Cryptic speciation at organic-rich marine habitats: a new bacteriovore annelid from whale-fall and fish farms in the North-East Atlantic

HELENA WIKLUND[1], ADRIAN G. GLOVER[2], PER J. JOHANNESSEN[3] and THOMAS G. DAHLGREN[1]*

[1]*Department of Zoology, Göteborg University, PO Box 463, SE-40530 Göteborg, Sweden*
[2]*Zoology Department, The Natural History Museum, Cromwell Road, London SW7 5BD, UK*
[3]*Institutt for Biologi, PO Box 7800, N-5020 Bergen, Norway*

***Vigtorniella ardabilia* sp. nov.**, a new chrysopetalid annelid, is described from a whale-fall in Sweden and from sediment samples collected beneath fish farms in Norway. The new *Vigtorniella* species is morphologically almost identical to *Vigtorniella flokati* from whale-falls in the Pacific Ocean, although molecular evidence from four genes shows that they are different species. Population genetic structure and phylogenetic relationships of ***V. ardabilia* sp. nov.** were assessed using molecular data from the nuclear genes 18S and 28S, and the mitochondrial 16S and cytochrome *c* oxidase subunit I (COI). High levels of gene flow are reported between contrasting organic-rich environments in the North Atlantic (fish farms and whale-fall). Observations of feeding biology and habitat suggest that *V. ardabilia* specializes on bacterial mats, rather than on whale-falls, although the two species of *Vigtorniella* for which data were available show very different feeding behaviours. Our results further showed an unexpectedly low divergence rate in *Vigtorniella* for the mitochondrial markers, suggesting stabilizing selection. Analyses carried out with parsimony, maximum likelihood, and MrBayes all placed the genus *Vigtorniella* as sister group to *Dysponetus*, suggesting a close evolutionary link to sediment-dwelling fauna. © 2009 The Linnean Society of London, *Zoological Journal of the Linnean Society*, 2009, **155**, 774–785.

ADDITIONAL KEYWORDS: chemosynthetic ecosystem – Chrysopetalidae – evolution – organic enrichment – Polychaeta – population genetics – ***Vigtorniella ardabilia* sp. nov.**

INTRODUCTION

A range of both geological and biological processes may give rise to ephemeral, organic-rich habitats on the seafloor. Well-studied geological phenomena are deep-sea hydrothermal vents and cold seeps (Van Dover *et al.*, 2002). Less well-studied biological processes may include the deposition of whale remains (or 'whale-falls'), wood-falls, fish-falls, kelp-falls, and anthropogenically influenced habitats such as the benthos of fish farms and sewage dumping sites. All of these environments, both in shallow and deep water, are punctuated in time and space, and as such serve as useful models for the study of biological processes

such as larval dispersal and speciation. Furthermore, new evidence is suggesting that there may be considerable overlap in species composition among these habitats, both in terms of the microbial (e.g. *Beggiatoa* mat) and macrofaunal communities (Smith & Baco, 2003).

Since the discovery of a specialized fauna associated with whale-falls (Smith *et al.*, 1989), there have been several reports of whale-fall associated polychaetes in both deep sea and shallower waters. Apart from the polychaete genus *Osedax*, Rouse, Goffredi & Vrijenhoek, 2004 which has its roots embedded within the bone, several free-living species have been found, e.g. the chrysopetalid *Vigtorniella flokati* Dahlgren *et al.*, 2004, the hesionid *Vrijenhoekia balaenophila* Pleijel *et al.*, 2008, and several polynoids (Pettibone,

*Corresponding author. E-mail: thomas.dahlgren@zool.gu.se

1993), among them *Bathykurila guaymasensis* Pettibone, 1989. The latter has also been found in association with hydrothermal vents (Pettibone, 1993). In an early whale-fall study, it was suggested that the bacterial mat covering the whale bones would discourage the activities of scavengers by producing a noxious envelope around carcasses (Allison *et al.*, 1991). Since then, several studies have shown that some animals can utilize the mat, e.g. limpets (Smith & Baco, 2003) and polychaetes (Glover *et al.*, 2005a), by grazing filamentous bacterial mats, such as formed by *Beggiatoa*.

Chrysopetalidae Ehlers, 1864 consists of 12 genera with around 50 species. They are small, errant forms, often distinguished from other polychaete families by the presence of golden or silver-coloured flattened notochaetae, or paleae, which in some species form fans covering the dorsum. Morphologically they may be divided into two groups based on the form of the paleae, those with flattened paleae like *Chrysopetalum* Ehlers, 1864, and those with more cylindrical notochaetae like *Dysponetus* Levinsen, 1879 and the carpet-worms, *Vigtorniella* (Kiseleva, 1992). Chrysopetalid species have often been recorded associated with the cracks and crevices of hard substrates such as coral reefs, rock reefs, rotting wood, and shells, although deep-water sediment-dwelling species are also known (Watson Russell, 2000). More recently, chrysopetalids have been found in sulphide-rich, redox habitats such as sunken wood, cold seeps, hydrothermal vents (Watson, 2001), and whale-falls (Dahlgren *et al.*, 2004). *Vigtorniella zaikai* (Kiseleva, 1992) was described from pelagic polychaete larvae from the Black Sea and raised to maturity in the laboratory. Larvae of this form were first reported by Kiseleva (1959), and occur in large numbers throughout the year. In 1994, live adults were collected for the first time in sediments at the oxic–anoxic boundary of the Black Sea (Sergeeva, Zaika & Kiseleva, 1997).

When *Vigtorniella flokati* was described from whale-fall habitats in the Pacific, its phylogenetic position was assessed from morphological data alone (Dahlgren *et al.*, 2004). In this study we describe a new whale-fall *Vigtorniella* species from Sweden and investigate the phylogenetic position of the genus *Vigtorniella* within Chrysopetalidae with molecular data from the nuclear genes 18S and 28S, and the mitochondrial genes 16S and cytochrome *c* oxidase subunit I (COI). The new species is morphologically very similar to *V. flokati*, despite them occurring in separate ocean basins and at disjunct depth ranges. To assess the level of genetic divergence between the new species and its congeneric sibling in relation to other congeneric species pairs within the same family, we also compared genetic divergence at the same four DNA loci in three other chrysopetalid genera.

