Two new European species of the marine genus Tubificoides (Annelida: Clitellata: Naididae) with notes on the morphology of T. pseudogaster (Dahl, 1960)

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Abstract

Two new species of Tubificoides (subfamily Tubificinae), T. charlotteae n. sp. and T. mackiei n. sp., are described based on morphological analyses. Both species were flagged as potentially cryptic in a previous investigation, based on both mitochondrial and nuclear sequence data. Tubificoides charlotteae n. sp., known only from an intertidal site in southern Spain, is characterized by the lack of cuticular papillation, the possession of several needle-like hair chaetae in dorsal bundles, a cone-shaped penis sheath, and a rather large, muscular penial sac. It strongly resembles the north-west European, largely sublittoral species T. amplivasatus, but differs from this species in terms of body size, width of vas deferens, and the shape and size of both the ejaculatory duct and penial sac. Tubificoides mackiei n. sp., collected from the southeast coast of Norway and the west coast of Sweden, is characterized by the lack of cuticular papillae and hair chaetae, and the possession of a rather long penis sheath with a wide terminal opening. It shares several morphological features with the sympatric species Tubificoides pseudogaster, but is distinguished from the latter by the detailed morphology and length of the penis sheaths, the width of the atrium, and the lower maximal number of bifid chaetae in dorsal precloacal bundles.

The utility of an integrative comparative approach, combining molecules and morphology, for the identification and delineation of new taxa within Tubificoides is briefly discussed.

Key words: Tubificoides, Tubificinae, pseudo-cryptic speciation, new species, genetic variation, integrative taxonomy

Introduction

The cosmopolitan clitellate genus Tubificoides Lastockin, 1937 (family Naididae sensu Erséus et al. 2008) currently harbors 59 nominal species and is one of the few genera within the subfamily Tubificinae that inhabits the marine environment; most other Tubificinae are found in freshwater. Virtually all naidids possess a demarcated section of the male genital ducts that is referred to as the “atrium”, and all members of Tubificoides are recognized by the subapical entry point of the vas deferens into the atrium, approximately opposite to the entrance of the prostate gland; the general condition in the remainder of Tubificinae is an apical entry point of the vas deferens into the atrium. Species within the genus are commonly distinguished from each other by unique combinations of characters, mainly pertaining to the presence or absence of needle-like hair chaetae, the number and detailed morphology of the bifid crotchets, the presence or absence of distinct cuticular papillation on the body surface, and the shape and size of the cuticular penis sheaths (Hrabě 1966; Holmquist 1978, 1979; Brinkhurst & Baker 1979; Brinkhurst 1985, 1986; Helgason & Erséus 1987; Milligan 1996; Kvist et al. 2008, 2010). So far, however, it has been difficult to trace the evolution of these morphological characters, and phylogenetic analyses based on morphology have so far failed to produce resolved hypotheses (Erséus, unpublished data). In addition, due to our poor understanding of the morphological characters that separate certain species within Tubificinae, the taxonomy of Tubificoides has been in flux with some species having been placed in several different genera (e.g. Limnodrilus Claparède, 1862, Tubifex Lamarck, 1816, Clitellio Savigny, 1820 and Peloscolex Leidy, 1851). By contrast, at least
one species, originally ascribed to *Tubificoides*, has since been subsumed by a different genus (*Troglodrilus* Juget, des Chatelliers & Rodriguez, 2006; see Achurra et al. 2012). Members of *Tubificoides* are predominately known from intertidal and littoral, marine habitats, although some species have adapted to brackish water [e.g. *Tubificoides pseudogaster* (Dahl, 1960) and *Tubificoides heterochaetus* (Michaelsen, 1926); see Dahl 1960; Laakso 1969; Baker 1980] or the deep sea, e.g. *Tubificoides aculeatus* Cook, 1969 and *Tubificoides blakei* Kvist, Dreyer & Erséus, 2008 (Cook 1969, 1970; Kvist et al. 2008).

