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Mitochondrial evidence supports a Nearctic origin for the spreading limicolous earthworm *Sparganophilus tamesis* Benham, 1892 (Clitellata, Sparganophilidae)

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Key words: aquatic megadriles, genetic divergence, Oligochaeta, recent introduction, *Sparganophilus eiseni*, synonymy

Abstract

We analysed samples of *Sparganophilus* taken at the corners of its distribution area in Europe (UK, Germany and Italy). No mitochondrial genetic divergence within and amongst them was found, neither in COI nor in 16S. Further, the COI haplotype was also identical to two sequences from Ontario, Canada in the Barcoding of Life Data System (BOLD) database. Our European COI and 16S sequences showed only minimal differentiation (only 1 or 2 substitutions) from specimens newly collected in Illinois and Washington states (USA), as well as from a COI haplotype from Tennessee (USA) in BOLD. An additional COI haplotype from Illinois (found in BOLD) is 2.1% different from the other haplotypes but clearly belongs to the same lineage of *Sparganophilus*. This geographically broad but genetically compact group fits the morphological diagnosis of *S. tamesis* Benham, 1892 as revised by Jamieson (1971) and is seen as evidence that all European populations 1) belong to the same species, 2) derive from a recent introduction, 3) are conspecific with the most widespread species of *Sparganophilus* in North America, and that 4) *S. tamesis* is a senior synonym of *S. eiseni* Smith, 1895. The single European haplotype does not refute the possibility of its spread from a single introduced source population.

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Introduction

The aquatic megadrile *Sparganophilus tamesis* Benham, 1892 (type species of the monotypic Sparganophilidae) was first discovered in River Thames south of Oxford, England, hence the type species name. However, in his description of this species, Benham suggested that cocoons may have been an introduction from North America, via the roots of water plants or attached to timber that had been shipped from the United States (Benham, 1892: p. 175; see Rota *et al*., 2014). The taxon was never recorded again in Europe until Černosvitov (1945), based on a rich collection of specimens from Windermere, England, pointed out possible errors in the original description – which might have led to misinterpretations and descriptions of synonyms such as *Pelodrilus cuenoti* Tétry, 1934 from France (see full list of synonyms in Rota *et al*., 2014). In particular, the interchaetal dorsal interval *dd*, illustrated by Benham (1892) as broader than half the body circumference, differed by being one-third the body circumference in the worms studied by Tétry (1934) and Černosvitov (1945).

In the meantime, *Sparganophilus* Benham, 1892 had been found also in North and Central America. Trusting Benham's account of *S. tamesis* (ventral position of the outer chaetae and lack of prostate-like glands), three new congeners had been described: one from Illinois, USA (*S. eiseni* Smith, 1895), and two

Table 1. List of the specimens of *Sparganophilus tamesis* Benham, 1892 included in the present study, with identification numbers, voucher numbers, collection data and sequence accession numbers. The barcode sequences under the line were retrieved from BOLD.

