

The first record of the north-west Pacific nemertean *Cephalothrix simula* in northern Europe

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The north-west Pacific nemertean Cephalothrix simula has been introduced to the Pacific coast of North America, to the Mediterranean Sea and to the Atlantic coast of the Iberian Peninsula. This paper describes the first records of Cephalothrix simula from northern Europe, revealing a broader range for this non-endemic palaeonemertean species. A tree-based species delimitation method (Poisson Tree Process) employing the mitochondrial cox1 gene fragment reveals that four of five Cephalothrix specimens collected from The Netherlands correspond to Cephalothrix simula and only one to the endemic Cephalothrix rufifrons. The Cephalothrix simula sequences from The Netherlands exhibit the highest similarity with sequences from the native area and those from the invaded area in southern Europe. Its co-occurrence with the native Cephalothrix rufifrons in the same habitat is reported. External characters potentially distinguishing live Cephalothrix simula from Cephalothrix rufifrons are given. This finding raises the number of recorded species in The Netherlands to 20.

Keywords: nemertean, *Cephalothrix simula*, introduction, northern Europe, diversity, surveys

Submitted 16 June 2014; accepted 29 December 2014

INTRODUCTION

Nemertean diversity of The Netherlands has received limited attention historically, with the first checklist published only recently (Faasse, 2003). Eleven species were discovered that were not previously recorded in The Netherlands, extending the total number of species to 18 (Faasse, 2003). (Gittenberger & Schipper, 2008). Some of these findings were the result of regular SCUBA surveys conducted to monitor the marine invertebrate fauna in The Netherlands (north-east Atlantic) (Gmelig Meyling & de Bruyne, 2003). Nemerteans are not usually identified below phylum level during routine ecological surveys, which means that dedicated surveys with a focus on species identification will likely provide additional important species distribution data. During one of these surveys at Zierikzee, we found *Cephalothrix* specimens that, externally, did not fit the description of the only native north-east Atlantic species *Cephalothrix rufifrons* (Johnston, 1837) reported for this area. This prompted planning of two additional dedicated nemertean surveys at Zierikzee and Sint Annaland. Species distinctions within the genus *Cephalothrix* are often based upon very subtle external and usually internal morphological differences, but DNA comparisons have proven a reliable alternative for species identification (Chen *et al.*, 2010; Fernández-Alvarez & Machordom, 2013; Leasi & Norenburg, 2014). Kajihara *et al.* (2013) demonstrate that *Cephalothrix hongkongiensis* Gibson 1990 and *Cephalothrix simula* (Iwata, 1952) are

morphologically indistinguishable, making DNA comparison the only reliable way to identify these species.

Here we report the first record of the non-indigenous nemertean *Cephalothrix simula* from northern Europe based on mitochondrial cytochrome oxidase I (*cox1*) gene sequence comparisons. This species is endemic to the north-west Pacific, but has been previously reported from the northern coast of Spain (Atlantic Ocean) and the Mediterranean and Adriatic coasts of Spain and Italy, respectively (Fernández-Alvarez & Machordom, 2013).

MATERIALS AND METHODS

Survey area

The south-eastern seaboard of the North Sea consists almost exclusively of soft sediments. The south-west of The Netherlands is an estuarine area of the rivers Rhine, Meuse and Scheldt on the south-eastern seaboard of the North Sea. In the mouths of the estuaries, deep channels have been cut out by currents. To protect the low-lying land, sea dykes have been built and sublittoral tidal channel slopes have been reinforced with boulders to prevent landslides and damage to the sea dykes. The Oosterschelde is a former estuary, closed off from riverine influence and protected against the sea with a storm-surge barrier. It is now an area important for shellfish culture, mainly mussels and recreational activities such as yachting. Due to shellfish imports and the presence of two nearby international ports, Antwerp and Rotterdam, many alien marine species have been introduced to the Oosterschelde (Wolff, 2005). Most introduced species

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are associated with the man-made coastal protection works. SCUBA surveys were conducted at two locations in the Oosterschelde, i.e. Zierikzee and Sint Annaland. Zierikzee is a recently disturbed location, where in 2009 steel slag and boulders were used for additional reinforcement of the subtidal slope to a depth of about 17 m below the low water mark. In Sint Annaland no recent reinforcements have taken place; the boulder slope at the sampling location gives way to a flat-tish sandy bottom at 14 m below the low-water mark.