Concurrent with the expansion of our knowledge concerning taxonomy, geographic boundaries, molecular phylogenetics and genetic variation within Clitellata, cryptic diversity within this taxonomic group has become more frequently exposed (e.g. Gustafsson et al. 2009; James et al. 2010; De Wit & Erséus 2010; Buckley et al. 2011; Matamoros et al. 2012; Martinsson & Erséus 2014, 2017; Liu et al. 2017). Cryptic species are those that are seemingly identical on a morphological basis and that have been incorrectly lumped into a single taxon (Bickford et al. 2007). Because of the morphological uniformity between cryptic species, molecular analyses have become vital components in uncovering hidden diversity (e.g. Hebert et al. 2004; Witt et al. 2006; Moura et al. 2008). Occasionally, the separation of taxa based on molecular data begets re-assessment of morphological characters, which may lead to the finding of inconspicuous morphological differences between "cryptic" taxa. These taxa are sometimes referred to as pseudo-cryptic (e.g. Kawauchi & Giribet 2014; Achurra et al. 2015). The use of molecular phylogenetics and DNA barcoding allowed Kvist et al. (2010) to uncover previously hidden diversity within two nominal species of *Tubificoides*. At first consideration, worms collected from the Atlantic coast of Spain conformed morphologically to *Tubificoides amplivasatus* (Erséus, 1975), and several worms collected from the west coast of Sweden seemed morphologically compatible to the description of *T. pseudogaster* (Kvist et al. 2010). However, molecular analyses, with emphasis on the DNA barcoding locus COI (cytochrome *c* oxidase subunit I), revealed an upper limit of genetic variation that was orders of magnitude greater than previous estimations of intraspecific variation (Hebert et al. 2003; Smith et al. 2005; Ratnasingham & Hebert 2007; Erséus & Kvist 2007). The mitochondrial genetic variation was therefore complemented by analyses of nuclear genes, revealing the same pattern of separation, albeit at lower magnitudes. Whereas this result in itself justifies the separation of the species, it also impelled a closer investigation into the detailed morphology of these specimens. Herein, we formally describe these two new species on the basis of morphological analyses.

**Material and methods**

**Specimen collection and morphological analyses.** Between 2005 and 2014, several specimens of *Tubificoides* were collected from the Atlantic coast of southern Spain (intertidal), the southeast coast of Norway (0.3–0.5 m depth) and the west coast of Sweden (0.5–2 m depth). Collection was carried out by hand and the retained sediment was composed of fine silt, sand or mud; on occasion, hypoxic conditions were encountered, judging from the black coloration and sulfurous odor. Material was then sieved by elutriation using a mesh size of 125–250 μm and specimens were sorted under a dissecting microscope. Samples were often associated with several other species of naidids [e.g. *Tubificoides benedii* (d’Udekem, 1855) and *Paranais* sp.] and small bivalves. The sampled worms were subsequently cut in two parts; the posterior parts were used for DNA analysis and were therefore stored in 95% ethanol, whereas the anterior parts, used for morphological analysis, were stored in 80% ethanol to prevent shrinking. The protocol for dehydration, staining, clearing and mounting followed Kvist et al. (2010) and the methods used are further described herein. Briefly, the specimens were stained with alcoholic paracarmine, cleared and dehydrated in a ethanol/xylene series, and mounted whole in Canada balsam on microscope slides. Morphological examinations were carried out using an Olympus BX50 stereomicroscope fitted with an Optem DC500U digital camera or on a Leitz Dialux 22 stereomicroscope, fitted with a SpotFlex 15.2 64 Mp digital camera. To avoid creation of junior synonyms, the lectotype and paralectotypes of *T. pseudogaster* (see Baker 1980) (Natural History Museum of Denmark, accessions ZMUC-OLI-000032-34), as well as several non-type specimens of both *T. amplivasatus* and *T. pseudogaster* were examined and compared to the new specimens. Types and other material of the new species are deposited in the Swedish Museum of Natural History (SMNH), Stockholm, Sweden and in the Zoological Museum, University of Bergen (ZMBN), Bergen, Norway.

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**Taxonomy**

*Tubificoides charlotteae* n. sp.