MATERIAL AND METHODS
SAMPLING

In the Koster area off the Swedish West coast (Fig. 1), two dead, stranded whale carcasses have been implanted at shallow depths, a Minke whale at 125 m (sunk in October 2003) and a Pilot whale at 30 m (sunk in January 2005) (Dahlgren *et al.*, 2006). From these whale-falls, bones have been retrieved using remotely operated vehicles (ROVs) and maintained in aquaria at Tjärnö Marine Laboratory, with the purpose of studying the associated fauna alive. The new chrysopetalid species from the Minke whale was first found in the aquarium tanks in November 2005, on bones that had been retrieved 5 months earlier. Later, the species was also found by the ROV *in situ*, on the remains of the Minke whale at 125 m. The new species has also been found in sediment samples taken beneath fish farms in a Norwegian fjord (Fig. 1), at depths between 80–150 m.
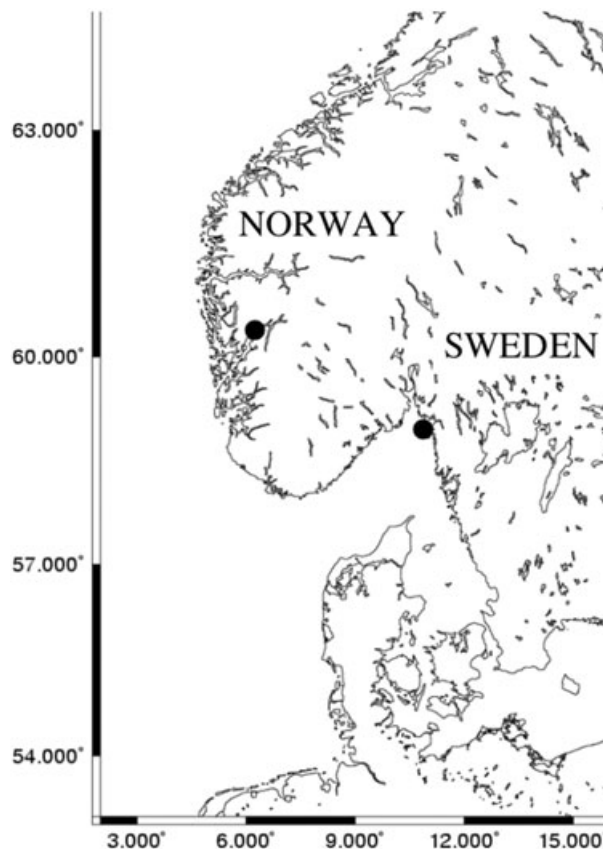

Figure 1. Map showing collection sites in Sweden and Norway for the new species.

Table 1. Taxa and *Vigtorniella ardabilia* cytochrome *c* oxidase subunit I (COI) haplotypes, with collection sites and NCBI GenBank accession numbers

Taxa/haplotype	Collected	18S	28S	16S	COI	Voucher
OUTGROUP						
Nereimyra punctata Müller, 1776	NCBI GenBank	DQ779661	DQ442606	DQ442577	DQ442566	
Ophiodromus flexuosus Delle Chiaje, 1827	Koster Area, Sweden	EU555039	DQ442607	DQ442578	DQ442567	SMNH97312
CHRYSOPETALIDAE						
Bhawania heteroseta Hartman, 1945	Florida, USA	EU555035	EU555025	EU555044	EU555053	SMNH97305
Bhawania reyssi Katzmann, Laubier & Ramos, 1974	Banyuls, France	EU555036	EU555026	EU555045	EU555054	SMNH97306
Chrysopetalum debile Grube, 1855	Banyuls, France	EU555037	EU555027	EU555046	AF221567	SMNH97307
Dysponetus caecus Langerhans, 1880	Koster Area, Sweden	AY839568	EU555028	EU555047	AF221568	
Dysponetus sp. NZ	New Zealand	EU555038	EU555029	EU555048	EU555055	SMNH97308
Paleanotus sp. LI	New Caledonia	EU555040	EU555030	EU555049	EU555056	SMNH97309
Paleanotus sp. NZ	New Zealand	EU555041	EU555031	EU555050	EU555057	SMNH97310
Vigtorniella flokati	California, USA	EU555043	EU555033	EU555034	EU555065	SMNH97311
***Vigtorniella ardabilia* sp. nov.**	Sweden & Norway	EU555042	EU555032	EU555051	EU555052	SMNH97313
Haplotype A	Norway	–	–	–	EU555058	
Haplotype B	Sweden & Norway	–	–	–	EU555059	
Haplotype C	Norway	–	–	–	EU555060	
Haplotype D	Sweden	–	–	–	EU555061	
Haplotype E	Sweden	–	–	–	EU555062	
Haplotype F	Sweden	–	–	–	EU555063	
Haplotype G	Sweden	–	–	–	EU555064	

MORPHOLOGICAL ANALYSIS

A total of 30 individual worms were obtained from material collected at the Swedish experimental whale-fall site in 2005–2007 and 15 individuals from environmental survey samples collected under the fish farms in Norway in 2005–2006. The animals were relaxed in 7% magnesium chloride in distilled water, photographed alive, and preserved for scanning electron microscopy (SEM), DNA analyses, and standard morphological examination. Specimens for SEM were fixed in 1% osmium tetraoxide in filtered seawater for 15 min, rinsed in distilled water, and stored in 70% ethanol, critical point-dried in ethanol, gold-coated, and imaged using a Hitachi S-4300. Specimens for DNA sequencing were preserved in 95% ethanol and stored at –20 °C. Specimens for standard morphology were fixed in 10% formalin in seawater for 1 day, and transferred to 70% ethanol. The live photographs, SEM, and light microscopy pictures were edited using Adobe Photoshop CS2, making the background black in the live photo and enhancing contrast in the SEM and light microscopy pictures.

