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COI variation in Scandinavian marine species of *Tubificoides* (Annelida: Clitellata: Tubificidae)

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Intra- and interspecific variation in a 658 bp long part of the cytochrome c oxidase subunit 1 (COI) gene of the mitochondrial genome, i.e. a suggested 'DNA barcode', was assessed in four north-west European species of the marine tubificid genus *Tubificoides*: *T. benedii*, *T. amplivasatus*, *T. heterochaetus* and *T. kozloffi*. Within species mean genetic distance was from 0.10% (*T. amplivasatus*) to 0.14% (*T. benedii*), between species from 19.3% to 22.9%. For *T. benedii* and *T. amplivasatus*, material collected in two separate areas, The Sound between Denmark and Sweden, and the Koster area about 330 km to the north along the Swedish west coast, showed a geographically random distribution of COI haplotypes, suggesting that each of these two species forms a continuous population in southern Scandinavia. We conclude that the COI gene is suitable as a barcode marker for the secure identification of these species, at least within the area investigated. *Tubificoides heterochaetus* is reported for the first time from Denmark.

INTRODUCTION

DNA barcoding (Hebert et al., 2003a,b; Hebert & Gregory, 2005) is meant to enable quick identification of an animal specimen by comparing a selected gene sequence ('barcode') of its genome with already known sequences of the same gene. Specifically, the aim of the Barcode of Life initiative (www.barcoding.si.edu) is to use large-scale screening of a part of the mitochondrial cytochrome c oxidase subunit 1 (COI), to create an extensive database covering millions of species (Savolainen et al., 2005). If successful and complete, this database will enable identification of animal specimens without using morphological characters, and it will also aid the search for and recognition of new taxa.

In an overview of COI divergences in congeneric species pairs of various animals (data available from GenBank and similar sources), Hebert et al. (2003b) showed that 88% of the annelid species compared are 8% or more different from their congeners; 70% being 16% or more different. While this is roughly equal to the intrageneric variation in most other major taxa, COI divergences lower than 8% are more frequent in groups such as insects, echinoderms and chordates, and there are groups with even lower variation. For instance, 94% of the congeneric species pairs of cnidarians compared are less than 2% different (Hebert et al., 2003b), and similar results have been obtained for sponges (e.g. Duran et al., 2004; Wörheide, 2006). Cnidarians appear to possess a unique repair system in the mitochondria resulting in low levels of variability (France & Hoover, 2002; Shearer et al., 2002), while a combination of long generation time and low metabolic rate has been suggested as the cause of slow mtDNA evolution in sponges (Wörheide, 2006). By and large, however, mitochondrial genes appear to be better than the nuclear ones for DNA barcoding, because of its lack of introns and its haploid mode of inheritance (Saccone et al., 1999). Moreover, the COI gene seems to possess a larger range of phylogenetic signal than all other mitochondrial regions (Knowlton & Weigt, 1998; Hebert et al., 2003a). Thus, COI has proved to be a good marker for the identification of already known (e.g. Hebert et al., 2004a) as well as morphologically cryptic species (e.g. Trewick, 2000; Hebert et al., 2004b).

Nevertheless, DNA-barcoding is frequently discussed and partly challenged in the recent literature of biological systematics. There are concerns about the practicability and cost (e.g. Cameron et al., 2006), and some authors have raised worries about traditional taxonomy becoming extinct and replaced by pure DNA work (e.g. Will & Rubinoff, 2004; Ebach & Holdrege, 2005; Will et al., 2005). Others, however, emphasize that the barcoding approach always will depend on, and supplement, morphological knowledge, and that this will be beneficial for taxonomy in biodiversity science (Hebert & Gregory, 2005; Schander & Willassen, 2005; Herre, 2006).