Figures 1, 2

*Tubificoides "amplivasatus II"; Kvist et al. 2010*

**Diagnosis.** This species can be distinguished from congeners on the basis of its possession of hair chaetae, its lack of cuticular papillation, its relatively small body size, and in the details of its male genital ducts. In comparison with its closest relative, *T. amplivasatus*, its body is smaller and its vas deferens narrower. In addition, whereas the ejaculatory duct is long and not incorporated in the penial sac in *T. amplivasatus*, the same structure is short and enclosed by the musculature of the penial sac in *T. charlotteae*. The average COI distance between *T. charlotteae* n. sp. and *T. amplivasatus* is 12.74% (see Kvist et al. 2010) and a total of 78 characteristic attributes (i.e., molecular synapomorphies; see Sarkar et al. 2002) separate the COI sequences of these species (Kvist et al. 2010).

**Holotype.** SMNH Type coll. 8952 (formerly SMNH 108958; specimen ID CE1247 in Kvist et al. 2010), stained, whole-mount on microscope slide with coverslip, sexually mature specimen, collected by C. Erséus, Nov. 21 2005; holotype COI sequence GenBank accession number HM460169; for other genetic information, see details in Kvist et al. (2010).

**Type locality.** San Pedro River, Puerto Real, Cadiz, Andalucía, Spain, intertidal (36°33'24"N, 06°12'23"W).

**Paratypes.** SMNH Type Coll. 8953 (formerly SMNH 108959; specimen ID CE1248 in Kvist et al. 2010) and 8954 (formerly SMNH 108960; specimen ID CE1249 in Kvist et al. 2010), stained, whole-mounts on microscope slide with coverslip, both specimens mature, collected at type locality by C. Erséus, Nov. 21 2005; for genetic information, see Kvist et al. (2010). Paratypes COI sequence GenBank accession numbers HM460170 and HM460171.

**Etymology.** Named in honor of Charlotte Calmerfalk Kvist - the first author is eternally grateful for her tireless support, patience and love during years of focus on clitellate research.

**Description.** All types incomplete, posterior ends used for DNA extraction. Holotype 1.58 mm long with anterior 13 segments (Fig. 1A), paratypes 1.5–2.4 mm long with anterior 12–22 segments. Width at XI 0.28 mm in holotype, 0.15–0.30 mm in remaining specimens; all specimens mounted and compressed. Prostomium bluntly conical in most specimens (Fig. 1A), more pointed in one paratype, slightly shorter than basally wide. Cuticle smooth in anterior portion, densely and finely granulate in posterior part, aspect somewhat "dusty" (Fig. 1A), but proper cuticular papillae nowhere developed. Clitellum poorly developed in segments X–XI of all type specimens.

In preclitellar segments, dorsal chaetal bundles with (1)2–3 needle-like hair chaetae (up to 100 µm long) and 1–3 bifid crotchets, 25–35 µm long (Fig. 2A). Bifids with straight teeth of equal length and approximately same width. Postclitellar dorsal bundles with 1–3 hair chaetae (about 50 µm long) and 1(2) bifid crotchets, 25–30 µm long (Fig. 2B); bifids straight, with teeth of equal length and approximately same width. Some bundles without bifids (tips broken off?). Precitellar ventral bundles (Fig. 2C) with 2–3 bifid crotchets (25–50 µm long), sometimes transitioning into a single bifid in "bundles" closer to genital region; teeth of equal length, upper tooth thinner than lower and teeth rather diverging. No ventral chaetae in segment XI of mature worms. Postclitellar ventral "bundles" (Fig. 2D) each represented by a single bifid crotchet (at least 20–25 µm long), both teeth becoming increasingly thinner posteriorly, with approximately same width; chaetae are in an oblique position. Nodulus inconspicuous in all bifid crotchets. Spermathecal pores paired, located slightly above ventral chaetae in X. Male pores paired, in line with or slightly above assumed ventral chaetal line in mid XI.