DNA ANALYSIS

In the molecular phylogenetic analyses, nine taxa from Chrysopetalidae and two outgroup taxa from Hesionidae were used (Table 1). The outgroup taxon choice was based on earlier phylogenetic analyses of nereidiform annelid relationships (Glasby, 1993; Pleijel & Dahlgren, 1998; Dahlgren *et al.*, 2000). The analyses were based on new data as well as sequences obtained from NCBI GenBank. Extraction of DNA was carried out with E.Z.N.A. Tissue DNA Kit (Omega Bio-tek) following the protocol supplied by the manufacturer. Around 1200–1800 bp of 18S, 350 bp of 28S D1-region, 400 bp of 16S, and 600 bp of COI were amplified using a range of primers (Table 2). PCR mixtures contained H_2O, 1 µL of each primer (10 µM), 2 µL template DNA, and puReTaq Ready-To-Go PCR Beads (Amersham Biosciences) in a mixture of total 25 µL. The temperature profile was as follows: 96 °C/240 s – (94 °C/30 s – 48 °C/30 s – 72 °C/60 s) * 45cycles – 72 °C/480 s. PCR products were purified with the E.Z.N.A. Cycle-Pure Kit (Omega Bio-tek). Sequencing was performed by

Table 2. PCR and sequencing primers

Primer	Sequence 5′-3′	Position	References
18SA	AYCTGGTTGATCCTGCCAGT	1–20	Medlin *et al.* (1988)
18SB	ACCTTGTTACGACTTTTACTTCCTC	1776–1800	Nygren & Sundberg (2003)
620F	TAAAGYTGYTGCAGTTAAA	618–636	Nygren & Sundberg (2003)
584R	ACGCTATTGGAGCTGGAAT		Persson, pers comm
860F	GAATAATGGAATAGGA	821–836	Turbeville, Field & Raff (1992)*
977R	AACCTCTGACTTTCGTTCTT		Persson, pers comm
1324F	GGTGGTGCATGGCCG	1284–1298	Cohen, Gawthrop & Cavalier-Smith (1998)
1324R	CGGCCATGCACCACC	1284–1298	Cohen *et al.* (1998)
16SarL	CGCCTGTTTATCAAAAACAT		Palumbi (1996)
16SbrH	CCGGTCTGAACTCAGATCACGT		Palumbi (1996)
28SC1′	ACCCGCTGAATTTAAGCAT		Lê, Lecointre & Perasso (1993)
28SC2	TGAACTCTCTCTTCAAAGTTCTTTTC		Lê *et al.* (1993)
LCO1490	GGTCAACAAATCATAAAGATATTGG		Folmer *et al.* (1994)
COI-E	TATACTTCTGGGTGTCCGAAGAATCA		Bely & Wray (2004)

Position numbers refer to the *Autolytus prolifer* 18S sequence (GenBank accession no. AF474295); F, forward; R, reverse.
*Modified from original primer by Nygren & Sundberg (2003).

the Macrogen Sequencing System in Korea on an ABI 3730XL DNA Analyser (Applied Biosystems).

Overlapping sequence fragments were merged into consensus sequences using SeqMan 4.0 (DNAStar), and aligned using CLUSTAL X (Thompson *et al.*, 1997) with default settings, 15/6,66 as gap/gap length penalties for the 16S, 18S, and 28S dataset alignments. All regions that could not be unambiguously aligned in those alignments were excluded. Two COI datasets were made, one for population genetic analyses with the 17 sampled individuals from the two *Vigtorniella* species, and one for phylogeny analyses using the same 11 taxa as in the other molecular datasets. Alignments are available at TreeBase, http://www.treebase.org. The computer program PAUP* 4.0b10 (Swofford, 2002) was used for the parsimony (PA), and maximum likelihood (ML) analyses, with heuristic search and tree bisection and reconnection (TBR) branch swapping. Clade support was assessed using nonparametric bootstrap with 5000 replicates and ten random additions in PA, and with 100 replicates in ML. Bayesian phylogenetic analyses (BA) were conducted with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Analyses were run three times for each dataset with four chains for 1 000 000 generations. 250 000 generations were discarded as burn-in. The results from each dataset analyses were compared, and when values approached similar mean values for all parameters they were considered to have converged. The evolutionary models used for the molecular data in BA were obtained by running the datasets in MrModelTest (Nylander, 2004). The model GTR+I+G was suggested as the best fit for 28S, whereas GTR+G was used for 18S and 16S. For COI,

the data were partitioned into codon positions, and position 1 followed SYM+I, GTR+G was used for position 2 and HKY+G for position 3. The datasets were tested for incongruence using the Shimodaira–Hasegawa (SH) test in PAUP*, with resampling estimated log-likelihood (RELL) and 1000 bootstrap replicates. The trees within the 95% confidence interval from the separate analyses made in MrBayes were used in the test. The SH test showed that the partitions were congruent, and all four datasets were combined. In the combined Bayesian analysis, the data were partitioned into four parts (28S, 18S, 16S, COI), and the evolutionary models mentioned above were applied to each partition respectively, and the parameters used for the partitions were unlinked. For the ML analysis, the four molecular datasets were combined and run in ModelTest (Posada & Crandall, 1998), which suggested GTR+I+G as the best model.

The range of genetic divergence at generic level within the family Chrysopetalidae was assessed between species pairs in four genera within the Chrysopetalidae at four loci (18S, 28S, 16S, and COI). The optimal mutation model was calculated for each locus using ModelTest (Posada & Crandall, 1998) and genetic divergence calculated using PAUP* (Swofford, 2002). Besides *Vigtorniella*, the genera for which the species pairs were sampled, *Bhawania* Schmarda, 1861, *Dysponetus*, and *Paleanotus* Schmarda, 1861 represent a sample of the known morphological diversity within the family Chrysopetalidae (Dahlgren *et al.*, 2000). These genera have been considered to be monophyletic based on previous morphological analyses (Perkins, 1985; Dahlgren & Pleijel, 1995; Dahlgren *et al.*, 2000).

The population genetic estimates are based on two samples from *Vigtorniella ardabilia* sp. nov. and one sample from *Vigtorniella flokati*. In the former, 14 specimens were genotyped for variation in the COI locus, of which eight were from the Koster sample in Sweden and six from Hardangerfjord in Norway, and in the latter, three specimens were genotyped from the Santa Cruz Basin population (Dahlgren *et al.*, 2004). A parsimony haplotype network analysis of the *Vigtorniella* haplotypes was made with TCS 1.21 (Clement, Posada & Crandall, 2000). The holotype and vouchers for the sequenced species in this study have been deposited at the Swedish Museum of Natural History (SMNH) in Stockholm, Sweden (voucher numbers in

Table 1). Paratypes are deposited at the Natural History Museum (NHM) in London, UK.