Scientifically more serious is that there is growing evidence that the 'barcoding gap', i.e. discrete distributions of intra- and interspecific COI variation, is not as clear-cut as originally thought. If there is considerable overlap between the intra- versus interspecific variation, barcoding is reliable only for taxonomically well-understood and thoroughly sampled clades, and it is also important that the data used to establish congeneric interspecific variation include comparisons between true sister species (see e.g. Moritz & Cicero, 2004; Meyer & Paulay, 2005). Thus, to fully evaluate the power of COI as a marker of species identity, extensive sampling in terms of individuals and geographical coverage is needed.

So far, no marine oligochaetous clitellates have been subjected to the molecular typification suggested by the Barcode of Life initiative. Therefore, the purpose of this study was to compare the partial COI sequence of individuals within a genus of marine oligochaetous clitellates, *Tubificoides* Lastockin (subfamily Tubificinae), to explore to what degree it may vary at inter- and intraspecific levels, and thus to get a first indication of the usefulness of COI for species identification in these marine worms. Four species were analysed: *T. benedii* (d'Udekem, 1855) [see Brinkhurst, 1986], *T. amplivasatus* (Erséus, 1975) [Erséus, 1975; see also Erséus, 1976; Erséus & Diaz, 1989], *T. heterochaetus* (Michaelsen, 1926) [redescribed by Baker, 1981; see also Brinkhurst, 1986] and *T. kozloffi* Baker, 1983 [Baker, 1983; see also Helgason & Erséus, 1987]. A fifth marine tubificine tubificid, *Clitellio arenarius* (Müller, 1776), was included as an 'outgroup' to make it possible to estimate phylogenetic relationships among the studied species of Tubificoides. All taxa considered here occur in north-west Europe, and all except *T. amplivasatus* are restricted to shallow waters. Specimens of *T. benedii* and *T. amplivasatus* were collected at different locations in Denmark and Sweden to establish whether these species show any geographical intra-specific variation within this part of Scandinavia.

Our material of *T. heterochaetus* from the Helsingör harbour appears to be the first record of this species from Denmark and the Kattegat region. The species was earlier known from other coasts of northern Europe, including the Baltic Sea (Michaelsen, 1926; von Bülow, 1957; Timm, 1965, 1970, 1999; Laakso, 1969), the Danube estuary in the Black Sea (Popescu-Marinescu et al., 1966) and several parts of North America (Baker, 1981; Brinkhurst, 1986).

MATERIALS AND METHODS

Collection of material

Two geographical areas were chosen for this study: (1) Helsingör (Elsinore), in The Sound between Denmark and southern Sweden (January 2006); and (2) the Koster archipelago near Strömstad, west coast of Sweden (February 2006). Subtidal sediment samples were dredged in both areas, and in the Koster area, barely subtidal material was collected by hand; sites and specimens are listed in Table 1. Specimens were sorted from sieved fractions of the samples, and each worm was cut into two pieces: the posterior part was preserved in 95% ethanol for DNA extraction, while the anterior end was preserved in 80% ethanol, later stained in alcoholic paracarmine and mounted in Canada balsam on a microscope slide, as a voucher. A few specimens of *T. benedii*, *T. amplivasatus* and *T. kozloffi*, earlier *in toto* preserved in 95% ethanol, were already available in the first author's collections; slide vouchers of these individuals were prepared too. All vouchers were deposited in the Swedish Museum of Natural History (SMNH), Stockholm.

Extraction, PCR amplification and sequencing

DNA was extracted using EZNA® Tissue DNA kits (Omega Bio-Tek) according to the manufacturer's protocol. A 658 bp region of COI was amplified using PuReTaq™ Ready-To-Go PCR beads (Amersham Biosciences) and 2 µl or (when this gave weak results) 3–4 µl DNA extract. The primers HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (Folmer et al., 1994) were used in quantities of 1 µl, with 21 µl of sterilized water added to each amplification. Either a PTC-100 (MJ Research) or an Eppendorf PCR machine was used, with 35 cycles of 30 s at 95°C, 30 s at 50°C, and 1 min at 72°C, with an additional initial step of 5 min denaturation at 95°C, and a final step of 8 min extension at 72°C.