Pharyngeal glands present in IV–V, undetectable in one specimen. Esophagus possibly modified in IX but difficult to discern (see below, in the Remarks section for *Tubificoides mackiei* n. sp.). Vas deferens (Figs. 1D, 2E; vd) 25 µm wide in holotype, about 320 µm long (only measurable in one paratype). Vas rather thin-walled and densely ciliated; entering atrium (Figs. 1B, 2E; at) subapically and opposite to entrance of rather small prostate gland (Figs. 1B, 2E; pr). Atrium about 265 µm long in holotype, 45–60 µm wide; bipartite with dense and finely granulated cellular matrix in mid and ectal (closest to the external pore) portions, and coarser glandular epithelium in ental cap portion (inner portion, furthest away from external pore). Ectalmost end of atrium with short ejaculatory duct, leading into a muscular, egg-shaped penial sac, the latter about 50 µm long, 35 um wide (Figs. 1C, 2E). Cuticular penis sheath cone-shaped with terminal opening (Figs. 2E, 2F; cu); about 20 µm long, 25 µm wide.
Poorly developed spermathecae visible in holotype and one paratype; spermatheca lightly pear-shaped with narrow duct (18 µm wide at narrowest point) leading to wider ental ampulla [about 170 µm long, 45 µm wide at widest part (Figs. 1E, 2G)]. All specimens pre-copulatory; spermatozeugmata not observed.

**Distribution and habitat.** Known only from type locality in southern Spain.

**FIGURE 1.** *Tubificoides charlotteae* n. sp., holotype. A. Anterior end of body. B. Male (and partly female) genital apparatus. C. Muscular penial sac, containing also a part of the ejaculatory duct (demarcated as a ring), and internally with a penial sheath. D. Vas deferens. E. Spermatheca at an early stage of development (comp. Fig. 2G). Abbreviations: at, atrium; pr, prostate gland; psm, penial sac musculature; sp, spermatheca; vd, vas deferens.

**Remarks.** *Tubificoides charlotteae* shares several features with, and seems closely related to, the north European species *T. amplivasatus* (see Kvist *et al.* 2010). First, the chaetal arrangement of *T. charlotteae* resembles that of *T. amplivasatus* inasmuch as both species possess dorsal hair chaetae and 1–3 bifid crotchets in anterior bundles. Second, the lack of cuticular papillation, but dense and fine granulation of the body wall, in the postclitellar region is reported for both species (Erséus 1975). Third, both species have unusually thin-walled vasa deferentia (Fig. 2E; Erséus 1975, Fig. 1 and Erséus 1976, Fig. 2), as well as cone-shaped penis sheaths. However, the new species differs from *T. amplivasatus* in its shorter body length (average of 0.2 mm per segment in *T. amplivasatus* versus 0.12 mm in *T. charlotteae*), lower number of dorsal hair chaetae (as many as four hair chaetae, reported in some bundles of *T. amplivasatus*, are nowhere found in *T. charlotteae*), in the width of the vas deferens (about 50 µm in *T. amplivasatus*, 25 µm in *T. charlotteae*), and in the shape and size of both the ejaculatory duct and penial sac. In *T. amplivasatus*, the ectal part of the male duct is a distinct ejaculatory duct (with a thin outer layer of circular musculature), and it is separated from the atrium proper by a constriction (Erséus 1976). This duct leads into the penis, which is located in a poorly developed penial sac (Erséus 1976, Fig. 2), and the latter does not enclose the ejaculatory duct. In *T. charlotteae*, however, the ejaculatory duct is short, its outer lining of muscles seen as a ring in Fig. 1C, and, here, the duct is enclosed by the strong musculature of the penial sac (Figs. 1C, 2E: psm).
NEW TUBIFICOIDES

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FIGURE 2. Tubificoides charlotteae n. sp., holotype. A. Preclitellar dorsal chaeta. B. Postclitellar dorsal chaeta. C. Preclitellar ventral chaeta. D. Postclitellar ventral chaeta. A–D distal ends of chaetae only; note that Fig. 2D is drawn in an oblique position, such that the length of the chaeta may not accurately reflect the true length, but that the bifurcation of the teeth is still accurate. E. Male genital apparatus and ovary (note that the ovary is not connected to any male structure, but abuts the atrium). F. Lateral contours of penis sheaths. G. Spermatheca at an early stage of development (comp. Fig. 1E). Abbreviations: at, atrium; bv, blood vessel; cu, cuticular penis sheath; psm, penial sac musculature; ov, ovary; pr, prostate gland; sa, spermathecal ampulla; sd, spermathecal duct; sf, sperm funnel; vd, vas deferens (only a short part of vd is shown).