Species	ID nos.	Museum Voucher nos.	Collection locality	Coordinates	Coll. date	Leg.	Accession nos. COI	16S
S. tamesis	CE20777	SMNH149684	England, Derbyshire, Smisby	52.7660°N, 01.4900°W	Aug 2013	S. Martinsson, K. Elliott	KT924124[1]	-
S. tamesis	CE13359	SMNH149679	Germany, Hamburg, Alster River	53.6817°N, 10.1153°E	Jul 2011	U. Graefe	KT924119[1]	-
S. tamesis	CE13360	SMNH149680	Germany, Hamburg, Alster River	53.6817°N, 10.1153°E	Jul 2011	U. Graefe	KT924120[1]	KT924175[1]
S. tamesis	CE13361	SMNH149681	Germany, Hamburg, Alster River	53.6817°N, 10.1153°E	Jul 2011	U. Graefe	KT924121[1]	KT924176[1]
S. tamesis	CE13362	SMNH149682	Germany, Hamburg, Alster River	53.6817°N, 10.1153°E	Jul 2011	U. Graefe	KT924122[1]	KT924177[1]
S. tamesis	CE13363	SMNH149683	Germany, Hamburg, Alster River	53.6817°N, 10.1153°E	Jul 2011	U. Graefe	KT924123[1]	KT924178[1]
S. tamesis	CE21088	MCZR0189	Italy, Mantua, Mincio River	45.2784°N, 10.7087°E	May 2014	A. Laini	KT924117[1]	KT924179[1]
S. tamesis	CE21089	MCZR0190	Italy, Mantua, Mincio River	45.2784°N, 10.7087°E	May 2014	A. Laini	-	KT924180[1]
S. tamesis	CE21090	MCZR0191	Italy, Mantua, Mincio River	45.2784°N, 10.7087°E	May 2014	A. Laini	-	KT924181[1]
S. tamesis	CE21091	MCZR0192	Italy, Mantua, Mincio River	45.2784°N, 10.7087°E	May 2014	A. Laini	KT924125[1]	KT924182[1]
S. tamesis	CE21092	MCZR0193	Italy, Mantua, Mincio River	45.2784°N, 10.7087°E	May 2014	A. Laini	KT924118[1]	KT924183[1]
S. tamesis	CE25922	MCZR0194	USA, Illinois, Rock Falls, I&M Canal trib.	41.7612°N, 89.6877°W	Oct 2014	M.J. Wetzel	-	KT924167[1]
S. tamesis	CE25923	MCZR0195	USA, Illinois, Rock Falls, I&M Canal trib.	41.7612°N, 89.6877°W	Oct 2014	M.J. Wetzel	KT924128[1]	KT924169[1]
S. tamesis	CE25924	MCZR0196	USA, Illinois, Rock Falls, I&M Canal trib.	41.7612°N, 89.6877°W	Oct 2014	M.J. Wetzel	KT924129[1]	KT924168[1]
S. tamesis	CE25925	MCZR0197	USA, Illinois, Rock Falls, I&M Canal trib.	41.7612°N, 89.6877°W	Oct 2014	M.J. Wetzel	KT924130[1]	KT924170[1]
S. tamesis	CE25926	MCZR0198	USA, Illinois, Rock Falls, I&M Canal trib.	41.7612°N, 89.6877°W	Oct 2014	M.J. Wetzel	KT924131[1]	KT924171[1]
S. tamesis	CE25927	MCZR0199	USA, Illinois, Rock Falls, I&M Canal trib.	41.7612°N, 89.6877°W	Oct 2014	M.J. Wetzel	KT924132[1]	KT924172[1]
S. tamesis	CE10914	SMNH149677	USA, Washington, Thurston, Summit Lake	47.0491°N, 123.1162°W	Sep 2009	S. Kvist	KT924126[1]	KT924173[1]
S. tamesis	CE10915	SMNH149678	USA, Washington, Thurston, Summit Lake	47.0491°N, 123.1162°W	Sep 2009	S. Kvist	KT924127[1]	KT924174[1]
S. tamesis		BIOUG08058-A12	Canada, Ontario		Sep 2013		RBNII393-13[2]	-
S. tamesis		BIOUG08058-B01	Canada, Ontario		Sep 2013		RBNII394-13[2]	-
S. tamesis			USA, Tennessee		Aug 2011		SPANA077-11[2]	-
S. tamesis			USA, Tennessee		Aug 2011		SPANA078-11[2]	-
S. tamesis			USA, Tennessee		Aug 2011		SPANA079-11[2]	-
S. tamesis			USA, Tennessee		Aug 2011		SPANA080-11[2]	-
S. tamesis			USA, Tennessee		Aug 2011		SPANA081-11[2]	-
S. tamesis			USA, Tennessee		Aug 2011		SPANA082-11[2]	-
S. tamesis			USA, Tennessee		Aug 2011		SPANA083-11[2]	-
S. tamesis			USA, Illinois		May 2011		SPANA143-11[2]	-

[1]GenBank accession numbers; [2]BOLD accession numbers.

more from California, Mexico and Guatemala (*S. smithi* Eisen, 1896 and *S. benhami* Eisen, 1896). Additional collections had soon extended the distribution of *S. eiseni* to Ohio, Michigan, Florida, Indiana and Canada (*e.g.*, Smith, 1896; Moore, 1906; Heimburger, 1915). Michaelsen (1918) refuted Moore's (1895) identifications of *S. tamesis* from Pennsylvania and New Jersey – suggesting instead that this species was a European autochthon.