PCR products were purified using the E.Z.N.A® Cycle-Pure kit (Omega Bio-Tek) following the manufacturer's protocol. Thereafter, sequencing-PCR was carried out in both directions using the primers as above, with addition of the DTCS Quick Start Mix (Beckman-Coulter), and 30 cycles of 20 s at 96°C, 20 s at 50°C and 4 min at 60°C. The sequencing was performed on a Beckman-Coulter CEQ 8000.

Sequence assemblage and alignment

Gene sequences were assembled using SeqMan II (DNAStar Inc.). Both ends of the sequence were manually trimmed to delete obvious misreadings made by the sequencer. The consensus sequences were aligned using Megalign (DNAStar Inc.), applying Clustal W alignment with the Slow-Accurate setting, gap penalty 15, and gap length penalty 6.66.

Data analysis

The genetic variation was assessed in PAUP*4.0b10 (Swofford, 2002) using the 'pairwise distance' application. For each sequence, the uncorrected ('p') distance to each of the other sequences was calculated. For mean values, standard deviation (±SD) was calculated using Microsoft Excel. A neighbour-joining (NJ) tree was created in PAUP with settings: neighbour-joining algorithm, random break ties and DNA/RNA distances. In addition, a (phylogenetic) bootstrap consensus tree was constructed using parsimony analysis of 500 bootstrap replicates, each consisting of 10 random addition-sequence replicates, and with settings: TBR branch swapping, collapse branch if minimum length is zero ('amb-'), and MulTrees on. To minimize the time needed to conduct the bootstrap analysis, specimens with identical COI haplotypes were reduced to be represented by a single terminal; thus, 17 of the worms listed in Table 1 were excluded from the analysis.

RESULTS

COI variation

The intraspecific variation within *Tubificoides benedii* was between 0 (in 52 of the 120 comparisons) and 0.49%, with a mean of 0.14% ±0.15. The highest difference was between CE1746 and CE1747, both collected in the Helsingör area, but separated geographically by several kilometres. Within *Tubificoides amplivasatus*, the variation was between 0 (in 49 of the 91 comparisons) and 0.68%, the mean being 0.10% ±0.13. The highest difference was between CE1728 and CE1731 (0.67%), also both from Öresund, but here the collection sites were closer to each other. *Tubificoides heterochaetus*

Table 1. *A list of the individuals collected for this study together with information on collection sites, senior author (C.E.) reference numbers, Genbank numbers of the partial COI sequence, and reference numbers of vouchers deposited at SMNH.*