Tubificoides mackiei n. sp.
Figures 3–4

Tubificoides pseudogaster; Brinkhurst & Baker 1979, partim?
Tubificoides pseudogaster; Brinkhurst 1986, partim?
Tubificoides "pseudogaster I"; Kvist et al. 2010

Diagnosis. This species can be distinguished from congeners on the basis of the lack of hair chaetae and cuticular
papillation, and the detailed morphology of the cuticular penis sheaths. In comparison with its closest known relative, *T. pseudogaster*, the new species is distinguished by its wider body at segment XI, its wider ental portions of the atria, its longer penis sheath, its lack of an outward-turned collar at the ental end of the penial tube, and its lower maximal number of preclitellar chaetae. In addition, the average COI distance between *T. mackiei* n. sp. and *T. pseudogaster* is 22.07% (see Kvist et al. 2010) and a total of 127 characteristic attributes (i.e., molecular synapomorphies; see Sarkar et al. 2002) separate the COI sequences of these species (Kvist et al. 2010).

**Holotype.** ZMBN 123768 (specimen ID CE21703), stained, whole-mount on microscope slide with coverslip, sexually mature specimen, collected by C. Erséus and M. Klinth, May 12, 2014; holotype COI sequence GenBank accession number MG652362.

**Type locality.** Gunnarsholmen Island (inside of island, close to marina), Kragerø, Telemark, Norway (58°51′53″N, 09°24′42″E), 0.3–0.5 m depth, anaerobic, coarse sand and gravel.

**Paratypes.** ZMBN 123769-123771 (specimen IDs CE21701, CE21702, CE21704); SMNH Type coll. 8955–8957 (specimen IDs CE21705–CE21707), stained, whole-mounts on microscope slide with coverslip, all specimens fully mature (except CE21707), collected by C. Erséus and M. Klinth, May 12, 2014, from type locality. Paratype COI sequence GenBank accession numbers MG652360, MG652361 and MG652363.

**Other material examined.** SMNH 108985, SMNH 108986, and SMNH 108988 (specimen IDs CE199-3, CE2077, CE3205 in Kvist et al. 2010), stained, whole-mounts on microscope slide with coverslip, one fully mature, one half-mature and one immature specimen, collected at Tjärnö Marine Biological Laboratory, Bohuslän, Sweden (58°52′ 29″N, 11°08′ 44″E) by C. Erséus, Sep. 2000, Oct. 2006, Sep. 2007; SMNH 108987 (specimen ID CE3107 in Kvist et al. 2010), half mature specimen, collected at Hanhalsholme, Halland, Sweden (57°26′ 58″N, 12°04′ 02″E) by S. Kvist and M. Lindström, Sep. 2007; for genetic information, see Kvist et al. (2010).

**Etymology.** Named after the esteemed polychaete systematist, Andrew (Andy) S.Y. Mackie, for his contributions to annelid systematics, and for his hospitality, generosity and aid during the first author’s collection of numerous naidids (from various localities in Wales in 2007), including *T. pseudogaster*, which partly enabled the separation of the new species.

**Description.** All specimens incomplete, posterior ends used for DNA extraction. Holotype 4.52 mm long, with anterior 20 segments (Fig. 3A), paratypes 1.7–4.1 mm long, with anterior 11–23 segments. Width at XI, 0.88 mm in holotype, 0.39–0.74 mm in paratypes; all specimens mounted and compressed. Holotype and all but one paratype sexually mature, other specimens in various stages of maturity. Prostomium rounded in most specimens, slightly more triangular in some, shorter than basally wide (Fig. 3A). Cuticle smooth, cuticular papillae nowhere present. Clitelleum present in X–XI in holotype and most paratypes, not developed in remaining specimens.