Survey methods

SCUBA surveys were performed during dives at two locations (Table 1). At both locations a transect about 2 m wide was carefully inspected by two divers, from the low-water mark to the end of the boulder slope and about 5 m further on the sandy bottom below. Nemertean specimens other than the well-known species of the area, *Lineus longissimus* (Gunnerus, 1770) or *Tubulanus superbis* (Kölliker, 1845), were collected at Zierikzee. In addition, a $10 \times 10 \text{ cm}^2$ sample of bryozoan/hydrozoan turf was collected. At Sint Annaland a $10 \times 10 \text{ cm}^2$ sample was taken from a mat of *Lanice conchilega* (Pallas, 1766) tubes on the sandy bottom. At Zierikzee a *Lanice* mat was absent.

Laboratory analyses

Two nemerteans from Zierikzee (28 July 2012) and six from Sint Annaland (15 June 2013) were preserved in 96% ethanol. The two specimens from Zierikzee and three from Sint Annaland were further analysed. Width measurements were taken of these five nemerteans (Table 2). Measurements were taken behind the mouth from preserved specimens, hence the position is approximate. *Cox1* sequences of the same five specimens were determined. Total DNA was purified from alcohol preserved tissue using the QIAamp DNA Mini Kit (Qiagen) and a fragment ($\sim 710 \text{ bp}$) of the mitochondrial cytochrome oxidase I (*cox1*) gene was PCR amplified with HCO2198 and LCO1490 primers (Folmer *et al.*, 1994) following standard protocols. Amplified DNAs were purified by incubating for 15 min at 37°C with Exo and SAP enzymes (USB[®]) and subsequently sequenced with the above-mentioned primers using the ABI BigDye[®] Terminator Cycle Sequencing Kit and an ABI 31310XL Genetic Analyzer. Sequences were aligned with 184 relevant *Cephalothrix* sequences downloaded from GenBank using MUSCLE (Edgar, 2004) and uncorrected pairwise distances (p-distances) were calculated using the PAUP* software package (Swofford, 2003). The five sequences from The Netherlands were deposited in GenBank (*Cephalothrix rufifrons* Zierikzee KP411243, *Cephalothrix simula* Zierikzee KP411244, *Cephalothrix simula* St. Annaland KP411245, KP411246, KP411247). For species delimitation, a tree-based criterion was employed. Nemertean outgroup sequences – *Tubulanus pellucidus* (Coe, 1895) and *Tubulanus sexlineatus*

(Griffin, 1898) – chosen based on the analysis of nemertean relationships (Andrade *et al.*, 2012), were added to the alignment and identical haplotypes were removed using the DNA collapser of FaBOX (Villesen, 2007). This resulted in a data set consisting of 102 *Cephalothrix* sequences plus the two outgroup sequences. A maximum likelihood (ML) tree was inferred using RAxML as implemented on the Cipres server (Miller *et al.*, 2010). Bootstrap analysis (100 pseudoreplicates) was employed to assess clade support. The Poisson Tree Process (PTP) method of species delimitation using a Bayesian approach as implemented on the bPTP web server (Zhang *et al.*, 2013) was employed using the RAxML tree as input. 500,000 MCMC generations were run for each of the three analyses using different seeds and convergence was monitored. The general mixed Yule-coalescent (GMYC) approach (Pons *et al.*, 2006) was not utilized, as it requires an accurate ultrametric tree (Zhang *et al.*, 2013; Tang *et al.*, 2014), which could not be generated from our RAxML input tree using the commonly employed r8s program (Sanderson, 2003), possibly because some branch lengths approached zero.

RESULTS

The specimens collected at Zierikzee were visible with the naked eye as single orange-coloured worms (Figure 1) on boulders between the bryozoan/hydrozoan turf and other invertebrates covering the boulders. Bryozoan/hydrozoan turf collected contained several juveniles of the terebellid polychaete worm *Nicolea zostericola* (Örsted, 1844). At Sint Annaland, no nemerteans were observed on the boulder slope. A mat of tubes of the terebellid polychaete worm *Lanice conchilega* covered several square metres of the sea bottom at a depth of 14 m, immediately below the boulder slope. In a $10 \times 10 \text{ cm}^2$ sample of *Lanice* tubes the only species observed were *Lanice conchilega* and orange-coloured *Cephalothrix*.