RESULTS

SYSTEMATICS

ANNELIDA LAMARCK, 1809

CHRYSOPETALIDAE EHLERS, 1864

VIGTORNIELLA ARDABILIA SP. NOV.

(FIGS 2–4)

Type material: Northern North Atlantic, coastal Skagerrak, 58°53.1′N; 11°06.4′E, female with eggs, 6 mm long, 35 segments, preserved in formaldehyde

Figure 2. *Vigtorniella ardabilia* sp. nov., live photo of specimen from whale-fall in Sweden. The worm is 6 mm long.
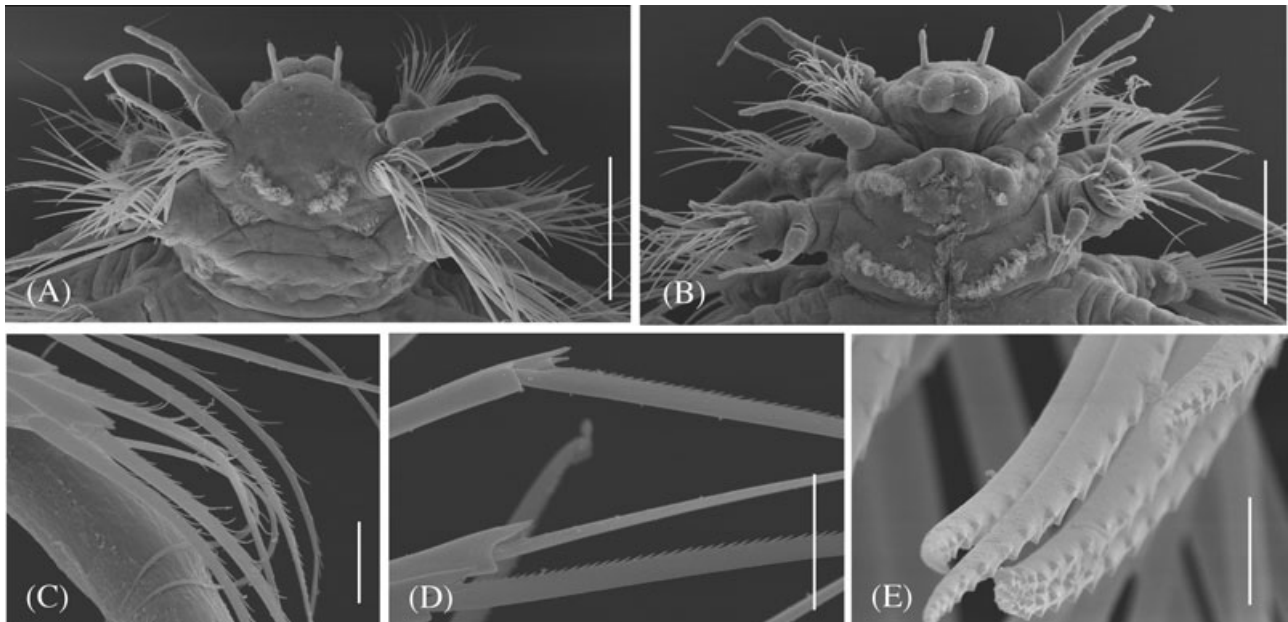

Figure 3. *Vigtorniella ardabilia* sp. nov., specimen from whale-fall in Sweden. SEM micrographs of (A) head region dorsal view, (B) head region ventral view, (C) fine neuropodial falcigers from segment 2, (D) neuropodial falcigers from mid-body region, and (E) notopodial spine. Scale bars in (A) and (B) are 150 μm, in (C) 7.5 μm, in (D) 15 μm and in (E) 6 μm.
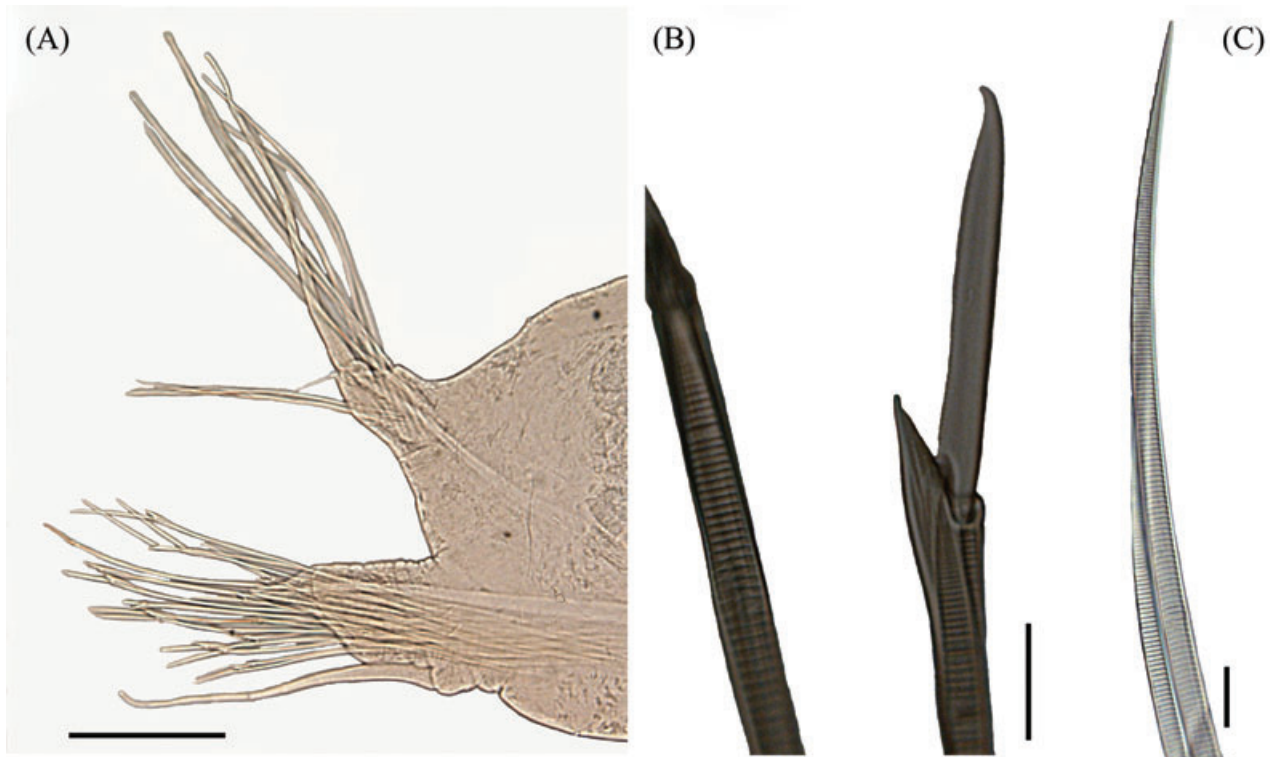
Figure 4. *Vigtorniella ardabilia* sp. nov., specimen from whale-fall in Sweden, light micrographs: (A) parapodia from mid-body region, (B) detail of neuropodial falcigers, (C) detail of notopodial spine. Scale bar in (A) is 100 μm, in (B) and (C) 10 μm.