In preclitellar segments, dorsal chaetal bundles with (2)3–5 bifid crotchets (Fig. 4A), 40–70 µm long. Bifids with teeth of about same length; upper tooth slightly thinner than lower. Postclitellar, dorsal bundles with 1–2(3) bifid crotchets (Fig. 4B), 35–55 µm long; upper tooth longer and slightly thinner than lower. Preclitellar ventral bundles (Fig. 4C) with (2)3–4 bifid crotchets (35–65 µm long); upper tooth thinner but about same length as lower. Postclitellar ventral bundles (Fig. 4D) with 1–2 bifid crotchets (35–40 µm long), upper teeth slightly thinner than lower; teeth of about same length, and rather diverging. No ventral chaetae in segment XI in mature specimens. Dorsal bifid chaetae weakly sigmoidal, some ventral bundles with heavily bent, sigmoidal chaetae (sometimes broken); nodulus inconspicuous in most specimens, somewhat more developed in holotype. Spermatiche pores paired, located slightly above ventral chaetae in X. Male pores paired, in line with assumed ventral chaetae about halfway into segment XI.

Pharyngeal glands present in segments IV–V. Vas deferens (Fig. 4E; vd) about 35 µm wide in holotype, full length not measurable in any specimen, but longer than 200 µm in one paratype; densely ciliated towards atrium insertion point. Prostate (Fig. 4E; pr) rather large, but possibly compressed. Atrium (Figs. 3B, 4E; at) about 430 µm long, about 100 µm wide at widest point; tripartite with coarse cellular matrix in ental cap-like portion, finer cellular structure in mid-portion epithelium, and denser epithelium in muscular ectal portion. Atrium terminating in cuticular penis sheath with slight inward turn at ectal end, but still rather large terminal opening (Figs. 3B, 4E, 4F; cu), about 90 µm long, 30–35 µm wide at base. Soft tissue of penis protruding through terminal opening of penis sheath in holotype and most paratypes. Spermatochaetae (Figs. 3C, 4G; sp) extending from rather large but non-swollen pore, with narrower ectal duct (Fig. 4G; sd) opening up into wider ampulla entally (Fig. 4G; sa), spermatochaetae much compressed in all specimens and often pressed into surrounding segments; duct about 80 µm long, 40 µm wide at widest point, ampullae about 400 µm long, 150 µm wide at widest part. Spermatozeugmata rather stout and often coiled (170 µm long, 40 µm wide) (Figs. 3C, 4G; sz).
**Distribution and habitat.** Known from the southeast coast of Norway and west coast of Sweden, in fine sand and silt, as well as coarse sand and gravel; intertidal and shallow subtidal.

**Remarks.** Molecular data support that *Tubificoides mackiei* and the north European *T. pseudogaster* are closely related (Kvist et al. 2010). The two taxa strongly resemble each other, both in general appearance and in some details of the chaetal morphology. In particular, *Tubificoides mackiei* and *T. pseudogaster* are similar in general body length from prostomium to segment XI, the lack of hair chaetae and cuticular papillation, and in the detailed morphology of dorsal chaetae (Dahl 1960; Baker 1980; Takashima & Mawatari 1996). However, the two species can be distinguished on the basis of body width, *e.g.* using the width at segment X (*T. mackiei* seems to be about twice as wide as *T. pseudogaster* in X [see Dahl 1960], although such a difference may depend on, or be exaggerated by, coverslip compression on a microscope slide), the shape of the atrium (wider ental portion in *T. mackiei*), and the maximal number of precloacal, dorsal chaetae (the reported [up to] six chaetae per bundle in *T. pseudogaster* [Dahl 1960] are nowhere found in *T. mackiei*). Also, the shape and size of the penis sheath (shorter in *T. pseudogaster*), and the details of the penial tube (with an outward-turned collar at the ental portion in *T. pseudogaster*) differs between the taxa. It should be noted that, with regard to the detailed morphology of the chaetae, in particular the length of the upper teeth in the dorsal bundles, Dahl’s (1960) original description and...
Baker’s (1980) re-description of *T. pseudogaster* report slightly different information. Dahl (1960) mentioned that the upper teeth in dorsal bundles are sometimes shorter than the lower teeth, whereas Baker (1980) suggested the opposite.