Černosvitov (1945), considering the large range of distribution of *S. tamesis* in England, its occurrence in France and its absence in America, suggested that the genus' distribution should be explained by the past geological relationships between the American and European continents rather than by accidental importation. Omodeo (1963) followed the trail and hypothesized that a *Sparganophilus* ancestor reached Europe from North America via a land-bridge across the Atlantic in a relatively ancient age, evolving to the European endemic *S. tamesis*.

Jamieson (1971), upon re-examination of the type-specimens of *S. tamesis*, left no morphological grounds for the distinction of North American *S. eiseni* – either as a specific or an infraspecific taxon – and considered the occurrence of *S. tamesis* at Kew Gardens as further support for the hypothesis of human transportation. To Gates (1982) it seemed a 'plausible view that sparganophilids were taken to England since 1500 A.D. and unwittingly by man', and Sims and Gerard (1985) concurred: 'as the British records can all be associated with gardens containing imported aquatic plants, *e.g.*, Goring-on-Thames, the type locality, is only a few miles downstream from the Botanic Gardens, Oxford'. Zicsi and Vaucher (1987) agreed on the proposed synonymy of *S. eiseni* with *S. tamesis* and could not see any other explanation for its occurrence in Europe (including their new record from Switzerland) than transplantation of aquatic plants from North America to England, and from there throughout the old continent. However, Reynolds (*e.g.*, 1980, 1995, 2008) has always maintained the synonymy of *S. tamesis* with *S. eiseni* to be unacceptable, claiming to have identified both species in specimens deposited in the Natural History Museum (London) that had been collected from artificial water habitats in England.

More recently, Bouché and Qiu (1998) have revived the view of the Sparganophilidae as a family suggestive of earlier connections between the two continents, and have even hypothesized the presence in Europe of two endemic species: the more widespread *S. tamesis*, postglacially re-expanded north of the Alps, and the Swiss *S. langi* Bouché and Qiu, 1998, which survived in the Rhone-Ebro basin during the glaciations and afterwards recolonized part of the Mediterranean. On the other hand, molecular phylogenetics (Jamieson *et al.*, 2002; James and Davidson, 2012) indicates that the family Sparganophilidae is sister taxon to Komarekionidae, a monospecific earthworm family living in forest soils in midwestern (Illinois, Indiana, Kentucky) and eastern United States (Georgia through Maryland) in and adjacent to the Appalachian Highlands region (Reynolds and Wetzel, 2008; Rota *et al.*, 2014a; Rota and de Jong, 2015).

Stimulated by the recent discovery of well-established populations of *Sparganophilus* in Germany (Graefe and Beylich, 2011) and Italy (Rota *et al.*, 2014) – in both cases morphologically conforming to the diagnosis of *S. tamesis* as revised by Jamieson (1971) – we analysed the mitochondrial genetic diversity (COI and 16S) of samples of *Sparganophilus* from the extreme corners (UK, Germany and Italy) of its distribution area in Europe (see map in Rota *et al.*, 2014: fig. 3) and from two well separated locations in the United States, one of which not far from the type locality of *S. eiseni*. The newly obtained COI sequences were compared to barcode sequences of North American *Sparganophilus* available in BOLD (Barcoding of Life Data Systems; Ratnasingham and Hebert, 2007), with the aims to find: molecular evidence on the identity/separation between *S. tamesis* and *S. eiseni*, arguments favouring/disproving recent introduction over old endemic distribution, and information about the possible provenience of the European specimens.