Cox1 sequences of specimens were compared with sequences from GenBank. The *cox1* sequences of one of the two specimens from Zierikzee (28 July 2012) and all three specimens sequenced from Sint Annaland (15 June 2013) were identical and showed the least divergence (p-distance = 0–8.34%) from vouchers published in GenBank for specimens that were identified as *Cephalothrix simula* with morphological and genetic data (Chen *et al.*, 2010; Fernández-Alvarez & Machordom, 2013; Kajihara *et al.*, 2013). The Netherlands sequences were identical to the sequence of the Japanese specimen of *Cephalothrix simula* deposited by Kajihara *et al.* (2013) and p-distances between The Netherlands sequences and those of southern European specimens of *Cephalothrix simula* submitted by Fernández-Alvarez & Machordom

Table 1. Details of observations of *Cephalothrix simula* (Iwata, 1952) in The Netherlands.

Date	Location	Number	Habitat
28/07/2012	Zierikzee	1	Boulder slope
15/06/2013	Sint Annaland	3	<i>Lanice conchilega</i> mat

Table 2. Width measurements of *Cephalothrix* used for molecular analysis.

Date	Location	Species	Width (mm)
28/07/2012	Zierikzee	<i>Cephalothrix rufifrons</i>	0.8
28/07/2012	Zierikzee	<i>Cephalothrix simula</i>	1.2
15/06/2013	Sint Annaland	<i>Cephalothrix simula</i>	1.9
15/06/2013	Sint Annaland	<i>Cephalothrix simula</i>	2.0
15/06/2013	Sint Annaland	<i>Cephalothrix simula</i>	2.2



Fig. 1. *Cephalothrix simula*, 28 July 2012, Zierikzee, The Netherlands, *in situ*, depth about 10 m, on the ascidian tunicate *Ciona intestinalis* (Linnaeus, 1767), with the bryozoan *Scrupocellaria scruposa* (Linnaeus, 1758) on the left.

(2013) ranged from 0 to 0.16%. Fernández-Alvarez & Machordom (2013) indicated that all sequences of the southern European specimens fall within network 11 of Chen *et al.* (2010). The *cox1* sequence of the second specimen from Zierikzee was most similar to GenBank vouchers for *Cephalothrix rufifrons* (p-distance = 0–0.78%). Uncorrected genetic distance (p-distance) between *cox1* sequences of *Cephalothrix simula* and *Cephalothrix rufifrons* ranges from ~14.68 to 15.48%.

Maximum likelihood analysis places three specimens from Sint Annaland and one from Zierikzee in a strongly supported clade (bootstrap value = 99%) containing other European specimens and those from Japan (including the topogenotype; Figure 2), all of which were previously identified as *Cephalothrix simula* using alternative delimitation methods (Chen *et al.*, 2010). This cluster, inclusive of the three Sint Annaland and one Zierikzee specimen, is inferred as a distinct species by PTP and is designated as *Cephalothrix simula sensu stricto*. The PTP analysis also infers two species that form consecutive sister taxa to *Cephalothrix simula sensu stricto* in the ML tree and this grouping of three entities is herein referred to as *Cephalothrix simula sensu lato* (Figure 2). One specimen from Zierikzee falls within a *Cephalothrix rufifrons* clade (bootstrap value = 100%) and this group is supported as a distinct species by PTP species delimitation (Figure 2).

Most of the nemerteans collected at Zierikzee and Sint Annaland were different in colour pattern and size from those that were hitherto identified with external characters as the native *Cephalothrix rufifrons*. In contrast to *Cephalothrix* collected in previous years, the whole body was tinged with bright orange, although becoming paler towards the rear. Sometimes a bright orange spot is present on the head. Often a dark grey core (digestive tract) of the body shimmers through (Figure 3). This form was never collected before 2012. Presumed native *Cephalothrix rufifrons* from previous years (Figure 4) also have an orange spot on the head, the rest of the body being uniformly pale straw-coloured, without any grey. In the specimens collected in 2012 and 2013 (Table 2) the body of *Cephalothrix simula* was conspicuously flatter in shape than the body of *Cephalothrix rufifrons*, a difference that has not been quantified. Furthermore, body width was larger in alcohol preserved specimens of

Cephalothrix simula than in *Cephalothrix rufifrons*: 1.8 mm (SD 0.4 mm) in *Cephalothrix simula* and 0.8 mm in *Cephalothrix rufifrons*. Unfortunately, only one specimen of *Cephalothrix rufifrons* was available and measurement of a larger sample of both species is desirable. According to Gibson (1974), the width of live *Cephalothrix rufifrons* is 0.5 mm or less, while Strand & Sundberg (2010) report a width of less than 1 mm. Our rough estimation of the width of live specimens of *Cephalothrix simula* is about 1 mm, which is consistent with measurements of live, relaxed specimens (Fernández-Alvarez & Machordom, 2013).