from experimental tank with bone material sampled with ROV from an implanted Minke whale carcass at 125 m water depth, holotype (SMNHType-7376); same location, two specimens, one female and one male, preserved in formaldehyde, paratypes (NHM2008.367 and 2008.368); same location, seven specimens preserved in formaldehyde, anterior parts of two specimens preserved in osmium for SEM, and several specimens preserved in ethanol for DNA extraction, all in first author's collection. Northern North Atlantic, Svåsand in Hardangerfjord, 84 and 150 m depth, six specimens preserved in ethanol; northern North Atlantic, Mele in Hardangerfjord, 60°21.27′N; 6°20.89′E, 104 m depth, nine specimens preserved in formaldehyde.

Description: Colour pale yellow in females with eggs (Fig. 2), males transparent, both with anterior five to six segments more reddish in live animals. Length up to 32 mm for 82 segments (male specimen). Body shape elongated, tapering slightly at anterior and posterior ends.

Prostomium ventrally displaced, not visible dorsally. Eye-pigment, four orange-red spots in a rectangular or trapezoidal arrangement, only visible in live animals. Median antenna and caruncle absent. Digitiform paired antennae inserted anteroventrally

(Fig. 3A). Palps spherical, partially fused, inserted ventrally directly anterior to mouth (Fig. 3B). Retracted proboscis visible through epidermis, terminating posteriorly in segment 5–6. Jaws absent.

Segment 1 reduced, achaetous with only tentacular dorsal cirri, partially fused with segment 2. Segment 2 with dorsal and ventral tentacular cirri, notopodia, and neuropodia, with chaetae (Fig. 3A, B). From segment 3, notopodia with dorsal cirri inserted distally, neuropodia with ventral cirri inserted basally (Fig. 4A). Neuropodia in mid-body segments longer than notopodia. Notochaetae serrated spines, partly surrounding dorsal cirrus, with very fine serration not visible in light microscopy (Figs 3E, 4A, C). Neurochaetae finely serrated compound falcigers with laddered shafts (serration not visible in the light microscopy picture), the three to four upper neurochaetae with long slender blades, lower blades gradually shorter and wider (Figs 3D, 4A, B). Neurochaetae on segment 2 compound spinigers with thinner and longer serrations compared to more posterior segments (Fig. 3C).

Pygidium with terminal anus and two pygidial cirri, unpaired appendage absent.

Distribution: Known from a whale-fall at 125 m depth in the Skagerrak, North Sea, and also found in

sediment samples taken beneath fish farms in Har-dangerfjord, Norway, at 80–150 m depth.

Etymology: Vigtorniella ardabilia sp. nov. is morpho-logically very similar to the 'carpet-worm' *Vigtorniella flokati*, and is named for the famous Ardabil Persian carpet, recently placed back on display at the Victoria and Albert Museum, London, UK.

Remarks: The species is morphologically almost iden-tical to *V. flokati*, which was recorded in large aggre-gations (up to 8000 m^{-2}) on a deep-sea whale-fall in the Santa Cruz Basin, California. One difference is that the antennae appear to be slightly longer on *V. ardabilia* compared to *V. flokati*; on the former they are as long as the palps, on the latter they are less than half the length of the palps. A further difference is the presence of eyes in live specimens of *V. ard-abilia* which are absent in *V. flokati*. As no live mate-rial of *V. flokati* was available for us to study, we cannot rule out the presence of eye spots in this species. *Vigtorniella flokati* have eggs in segments 6–9 only, whereas in *V. ardabilia* the eggs occur from segment 6 and backward in all segments to the end of the body. *Vigtorniella ardabilia* is different from *V. zaikai* in the presence of an achaetous tentacular segment (segment 1) which is not reported for *V. zaikai*, and in the absence of specialized whip-like chaetae and jaws. The neurochaetal spinigers that in *V. ardabilia* occur on segment 2 are not mentioned in the description of *V. flokati* (Dahlgren *et al.*, 2004) but must have been overlooked by the authors as they are present in their SEM micrograph (fig. 3A in Dahlgren *et al.*, 2004). *Vigtorniella flokati* juveniles were found to have jaws, but jaws were not found in any of the adults. No juveniles of *V. ardabilia* have been found.

POPULATION GENETICS AND SPECIATION

The resulting COI dataset consists of 585 characters of which 318 are variable, and 277 are parsimony-informative. The Kimura 2 Parameter (K2P) nucle-otide distance between *V. flokati* and *V. ardabilia* is 11.7%. Of the 63 substitutions separating *V. flokati* from *V. ardabilia* in the studied COI fragment, one occurs in a second position and six in first positions. Of these substitutions, five result in amino acid changes. The parsimony network constructed with TCS using a 95% connection limit separated the two species into two networks. The three sequenced *V. flokati* specimens all had identical COI-haplotypes, whereas seven different haplotypes were observed in the 14 sequenced *V. ardabilia* specimens from Norway and Sweden (Fig. 5). Within *V. ardabilia*, the most common haplotype group (B) was shared by six individuals, with the remaining haplotypes present in
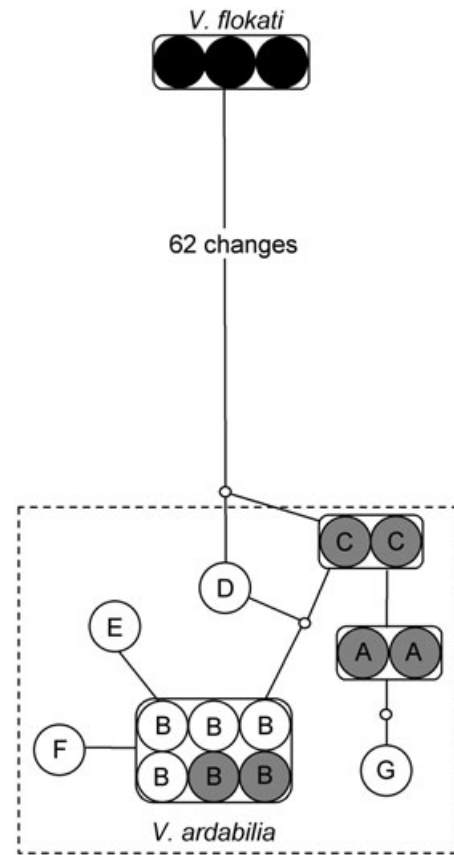