Material and methods

Nineteen newly-sequenced specimens of *Sparganophilus tamesis* from three European (England, Germany and Italy) and two North American (Illinois and Washington states) populations (see Table 1 for details) were included in the study. The morphology of adults from Italy, Germany and Illinois fits the diagnosis of *S. tamesis* by Benham (1892) as revised by Jamieson (1971). For a detailed morpho-anatomical study of the Italian worms see Rota *et al.* (2014). The specimens from England and Washington were immature and were identified through DNA barcoding. DNA was extracted from a piece of body wall from the posterior region of the worms. The extractions were performed with either Qiagen's DNeasy Blood & Tissue Kit or Epicentre QuickExtract DNA Extraction Solution 1.0,

following the manufacturer's instructions. Parts of two mitochondrial markers, 16S ribosomal RNA (16S), and cytochrome c oxidase subunit I (COI), were amplified. 16S was amplified using the primers AnnF and AnnR (Sjölin *et al*., 2005), and the following program: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 45°C for 30 s and 72°C for 1 min and a final extension step at 72°C for 8 min; COI using the primers LCO1490 and HCO2198 (Folmer *et al*., 1994) or COI-E (Bely and Wray, 2004), and the following program: 95°C for 5 min, followed by 35 cycles of 95°C for 40 s, 45°C for 45 s and 72°C for 1 min and a final extension step at 72°C for 8 min. After amplification, existence of the target genes was confirmed using 1% agarose gel electrophoresis. PCR products were purified using exonuclease I (Fermentas, Burlington, Canada) and FastAP thermosensitive alkaline phosphatase (Fermentas) (Werle *et al*., 1994) following the protocol provided by the producer (Fermentas, Burlington, Canada). Sequencing was performed by Macrogen Inc. (Seoul, Korea) and Eurofins MWG Operon (Ebersberg, Germany). Sequences were assembled and trimmed to the same length (334 bp for 16S and 531 bp for COI) in GENEIOUS PRO v. 7.1 (Biomatters Ltd., Auckland, New Zealand). Ten additional COI sequences belonging to *S. tamesis* were found in the BOLD database (accessed 12 Oct 2015) by using one of our COI sequences as query for an identification request. The BOLD sequences were labelled as *Sparganophilus* from Ontario (Canada) or as unspecified Haplotaxida from Illinois and Tennessee (USA), and belong to BIN BOLD:ABA8326 (BINs are clusters of close barcode sequences that are assumed to correspond to species; Ratnasingham and Hebert, 2013), and we included them in our COI dataset. Sequences were aligned using MAFFT v7.017 (Katoh *et al*., 2002) as implemented in Geneious using the auto algorithm.

Haplotype networks were constructed for both genes in PopART v1 (Leigh and Bryant, 2015) using statistical parsimony (Templeton *et al*., 1992; Clement *et al*., 2002).

We also compared our 16S and COI data to the data in GenBank by performing BLAST (Altschul *et al*., 1990) searches; the searches were performed as Standard Nucleotide BLAST (blastn) against the Nucleotide collection (nr/nt) database using Megablast (http://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed 12 Oct 2015).

The low mitochondrial divergence (see Results and discussion section) made us decide not to include any nuclear markers, as even a fast evolving marker such as ITS has less or the same amount of variation as COI, at least in clitellates (*e.g*. De Wit and Erséus, 2010; Martinsson *et al*., 2013; Timm *et al*., 2013).

All new 16S sequences and COI barcodes are deposited in GenBank (Table 1). Voucher specimens as well as non-sequenced specimens from the same localities are deposited in the Museo Civico di Zoologia di Roma, Italy (MCZR), and the Swedish Museum of Natural History, Stockholm (SMNH) (Table 1). Additional reference specimens from the studied sites are deposited in the Illinois Natural History Survey (INHS) Annelida Collection, Champaign, Illinois, USA, and the Zoological Museum Hamburg, Germany (ZMH).