DISCUSSION

The PTP delimitation approach strongly suggests that one specimen from Zierikzee and three from Sint Annaland are *Cephalothrix simula*. In the ML tree sequences of these individuals are positioned in the clade containing specimens previously inferred as *Cephalothrix simula* (see Chen *et al.*, 2010; Fernández-Alvarez & Machordom, 2013; Kajihara *et al.*, 2013). This clade, supported as a species by PTP, is equivalent to network 11 of Chen *et al.* (2010), and Kajihara *et al.* (2013) stated that, based on their revised analyses, specimens in this network should ‘always’ be designated as *Cephalothrix simula*. Two other entities inferred by PTP corresponding to networks 6 and 8 of Chen *et al.* (2010) form successive sister groups to *Cephalothrix simula sensu stricto* (network 11 of Chen *et al.* (2010) and these authors implied that networks 6 and 8 also corresponded to *Cephalothrix simula*. However, Fernández-Alvarez & Machordom (2013) stated that the barcoding gap between these three networks suggests cryptic speciation, which is congruent with our PTP inference. It is noteworthy that our PTP delimitation results are consistent overall with those of Leasi & Norenburg (2014) for cephalothricids, but consideration of other *Cephalothrix* species falls outside the scope of this contribution.

Cephalothrix simula originates from the north-west Pacific (Kajihara, 2007). Chen *et al.* (2010) reported its presence in Italy and Foss (2011) reported the introduction of *Cephalothrix simula* to the Pacific coast of North America. The identity of *Cephalothrix simula* from The Netherlands was established by molecular analysis in December 2012. Soon after, the presence of *Cephalothrix simula* was reported from the Mediterranean and Atlantic coasts of Spain (Fernández-Alvarez & Machordom, 2013). The latter authors concluded that *Cephalothrix simula* was introduced by anthropogenic transport, based on presumed natural dispersal limitations. From our limited data it is impossible to infer whether multiple introductions to southern and northern Europe, respectively, were involved. However, it is noteworthy that all northern European *cox1* sequences were identical to that of a Japanese specimen, unlike some *cox1* sequences from southern Europe, leaving open the possibility of multiple introductions. Population genetic studies with large sample sizes will be necessary to clarify this question.

In the years 2001–2002 an extensive survey of the nemertean fauna of hard substrates on the coast of The Netherlands (Faasse, 2003) more than doubled the number of nemertean species known from the area. *Cephalothrix rufifrons* was reported from a few locations. However, during the 2001–2002 survey and subsequent investigations (Gittenberger & Schipper, 2008) *Cephalothrix simula* was

