dif-
ferently between them. This happens with the different pattern of umbilicate series in RG (4+3) and VL, LA and VB (4+2), which among other differences might be evidence towards future species-level differentiation. As well, T. coenobita + T. anachoreta, estimated to have split about 5 Mya, could be considered a “macrospecies” (SERISH BROOKS & MCELLENAN 2002), where both lineages have recently diverged.

There is increasing evidence that similar situations could be frequent within the whole lineage of Typhlocha-
rina (SERRANO & AGUIAR 2000, 2002, 2014; PÉREZ-
GONZÁLEZ et al. 2013, PÉREZ-GONZÁLEZ et al. 2017). This is not surprising considering the great bias derived from the difficulties in the sampling of these minute beetles, where we only get small glimpses of the real diversity of the lineage. The presence of T. mende-
si far away of the distributional core of the three new species, strongly sug-

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Electronic Supplement Files

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File 1: perezgonzalez&al-carabidaeyplocharis-asp2018-electronic-supplement-1.doc — Appendix S1. — Table S1.1. List of vouchered specimens and Genbank accession numbers. From Pérez-González et al. (2017) and Andújar et al. (2017). — Table S1.2. List of characters and character transformation series. Adapted from Pérez-González et al. (2017). — Table S1.3. “Specimen-level” matrix of morphological data for the 44 hologenophores. In grey, characters recovered as significant predictor variables in the Discriminant Analysis. — Table S1.4. “Population-level” matrix of morphological data. *Species hypothesis used in the Discriminant Analysis: A, B and C equivalent to "T. sp. 6", "T. sp. 7" and "T. sp. 6" respectively, from Pérez-González et al. (2017). In grey, characters recovered as significant predictor variables in the Discriminant Analysis. — Table S1.5. Matrix of morphological data for the 43 additional specimens coded to test the performance of the classification function coefficients obtained by the Discriminant Analysis. Highlighted in red, characters recovered as significant predictor variables.

File 2: perezgonzalez&al-carabidaeyplocharis-asp2018-electronic-supplement-2.doc — Appendix S2. UPGMA Analysis. — Table S2.1. Distance matrix based on Euclidean coefficient for the 44 vouchered specimens. — Fig. S2.1. “Specimen-level” UPGMA dendrogram clustering the 44 vouchered specimens by morphological similarity according to 23 characters. Numbers at each node represent bootstrap values (over 100). — Table S2.2. Distance matrix based on Euclidean coefficient for the 6 studied populations. — Fig. S2.2. “Population-level” UPGMA dendrogram clustering the 6 studied populations by morphological similarity according to 23 characters. Numbers at each node represent bootstrap values (over 100). — Appendix S3. Discriminant Analysis. — Table S3.1. Results of applying the Classification Functions obtained by the Discriminant Analysis to the 43 additional specimens coded in Table S5. In grey, characters recovered as significant predictor variables. In red, values of the predicted group for each specimen. — Appendix S4. AMOVA Analysis.

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