Results and discussion

COI was successfully sequenced from 16 individuals, representing all populations sampled by us, and – with the 10 additional specimens retrieved from BOLD – the COI dataset comprised 26 sequences; whereas we obtained 16S sequences from 17 individuals from four of the five included populations, as we failed to amplify 16S from the individual from England. In GenBank we

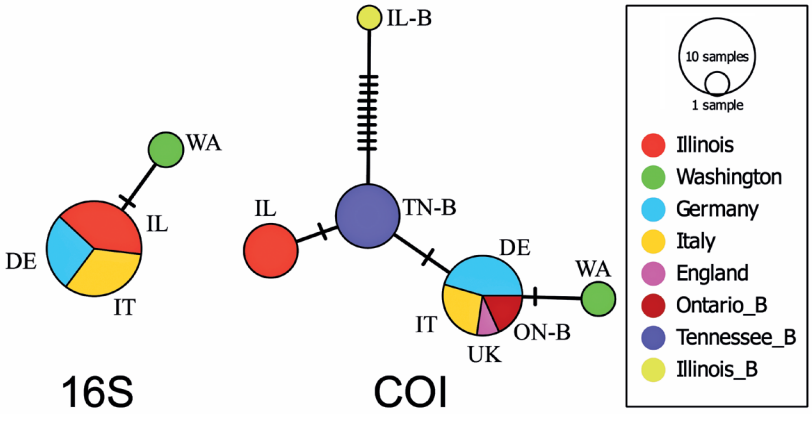

Fig. 1. Haplotype networks illustrating the patterns of divergence of the sampled populations of *Sparganophilus tamesis* Benham, 1892 from Illinois and Washington states (USA), Germany, Italy, and England based on 16S and COI sequences. In the COI network, additional North American specimens retrieved from BOLD as belonging to the same barcode cluster are included. The colours represent geographical origin; localities followed by 'B' indicate BOLD data. Hatch marks correspond to nucleotide substitutions.

found no sequences of COI or 16S that were close to our *S. tamesis* sequences.

In the gathered dataset genetic variation is low and all individuals clearly belong to the same species. In the newly sequenced material we found 3 COI haplotypes, separated by 1-3 substitutions: one in each of our American populations (from the states of Illinois and Washington, USA) and one shared by all the European worms. With the sequences from BOLD, the number of haplotypes increased to 5, with one additional haplotype from Illinois and one from Tennessee, while the sequences from Ontario, Canada belong to the same haplotype as our European specimens. The Illinois haplotype from BOLD differs by 10-12 substitutions from the other haplotypes, which differ from each other by only 1-3 substitutions. For 16S, in our material we found 2 haplotypes, separated by one substitution, one unique for the population from Washington and one shared between the European populations and the population from Illinois (see Fig. 1).

We found no mitochondrial genetic divergence among the European populations, and only a minimum differentiation from the specimens newly collected in Illinois and Washington states (1 or 2 substitutions in the COI gene, 0 or 1 substitution in the 16S gene, respectively), as well as from the specimens from Tennessee and Ontario retrieved from BOLD (0-1 substitutions in COI). The additional haplotype from Illinois is more distant (11 substitutions), but still closely related (2.1% different) to the others. This geographically broad but genetically compact group fits the morphological diagnosis of *S. tamesis* as revised by Jamieson (1971) and is seen as evidence that all European populations: 1) belong to the same species, 2) derive from a recent introduction, and 3) are conspecific with the most widespread and northerly expanded lineage of *Sparganophilus* in North America (sampled from sites as far apart as Ontario, Tennessee, Illinois, and Washington) as the worm commonly referred to as '*S. eiseni*' should be; and that 4) *S. tamesis* is a senior synonym of *S. eiseni*. All other North American *Sparganophilus* sequences recorded in BOLD fall into clusters that are well separated from *S. tamesis*. In GenBank, 16S sequences of other unidentified species of *Sparganophilus* were found, the closest being 7% different from *S. tamesis*.

Our new North American samples were collected from two well-separated localities (Table 1). The Illinois site – 160 km to the NNE of the type locality of *S. eiseni* (north of Havana, in Mason County) – is located near the town of Rock Falls in Whiteside County, along a small tributary of the Illinois and Mississippi (I & M) Canal, which flows NNE for 3 km before its confluence with the Rock River. Both the Rock and Illinois rivers drain into the Mississippi River, although their confluences are located 420 river km apart. According to Reynolds and Wetzel (2011: p. 62), '*S. eiseni*' is the only species in the family known to occur in Illinois.