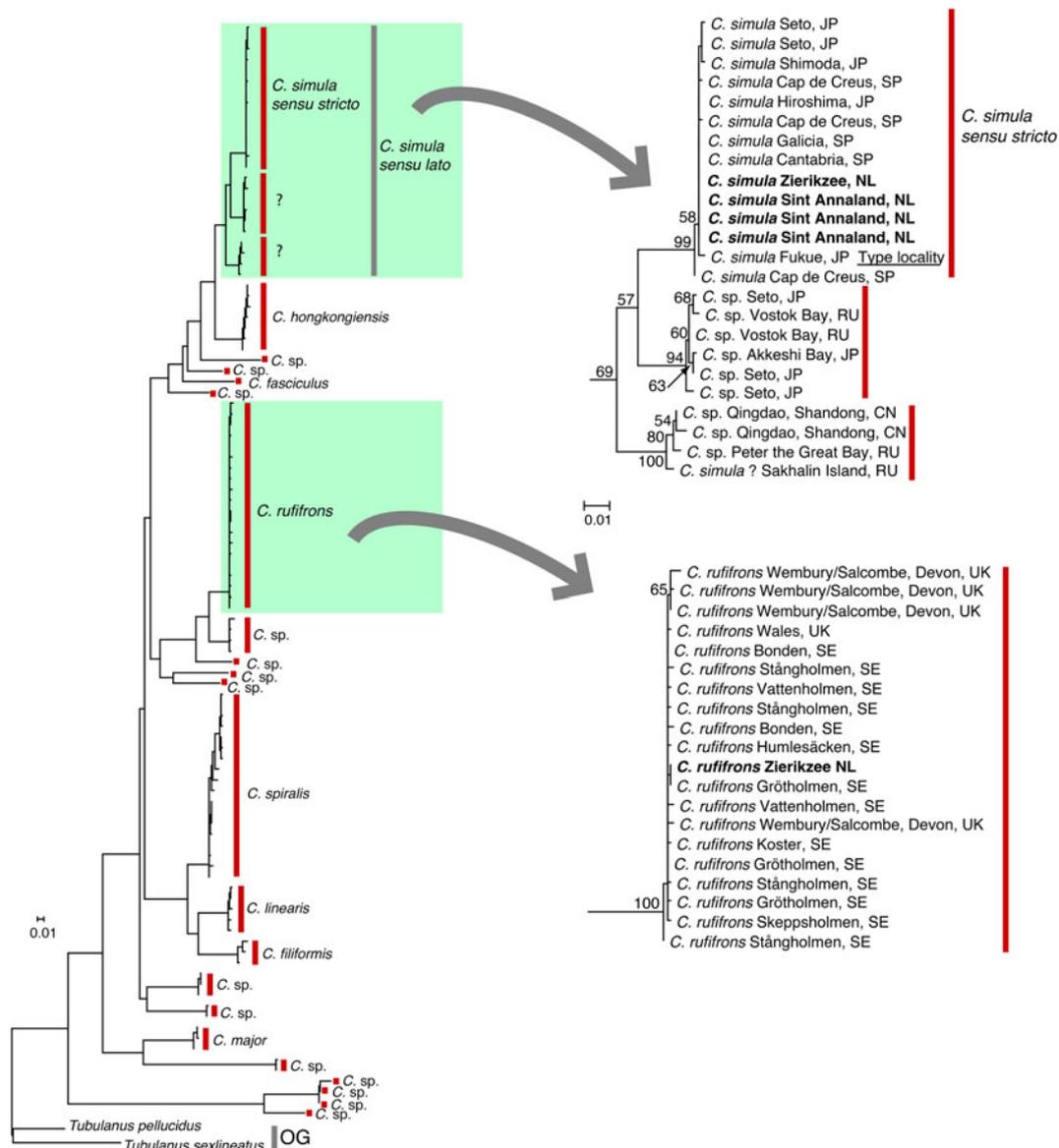


Fig. 2. Maximum likelihood tree of *cox1* haplotypes. Bootstrap percentages $\geq 50\%$ are indicated at corresponding nodes. *Cephalothrix simula sensu stricto* contains sequences from Japan, including the type locality, Fukue, Japan (JP) and sequences from Europe. Haplotypes from Korea, China and Europe identical to those contained in this clade were excluded as required by RAxML prior to analysis. Red bars indicate the 24 species inferred by PTP/bPTP analysis. *C. simula sensu lato* contains two clades (marked with ?) that are inferred as separate species (see text). Abbreviations: CN, China; JP, Japan; NL, The Netherlands; OG, Outgroup; RU, Russia; SE, Sweden; SP, Spain; UK, United Kingdom.

not recorded, leaving open the possibility that this species was introduced to The Netherlands during the last decade. Although the specimens were collected from the Oosterschelde, an area with extensive shellfish culture and receiving seed mussels imported from surrounding countries, the introduction vector of *Cephalothrix simula* is not known with certainty. The area is situated between the two large international ports of Antwerp and Rotterdam and this situation results in many species introductions by shipping (Wolff, 2005). The similarity between *Cephalothrix rufifrons* and *Cephalothrix simula* and the absence of the latter in regional identification keys and checklists (e.g. Howson & Picton, 1997) are reasons to point out the presence of *Cephalothrix simula* at different locations in Europe. Our findings also show that *Cephalothrix simula* sometimes co-occurs with *Cephalothrix rufifrons*. As an introduced species and predator, *Cephalothrix simula* may affect food chains in

invaded areas. Fernández-Alvarez & Machordom (2013) found *Cephalothrix simula* to represent 28% of all nemerteans sampled at one of their two Mediterranean sampling stations, while no native *Cephalothrix* were present in the samples. A lack of quantitative data on native *Cephalothrix* precludes an evaluation of potential competitive exclusion (Fernández-Alvarez & Machordom, 2013).