Species	Site (depth)	CE#	GenBank No.	Voucher#
T. benedii	H, Ellekilde have, near shore (6 m)	CE 1744	EF675192	SMNH85158
T. benedii	H, Ellekilde have, near shore (6 m)	CE 1745	EF675193	SMNH85159
T. benedii	H, Ellekilde have (10 m)	CE 1746	EF675194	SMNH85160
T. benedii	H, S of Helsingör harbour (16 m)	CE 1747	EF675195	SMNH85161
T. benedii	K, Saltö, mudflat (0.5 m)	CE 1754	EF675196	SMNH85162
T. benedii	K, Saltö, mudflat (0.5 m)	CE 1755	EF675197	SMNH85163
T. benedii	K, Saltö, mudflat (0.5 m)	CE 1756	EF675198	SMNH85164
T. benedii	K, Saltö, mudflat (0.5 m)	CE 1758	EF675199	SMNH85165
T. benedii	K, Saltö, mudflat (0.5 m)	CE 1759	EF675200	SMNH85166
T. benedii	K, Saltö, mudflat (0.5 m)	CE 1760	EF675201	SMNH85167
T. benedii	K, Persgrunden (17 m)	CE 1269-1	EF675202	SMNH85168
T. benedii	K, Persgrunden (17 m)	CE 1269-2	EF675203	SMNH85169
T. benedii	K, Persgrunden (17 m)	CE 1269-3	EF675204	SMNH85170
T. benedii	K, Ursholmen (3 m)	CE 539-1	EF675205	SMNH85171
T. benedii	K, Ursholmen (3 m)	CE 539-2	EF675206	SMNH85172
T. benedii	K, Ursholmen (3 m)	CE 539-3	EF675207	SMNH85173
T. amplivasatus	H, E of Ellekilde have (27 m)	CE 1724	EF675208	SMNH85174
T. amplivasatus	H, E of Ellekilde have (27 m)	CE 1726	EF675209	SMNH85175
T. amplivasatus	H, E of Ellekilde have (27 m)	CE 1727	EF675210	SMNH85176
T. amplivasatus	H, E of Ellekilde have (27 m)	CE 1728	EF675211	SMNH85177
T. amplivasatus	H, E of Ellekilde have (27 m)	CE 1729	EF675212	SMNH85178
T. amplivasatus	H, Ålsgårde (27 m)	CE 1730	EF675213	SMNH85179
T. amplivasatus	H, Ålsgårde (27 m)	CE 1731	EF675214	SMNH85180
T. amplivasatus	K, Koster fjord trench (250 m)	CE 541-1	EF675215	SMNH85181
T. amplivasatus	K, Koster fjord trench (250 m)	CE 541-2	EF675216	SMNH85182
T. amplivasatus	K, Koster fjord trench (250 m)	CE 541-3	EF675217	SMNH85183
T. amplivasatus	K, Koster fjord trench (250 m)	CE 541-4	EF675218	SMNH85184
T. amplivasatus	K, Inner Koster archipelago (<50 m)	CE 551	EF675219	SMNH85185
T. amplivasatus	K, Inner Koster archipelago (<50 m)	CE 552	EF675220	SMNH85186
T. amplivasatus[1]	Ha, Morups bank (30 m)	CE 1274	EF675221	SMNH85187
T. heterochaetus[2]	H, S of Helsingör harbour, 56.17°N 12.60°E (16 m)	CE 1732	EF675222	SMNH85188
T. heterochaetus	H, S of Helsingör harbour (16 m)	CE 1734	EF675223	SMNH85189
T. heterochaetus	H, S of Helsingör harbour (16 m)	CE 1735	EF675224	SMNH85190
T. heterochaetus	H, S of Helsingör harbour (16 m)	CE 1736	EF675225	SMNH85191
T. heterochaetus	H, S of Helsingör harbour (16 m)	CE 1740	EF675226	SMNH85192
T. kozloffi[1]	Ha, Fladen (15–20 m)	CE 1064	EF675227	SMNH95193
Outgroup: *Clitellio arenarius*	K, Saltö, mudflat (0.5 m)	CE 1762	EF675228	SMNH85194

[1], Individuals collected by Pierre de Wit, in 2005. [2], First record of species from Denmark. Therefore, geographical coordinates are given for the collection site. In the site column, H, Helsingör area (Denmark); K, Koster area (Sweden); and Ha, the province of Halland, Sweden.

showed an intraspecific variation ranging between 0 (in 7 of the 10 comparisons) and 0.54%, with a mean of 0.11% ±0.18. All specimens of this species came from the same site. No variation was calculated for *T. kozloffi* and *C. arenarius*, as only one specimen of each species was analysed.

The intraspecific variation between specimens from the two main areas (Helsingör and Koster) was 0.20% ±0.15 for *T. benedii*, and 0.05% ±0.08 for *T. amplivasatus*; *T. heterochaetus* was found only in Helsingör. Further, the intraspecific variation within the main areas was as follows: for *T. benedii*, 0.09% ±0.11 in the Koster area, and 0.23% ±0.21 at Helsingör; for *T. amplivasatus*, 0.04% ±0.08 in the Koster area, and 0.17% ±0.20 at Helsingör.