Figure 5. *Vigtorniella* spp. Haplotype network of cyto-chrome *c* oxidase subunit I (COI). Each large circle represents a sequence from an individual of *Vigtorniella ardabilia* that has been collected in Sweden (white) or Norway (grey). Black circles represent *V. flokati*, collected in the Pacific Ocean, three specimens sampled sharing the same haplotype. Letters in circles, A-G, represent haplo-types of *V. ardabilia* sampled in this study (Table 1). Each line represents a mutation and small empty circles are inferred haplotypes not present in the current study.

either one or two individuals (Fig. 5). Two haplotype groups (A, C) were present only in individuals col-lected from the Norwegian fish farm, but the most common haplotype group (B) was present in individu-als from both localities, implying gene-flow between the Norwegian and Swedish populations.

A comparison was made of species pair divergence (K2P distances) for four genetic markers (18S, 28S, 16S, and COI) within four congeneric species pairs within Chrysopetalidae (Fig. 6). Within *Bhawania*, *Palaeonotus*, and *Dysponetus*, the congeneric pairs show distinct morphological differences, whereas within *Vigtorniella*, the speciation is morphologically cryptic. This cryptic speciation process in a high sul-phide habitat has a different distribution of genetic divergence. Whilst the other species pairs follow the
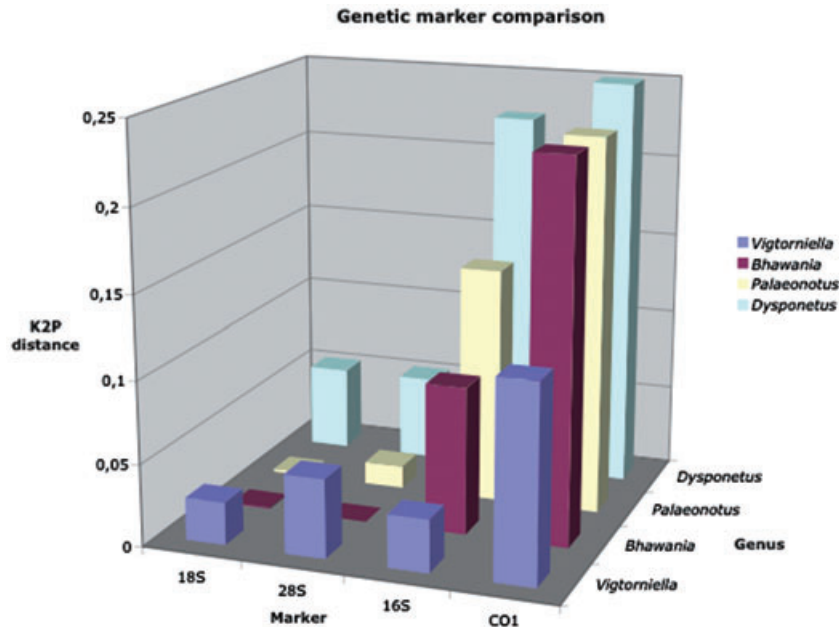
Genetic marker comparison

Figure 6. Diagram showing Kimura 2 Parameter (K2P)-distances between congeneric species in four chrysopetalid genera, for two nuclear genes (18S, 28S) and two mitochondrial genes (16S, cytochrome *c* oxidase subunit I (COI)).

convention of much greater divergence in mitochondrial markers (16S and COI) compared with nuclear markers (18S and 28S), *Vigtorniella* is different. These congeners show much higher nuclear gene distances relative to the other species pairs, with higher nuclear 28S divergence than mitochondrial 16S divergence.

PHYLOGENETIC POSITION

The combined dataset consists of 2917 characters, 810 variable, of which 604 are parsimony-informative. With high levels of support, all our analyses suggest that *V. ardabilia* is a sister taxon to *V. flokati* (Fig. 7A, B). The tree topology from the BA and ML analyses differed only in the position of *Chrysopetalum debile*, which in the ML analysis was sister to *Bhawania*, although with low bootstrap support (not shown). In the BA, *Chrysopetalum* was instead sister to *Paleanotus* (Fig. 7A). Both analyses suggest that the genera *Dysponetus* and *Vigtorniella* are sister taxa, and form a sister group to other chrysopetalid taxa (Fig. 7A). Also in the PA, *Dysponetus* and *Vigtorniella* were sister taxa, while the relationships of the other worms in the Chrysopetalidae group were unresolved (Fig. 7B).

ECOLOGY AND LIFE HISTORY

The ability to culture *V. ardabilia* on aquarium-maintained whale bones has allowed us to make some further observations on feeding and reproductive biology. Live observations indicated that the worms move actively over the surface of the whale bones,

sometimes crawling inside holes in the bone matrix. We found the worms most frequently associated with white bacterial mat, composed of several undescribed species of giant filamentous sulphide-eating bacteria, including *Beggiatoa* (Hans Roey, pers. comm.). The worms were found on the same bones as *Osedax mucofloris* Glover *et al.*, 2005a and several undescribed new species of *Ophryotrocha* Claparède & Mecznikow, 1869 (H. Wiklund, unpubl. data). *Vigtorniella ardabilia* was observed actively feeding on white bacterial mat, using its pharynx to package the mat into gut pellets. On bones that had been heavily infested with *Osedax, V. ardabilia* was able to move inside the soft, burrowed bone tissue, possibly to avoid predators.