Our locality in the state of Washington, USA is on the southern bank of Summit Lake in Thurston County (just west of the city of Olympia) (Table 1); this site is 2741 km WNW of the *S. eiseni* type locality near Havana, Illinois, and 2700 km WNW of the site at Rock Falls, Illinois. Summit Lake is oligotrophic, characterized by low nutrient levels, low algae growth, and good water clarity. Land uses are commercial forest and dense residential development (approximately 400 homes) along the shoreline. Primary water uses are domestic water supply, fishing, boating, swimming, and other water sports – none of which are impeded by aquatic weeds or algal growth; water quality is classified as excellent (http://www.co.thurston.wa.us/health/ehadm/swimming/SummitLake.html). Thus the new site does not seem affected by invasions of alien aquatic plants, as many other places in the Pacific Northwest (http://www.ecy.wa.gov/programs/eap/lakes/aquaticplants/index.html#annualsurvey). In any case, the low genetic divergence from the eastern American samples (new and in BOLD) would suggest that this population is of recent introduction.

For some reason, the two previous records of *Sparganophilus* (juvenile specimens) reported from the state of Washington (Reynolds, 1980: 'Jefferson Co., Kalaloch, near lake Crescent'; and 'Pacific Co., South bend of Tokeland'), and the collections of juveniles in southern Alaska (S of Ketchican) and northern British Columbia (E of Prince Rupert Island) (Reynolds, 1980), were not mentioned later when Reynolds (2008) revisited the North American distribution of the family. Altman (1936) did not mention Sparganophilidae in his monograph on the Oligochaeta of Washington. Our new locality of *S. tamesis* in the state of Washington confirms the occurrence of these worms north of Oregon and hints at the possibility that they presently may be as well represented in suitable substrates in the Pacific Northwest as they are in those of the Atlantic Maritime region of Canada (McAlpine *et al*., 2001).

The single European COI haplotype does not refute the possibility of a spread throughout the old continent from a single introduced source population, as suggested by Zicsi and Vaucher (1987) and Rota *et al*.

(2014). Interestingly, in Europe *Sparganophilus* cocoons have thus far been recovered only from amongst the roots of *Sparganium ramosum* Huds. (= *erectum* L.) (Benham 1892), *Sagittaria sagittifolia* L. (Benham, 1892: p. 156, footnote 2), and *Vallisneria spiralis* L. (Rota *et al*., 2014) – all aquatic plants with tape-like submerged foliage. These plant species also share the morphology of the root, comprising a mass of tiny root hairs (and not a single rhizome) among which cocoons can be laid and anchored. Furthermore, by leaking photosynthetically produced oxygen (*e.g*., Soana and Bartoli, 2013), the roots of these plants create an oxic rhizosphere, which makes sediments less inhospitable for the worm's eggs and hatchlings (Rota *et al*., 2014). *Sagittaria* is also present in the Alster site near Hamburg, Germany (U. Graefe, pers. obs.), and in the Rock Falls site, Illinois, USA (M.J. Wetzel, pers. obs.). It does appear, however, that – at least in North America – the habitats of reproduction of *S. tamesis* are more diverse (*e.g*., Harman, 1965). Within the limits of our sampling, the identity of the 16S sequences points towards the Illinois samples being part of the pool from which the worms introduced to Europe originated. However, the DNA data indicate that neither of our two sampled American populations are the source of the introduction. With better sampling across the geographical range of this species, perhaps combined with the use of a faster evolving marker, *e.g*., microsatellites (Cristescu, 2015), it may be possible to find the source population, or at least define a smaller area from where the introduction originated. However, since the European COI haplotype is identical with sequences from Ontario found in BOLD, it seems likely that the European populations have their origin somewhere in the northern part of the species' North American distribution.

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