Dahl (1960) reported the following for *T. pseudogaster*: "In segment 9 the intestinal walls are thickened dorsally and laterally with a glandular structure". These structures are indicated in the scientific name for the species (Gr. pseudēs meaning false, and Gr. gastēr meaning stomach). All of our slide-mounted, non-juvenile specimens of both *T. pseudogaster* and *T. mackiei* display a feature very similar to that detailed by Dahl (1960), yet it is difficult to discern if the structures are of digestive epithelial origin or chloragogenic cells, and whether they are directly related to the intestinal tract or free in the coelomic cavity. Regardless, because both species seem to have this structure, it may be a synapomorphy for them (and perhaps additional, closely related taxa).

![Image of Tubificoides mackiei n. sp., holotype.](image)

**FIGURE 4.** *Tubificoides mackiei* n. sp., holotype. A. Preclitellar dorsal chaeta. B. Postclitellar dorsal chaeta. C. Preclitellar ventral chaeta. D. Postclitellar ventral chaeta. A–D distal ends of chaetae only. E. Male genital apparatus (for clarity, penial sac is not drawn in figure) and ovary (note that the ovary is not connected to any male structure, but abuts the atrium). F. Lateral contours of penis sheaths (broken lines indicate protruding soft parts). G. Spermatheca with spermatozeugmata. Abbreviations: at, atrium; cu, cuticular penis sheath; ov, ovary; pr, prostate gland; sa, spermathecal ampulla; sd, spermathecal duct; sz, spermatozeugma; vd, vas deferens (only a short part of vd is shown).
**On the penis morphology of *T. pseudogaster***. Historically, delineations of species within *Tubificoides* have relied heavily on the shape and size of penis sheaths (Brinkhurst & Baker 1979; Brinkhurst 1981, 1985, 1986; Baker 1983; Helgasson & Erséus 1987); this character also seems to separate *T. mackiei* from *T. pseudogaster*. Although the resolution of Dahl’s (1960) figure showing the penis sheath of *T. pseudogaster* is less than ideal, the language in her description is clear. Dahl (1960), when describing the penis sheath, states that: "Its length is about twice its breadth" and that "Distally the penial tube has an outward turned collar". In addition, the figures supporting Baker’s (1980) and Takashima & Mawatari’s (1996) re-descriptions of the species clearly show a "cone-shaped" penis sheath, with an outward turn at the ental end [referred to as "ental flange" in Takashima & Mawatari (1996)]. In order to aid future morphological identifications of *T. pseudogaster* (see also above regarding the DNA barcodes for this species) and owing to the low quality of the figures presented by Dahl (1960), we here present a photograph of the penis sheath from the lectotype of *T. pseudogaster* (Fig. 3D). The structure is morphologically compatible with the detailed description by Dahl (1960), as well as both the text and figures presented in Baker’s (1980) re-description, and we hope that these new photographs, in combination with the genetic data, will mediate the difficulty of delimiting species that are morphologically similar to *T. pseudogaster*.

**Discussion**

Using both mitochondrial and nuclear sequence data, Kvist et al. (2010) were able to unequivocally separate both *Tubificoides charlotteae* and *T. mackiei* from congeners. Indeed, *T. charlotteae* differed from its sister taxon, *T. amplivasatus*, by an average of 12.74% ± 1.50 in COI and 3.85% ± 1.00 in the nuclear internal transcribed spacer region (ITS). Remarkably, *T. mackiei* differed from its closest related, included congener, *T. pseudogaster*, by an average of 22.07% ± 2.01 in COI, yet only by 1.26% ± 0.63 in ITS. This is despite the fact that the species within each pair are very similar morphologically; the molecular tools that alerted to them possibly being separate species were vital in unveiling this unexpected diversity. Much like in other groups of organisms, hidden diversity is currently being revealed at a high rate within Annelida in general, and Clitellata in particular, due to the wider use of sequence data (Erséus & Gustafsson 2009; James et al. 2010; Nygren 2014; Martinsson et al. 2013; Martinsson & Erséus 2014, 2017; Liu et al. 2017). Although the species described above are only pseudo-cryptic (see Achurra et al. 2015 and references therein), the uncovering of their defining and delineating morphological characters required detailed investigation.