Comparisons between *T. benedii* and *T. amplivasatus* showed a mean genetic difference of 20.5% ±0.4. Between *T. amplivasatus* and *T. heterochaetus*, the corresponding figure was 22.9% ±0.4, between *T. benedii* and *T. heterochaetus*, 20.6% ±0.8, between *T. kozloffi* and *T. amplivasatus*, 19.3% ±0.1, between *T. benedii* and *T. kozloffi*, 20.4% ±0.2, and between *T. kozloffi* and *T. heterochaetus*, 21.1% ±0.2. Thus, among these congeneric species the mean variation ranged between 19.3 and 22.9%. The total range of interspecific differences in the pairwise comparisons was 19.0–24.8 %.

The outgroup species, *Clitellio arenarius*, showed the highest mean divergence when compared to *T. amplivasatus* (24.6% ±0.2), the lowest when compared to *T. benedii* (21.7% ±0.3).

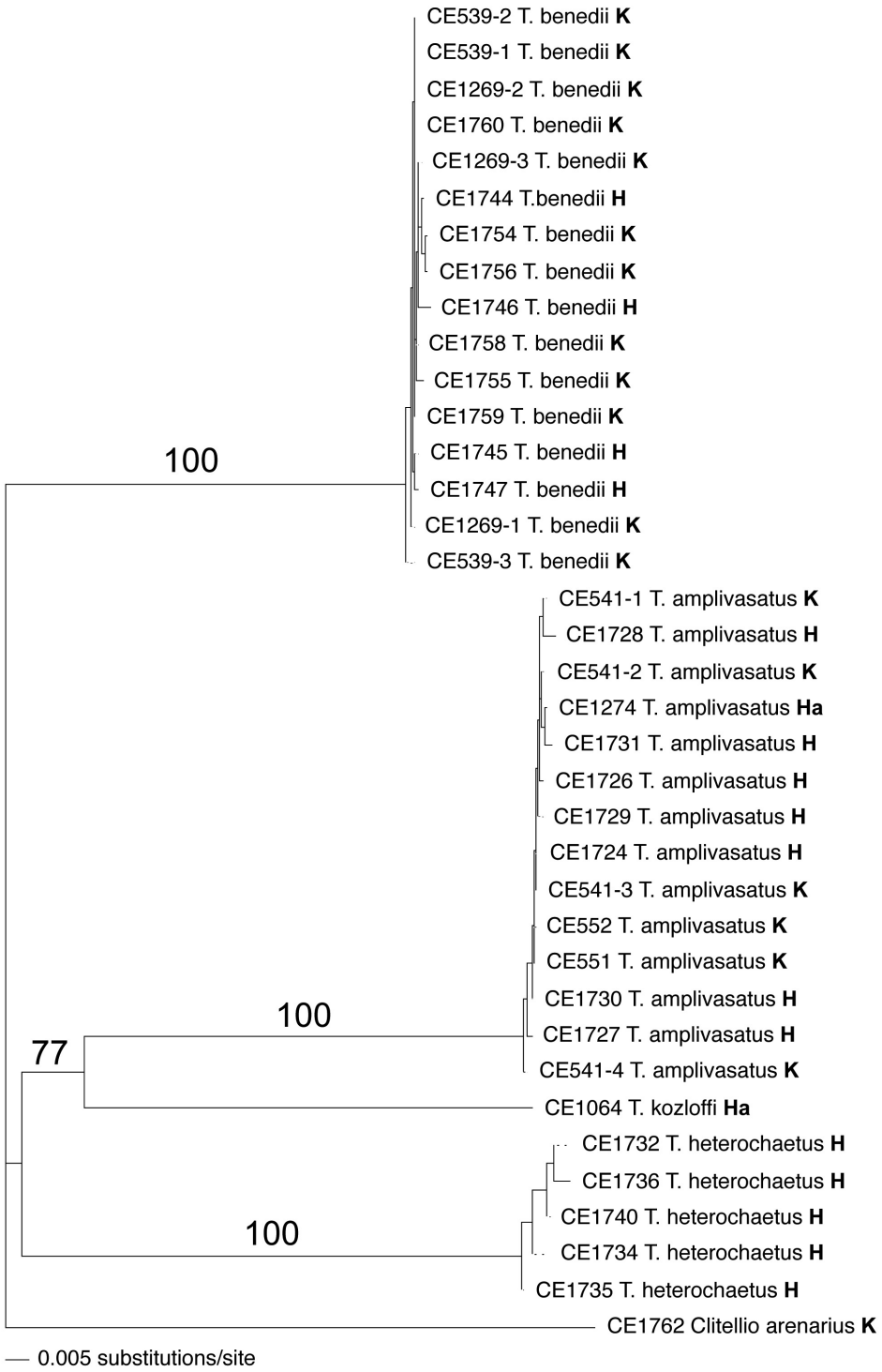

Figure 1. Neighbour-joining (NJ) tree derived from the same alignment as the pairwise distance analysis. The tree is rooted at *Clitellio arenarius*. Bootstrap values (%) are taken from the parsimony analysis of the same dataset; in a strict sense they do not refer to the NJ tree as such. The bold face letters following the specimens indicate in which area they were collected: H, Helsingör, Denmark; K, Koster area, Sweden; Ha, the province of Halland, Sweden.

Neighbour-joining (NJ) tree

The NJ tree (Figure 1) showed a much longer branch-length (number of substitutions) for the different species than for the individuals within the same species; i.e. as expected, the tree coincided largely with the values in the pairwise distance comparisons. It also showed a largest number of substitutions per site for the outgroup taxon, *Clitellio arenarius*.

Phylogenetic analysis

The bootstrap analysis revealed strong support (100%) for each of *T. benedii*, *T. amplivasatus* and *T. heterochaetus* being its own clade. The bootstrap tree topology is not illustrated here, but the support values are superimposed on the NJ tree (Figure 1), which is congruent with it. Of the four species investigated, *T. kozloffi* and *T. amplivasatus*

appear to be the two most closely related to each other (with 77% support). Within the clades of *T. benedii*, *T. amplivasatus* and *T. heterochaetus*, respectively, the bootstrap tree showed no further resolution to suggest phylogenetic separation of intraspecific lineages. That is, neither *T. benedii* nor *T. amplivasatus* showed any pattern of variation that would suggest that the Helsingör worms are genetically separated from those of the Koster area.