Although reliable identification of *Cephalothrix simula* is presently only possible by comparison of *cox1* sequences with GenBank sequences, we have given a short description of colouration and width measurements, to alert researchers collecting similar specimens to the possibility they have collected *Cephalothrix simula*. It will be possible to identify *Cephalothrix simula* with external characters in the future only if the differences described can be confirmed by observing and measuring a large number of specimens and correlated with sequence-based species delimitation.



Fig. 3. *Cephalothrix simula*, 15 June 2013, from Sint Annaland, The Netherlands, *ex situ*.

Although the intercontinental and transoceanic introduction of marine organisms to other parts of the world is a widespread phenomenon, reported instances of nemertean introductions are almost non-existent. This is unlikely to reflect the real share of nemerteans in marine species introductions. As mentioned in the Introduction, in routine ecological monitoring programs nemerteans are not usually identified below phylum level, which virtually precludes early detection of introduced nemerteans. Three regions where the composition of the nemertean fauna has been investigated for several years each yielded a nemertean species introduced from a different part of the world. First, Turbeville (2011) reported the north-east Atlantic species *Emplectonema gracile* (Johnston, 1837) from South Carolina, USA (north-west Atlantic). Second, the north-west Pacific *Cephalothrix simula* was recorded from Spain and Italy (southern Europe) (Fernández-Alvarez & Machordom, 2013) and from The Netherlands (northern Europe) (present paper). Fernández-Alvarez & Machordom (2013) question the non-native status of *E. gracile* in the north-west Atlantic, postulating a high dispersal capacity of this species based on its present distribution on both sides of the North Pacific and North Atlantic, disregarding that many cosmopolitan distributions are in fact anthropogenic (Carlton, 1989).



Fig. 4. *Cephalothrix rufifrons*, 14 March 2001, from the Goesse Meer, The Netherlands, *ex situ*, on the bryozoan *Cryptosula pallasiana* (Moll, 1803).

Turbeville (2011) refers to many comprehensive faunal surveys in the north-west Atlantic, inclusive of nemerteans, published in the 19th and 20th Centuries that do not mention this conspicuous intertidal species, suggesting a recent introduction to the Atlantic coast. It is interesting to note in this regard that Chernyshev (2014) mentions the presence of *Cephalothrix* larvae in the ballast water of ships in Port Vladivostok, Peter the Great Bay, north-west Pacific.

During spring 2012, unusually large numbers of the terebellid polychaete worm *Nicolea zostericola* were observed at Zierikzee, the first collection locality of *Cephalothrix simula*. Densities were so high that they attracted the attention of several recreational divers/photographers (http://www.natuurbericht.nl/?id=7992&cat=strand_en_zee). Bryozoan/hydrozoan turf collected with nemerteans on 28 July 2012 at Zierikzee contained numerous juveniles of *N. zostericola*. The association of *Cephalothrix simula* with the terebellid *N. zostericola* at Zierikzee and its occurrence in a mat of the terebellid *Lanice conchilega* at Sint Annaland merit further investigation into the food preference of *Cephalothrix simula*. *Cephalothrix simula* is known to eat polychaetes and oligochaetes in the laboratory (Wang *et al.*, 2008) and Chernyshev (2014) found small polychaetes, amphipods, isopods and nemerteans in the gut contents.

It is also worthwhile to point out the potentially extreme toxicity of *Cephalothrix simula*. Asakawa *et al.* (2013) found that in one case a single worm exceeded the minimum human lethal dose of the neurotoxin tetrodotoxin and that 48% of the worms contained more than 20% of the minimum human lethal dose.

ACKNOWLEDGEMENTS

We thank Marianne Ligthart (Stichting Anemoon, Bennebroek, The Netherlands) for assistance with the SCUBA surveys and Chris Friedline and Andrew Eckert for advice regarding ultrametric tree inference. Our manuscript benefited from the comments of an editor and two anonymous reviewers.

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