*Tubificoides mackiei* occurs sympatrially with *T. pseudogaster* (Baker 1980, 1984; Kvist et al. 2010), at least along the west coast of Sweden and in southern Norway. It is worth noting that *Tubificoides pseudogaster* has been recorded from across Scandinavia and northern Europe, including the British Isles (Baker 1984; Brinkhurst 1986; Reise et al. 1994), the Atlantic and Pacific coasts of North America (Brinkhurst & Baker 1979), and Far East Russia and Japan (Takashima & Mawatari 1996 and references therein). Indeed, Baker (1984) noted that several different morphotypes, similar to *T. pseudogaster*, seem to occur along its geographical range. The lack of distinct external or internal morphological characters that clearly separate *T. pseudogaster* from congeners (i.e., its size, which is typical of *Tubificoides*, its lack of cuticular papillation and hair chaetae, and the inconspicuous arrangement of its bifid chaetae) perhaps makes it more prone to lumping than other, more distinct species within the genus. Noting that the geographic range of *T. mackiei* partially overlaps with that of *T. pseudogaster* (it is still unclear whether or not *T. mackiei* also occurs in North America) and owing to their morphological similarity, it is possible that *T. mackiei* has been systematically confused with *T. pseudogaster* in the past. As a result of the separation of *T. mackiei* and *T. pseudogaster* by the present study, the COI sequences recovered by Kvist et al. (2010) can now be used as authoritative DNA barcodes for both *T. pseudogaster* (GenBank accession numbers HM460206–HM460215) and *T. mackiei* (GenBank accession numbers MG652360–MG652363, and HM460202–HM460205). This will hopefully minimize future taxonomic confusion as morphological identifications of specimens pertaining to *T. pseudogaster* s.str. can be corroborated by molecular data. This will likely also resolve the identities of morphologically similar species that may occur across the relatively wide geographical range of *T. pseudogaster*.

*Tubificoides amplivasatus* was described from the west coast of Norway (Erséus 1975, 1976) and its currently known geographic distribution covers Scandinavia and the British Isles (Bamber & Spencer 1984; Kvist et al. 2010). The species is sometimes recovered at greater depths than other species within the genus (occasionally
below 200 meters; Erséus 1976) but it is also found in intertidal and shallow subtidal environments. However, as the range of *T. amplivasatus* seems to be restricted to Northern Europe, there is no evidence that it occurs sympatrically with *T. charlotteae*, which is only known from the type locality off the Atlantic coast of southern Spain. Therefore, we anticipate less difficulty in identifying (separating) these morphologically similar species.

Morphological examinations, in combination with DNA sequence analyses, continue to be pivotal as a sound basis for taxonomic decision making within the genus *Tubificoides*. As there are several morphologically similar forms within this taxon, DNA sequences hold the power to deliver an initial alert when hidden diversity exists. Similar approaches have revealed cryptic diversity across a range of oligochaetous clitellate taxa (e.g. Chang et al. 2008; James et al. 2010; De Wit & Erséus 2010; Matamoros et al. 2012; Achurra et al. 2012, 2015; Donnelly et al. 2013; Martinsson et al. 2013; Martinsson & Erséus 2014, 2017; Liu et al. 2017) and several additional, separately evolving lineages (i.e., species *sensu* de Queiroz 2007) may yet be evinced using integrative and comparative approaches. To this end, it is worth underscoring the utility of a combination of mitochondrial and nuclear markers for robust separation of species pairs or complexes.

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