Gonochorism was observed, with females containing eggs from segment 6 through to the posterior end of the body and males with spermatids in segments 6–8. Eggs were 40–50 μm in size. Of 15 specimens measured, size ranged from 5 to 35 mm and the number of segments from 28 to 82, with no statistical difference between the size of female vs. male specimens. Eggs were observed in eight of the 15 specimens examined. A comparison with *V. flokati* in size and number of segments showed that there is no size difference between the two species (Fig. 8).

DISCUSSION

One of the central problems in studies of cryptic speciation is the question of the level of morphological
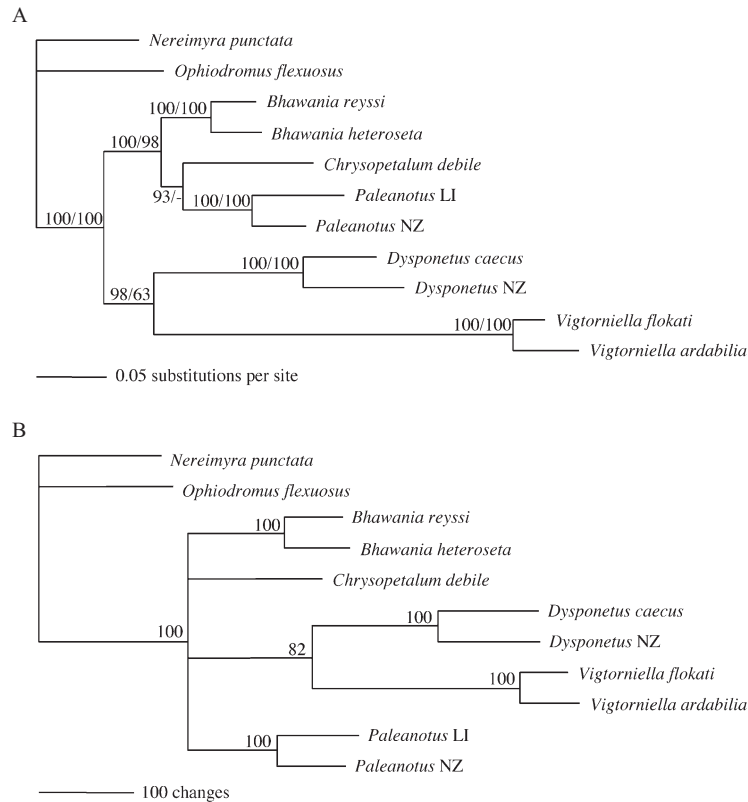
A

B

Figure 7. *Vigtorniella ardabilia*, molecular phylogenetic analyses of four genes with nine chrysopetalid taxa, and two hesionids as outgroup: (A) majority rule consensus tree from the Bayesian analyses with posterior probability /bootstrap values from the analyses in MrBayes/Maximum Likelihood respectively; (B) majority rule consensus tree with bootstrap values from the parsimony analyses in PAUP.
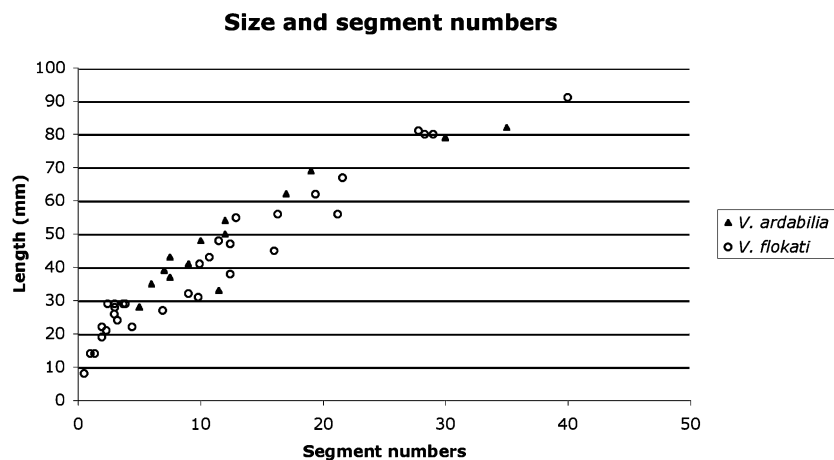

Figure 3. Relationship between the number of chaetigers and total bodylength in 15 specimens of *Vigtorniella ardabilia* and 33 specimens of *V. flokati*.

or genetic divergence that defines a biologically relevant taxon (Blaxter, 2004; Rubinoff, Cameron & Will, 2006). Our analyses suggest that genetically well delineated species found in separate ocean basins at vastly different depths, and with different behaviour patterns can be morphologically similar, even at the level of electron microscopy. Without genetic analyses the species pair in this study would probably have

been described as one cosmopolitan species, thus underestimating biological diversity (Knowlton *et al.*, 1993).

Although the morphological differences between the *Vigtorniella* species are minor, the molecular analyses suggest that the shallow-water Atlantic and deep-sea Pacific *Vigtorniella* belong to different species. Differences of 11.7% in COI and 3.2% in 16S between the two *Vigtorniella* species are comparable with, for example, differences of 5–24.8% in COI among oligochaete congeneric species (Bely & Wray, 2004), 7–14% in COI between sister species of vestimentiferan annelids (Chevaldonné *et al.*, 2002), and from 3% in 16S among congeneric species in *Ophryotrocha* (Dahlgren *et al.*, 2001). Hence, our data suggest that while *Vigtorniella* species may be highly divergent in terms of genetic distance, habitat, depth, feeding ecology, and behaviour, the genus is highly conserved morphologically.

Within the Atlantic species, shared haplotypes between populations from a Norwegian fjord and the whale-fall site in Sweden indicate considerable gene flow at the 100–500 km scale. We hypothesize a stepping-stone model of *V. ardabilia* dispersal along the continental margin, using both whale-falls and/or organic-enriched fish farm benthic habitats. It is possible that *V. ardabilia* may also be able to use other organic-rich habitats; further sampling may reveal this. The distribution of the new species from the Norwegian fish farms is patchy in time, one explanation for this is a possible dynamic ecology of bacterial mat communities, with rapid succession of microbial strains, as has been observed on whale-bones maintained in aquaria (H. Roey, unpubl. data).