DISCUSSION

Our investigated species of *Tubificoides*, which in southern Scandinavia all appear morphologically rather constant, revealed a COI variation pattern with a wide barcoding 'gap', the interspecific variation (19–25%) exceeding the intraspecific one (<1%) by two orders of magnitude. This seems to support the standard sequence threshold approach proposed by Hebert et al. (2004a): i.e. the interspecific variation is more than ten times the mean intraspecific variation for the group under study. Our results thus suggest that the COI gene may be used for the identification of these species, and the large barcoding gap may even be useful for detection of new and/or cryptic species of *Tubificoides*. The genus already contains 55 nominal species (C. Erséus, unpublished compilation), some of which are widely distributed in the world but reported as highly variable, and therefore, may include several composite taxa. However, considering the limited scope of the present study, and that work on other animal groups has shown that extensive sampling may result in a broad overlap between levels of intra- and interspecific variation (Moritz & Cicero, 2004; Meyer & Paulay, 2005), a more comprehensive investigation will be necessary to fully evaluate the usefulness of COI as a general barcode for marine Tubificidae.

One additional conclusion, however, can be drawn from the present study. Comparing the intraspecific variation within and between the two main areas investigated, both *Tubificoides benedii* and *T. amplivasatus* showed a higher mean divergence within the Helsingör area than between Helsingör and the Koster area. Further, the NJ and parsimony bootstrap trees were congruent with the pairwise distance results, directing each species to its own separate cluster/clade, and for both *T. benedii* and *T. amplivasatus* there was a random distribution of haplotypes from the two main areas. It thus seems likely that at least these two species each forms a continuous population in southern Scandinavia.

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