The first described *Vigtorniella* species, *V. zaikai*, was recorded in sulphide-rich sediment at around 120–150 m depth close to the oxic–anoxic boundary of the Black Sea (Sergeeva *et al.*, 1997). *Vigtorniella flokati* was discovered at a depth of 1600 m on the bones of a Grey whale skeleton in the North Pacific Ocean (Dahlgren *et al.*, 2004). The third species of this genus, *V. ardabilia*, has now been described from the bones of a Minke whale-fall at 125 m and at depths below 80 m in sulphidic sediments in a Norwegian fjord. To date, *Vigtorniella* has not been recorded on the pilot whale-fall at the Swedish experimental site in Kosterfjord, despite abundant bacterial mat and the presence of *Osedax mucofloris* on this whale (Dahlgren *et al.*, 2006). The Pilot whale carcass was sunk at 30 m depth, suggesting that the new species might be limited in distribution to depths below the thermocline.

The remarkable distribution of the *Vigtorniella* clade lends support to the theory that *Vigtorniella* is neither a sediment-dwelling or a hard-substrate bone-dwelling genus, but is in fact dependent on a readily available supply of bacterial mat, which is known to form both at the sediment–water interface and on hard substrates (Teske *et al.*, 2000). This could be an explanation to the findings of the same species beneath fish farms, as bacterial mats consisting of *Beggiatoa* spp. sometimes cover the sediments beneath fish cultures (Holmer & Kristensen, 1996). Observations of feeding in live *V. ardabilia* maintained on whale bones show them actively feeding on giant filamentous bacterial mat, although they may also be taking up other organic compounds that are not visible to the naked eye. However, *V. flokati* was not observed feeding on bacterial mat at the deep Pacific whale-fall. This may be a result of the limited resolution of the deep-sea submersible cameras. Another possibility is that *V. flokati* is using a very different feeding strategy, utilizing dissolved organic matter leaking directly from the bones themselves (Dahlgren *et al.*, 2004). Contributing to this mystery is the complete absence of *V. flokati* from the deep Pacific whale-falls in subsequent sampling expeditions when bacterial mat growth was much more intense (Glover *et al.*, 2005a), which is the opposite of what we might expect if all *Vigtorniella* were grazers on bacterial mat. At that time point, the dominant bacterial mat grazer was the polynoid polychaete *Bathykurila guaymasensis*, which may have outcompeted other grazers.

Sequence data using the COI barcode gene from an organism may indicate if it is a new species or not, but are less useful for putting that species into a meaningful evolutionary framework (Rubinoff *et al.*, 2006). In all three of our molecular analyses using a combined four-gene dataset, *Vigtorniella* was sister taxa to *Dysponetus*, and in the BA and ML, these two genera form a group apart from the other chrysopetalids (Fig. 7A), whereas in the PA, the position of the taxa within Chrysopetalidae is unresolved (Fig. 7B). That *Vigtorniella* is morphologically similar to *Dysponetus* was noted by Kiseleva (1992) when she described the first *Vigtorniella* species (Kiseleva, 1992). *Vigtorniella* and *Dysponetus* share morphological similarities, such as a reduction in cephalic specialization, and smaller, cylindrical notochaetae that do not form a dorsal cover to the worm, as in other chrysopetalid taxa. *Dysponetus* is abundant in a wide range of deep-sea sediment samples from both the North Atlantic and Pacific (A.G. Glover, unpubl. data). One possible evolutionary pathway for *Vigtorniella* is from sediment dwelling *Dysponetus*-like forms to organic-enriched sediments, to whale-falls. This would contrast with the evolutionary pathway for some other whale-fall specialists, which appear to be more closely related to hydrothermal vent or seep specialists (Smith & Baco, 2003; Jones *et al.*, 2006).

In their study of hydrothermal vent worms, Chevaldonné *et al.* (2002) used an inferred time of divergence to estimate substitution rates for COI for pairs of polychaete species. They suggested an average substitution rate of 0.2% per million years (Myr) for their hydrothermal vent polychaete species (Chevaldonné *et al.*, 2002). Using their substitution rate, a calculated time of divergence for the two sister species of *Vigtorniella* is 29 Mya, which is more recent than that estimated for *Osedax* (42 Mya) (Rouse *et al.*, 2004), but within the range of the evolution of large whales. However, given that *Vigtorniella* is not a whale-fall endemic, it may not have co-evolved with whales in the way that has been proposed for *Osedax* (Rouse *et al.*, 2004; Glover *et al.*, 2005b; Fujikura, Fujiwara & Kawato, 2006).

In contrast to the other congeneric species pairs investigated, genetic divergence in the *Vigtorniella* sibling pair diverges from the expected result of a tenfold higher rate at mitochondrial loci compared to nuclear loci (Brown, George & Wilson, 1979). Although the *Vigtorniella* species pairs were the most similar morphologically, they showed a high level of nuclear DNA divergence (Fig. 6), and their levels of mitochondrial DNA divergence were anomalously low given those rates of nuclear divergence. This lower than expected mitochondrial divergence lends support to the hypothesis that stabilizing selection at mtDNA loci may interfere with the phylogenetic signal in taxa at chemosynthetic environments (Glover *et al.*, 2005a).

ACKNOWLEDGEMENTS

REFERENCES

Allison PA, Smith CR, Kukert H, Deming JW, Bennett BA. 1991. Deep-water taphonomy of vertebrate carcasses – a whale skeleton in the bathyal Santa-Catalina basin. *Paleobiology* **17:** 78–89.

Bely AE, Wray GA. 2004. Molecular phylogeny of naidid worms (Annelida : Clitellata) based on cytochrome oxidase I. *Molecular Phylogenetics and Evolution* **30:** 50–63.

Blaxter ML. 2004. The promise of a DNA taxonomy. *Philosophical Transactions of the Royal Society of London Series B – Biological Sciences* **359:** 669–679.

Brown WM, George M, Wilson AC. 1979. Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences, USA* **76:** 1967–1971.

Chevaldonné P, Jollivet D, Desbruyeres D, Lutz RA, Vrijenhoek RC. 2002. Sister-species of eastern Pacific hydrothermal vent worms (Ampharetidae, Alvinellidae, Vestimentifera) provide new mitochondrial COI clock calibration. *Cahiers De Biologie Marine* **43:** 367–370.

Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9:** 1657–1659.

Cohen BL, Gawthrop A, Cavalier-Smith T. 1998. Molecular phylogeny of brachiopods and phoronids based on nuclear-encoded small subunit ribosomal RNA gene sequences. *Philosophical Transactions of the Royal Society of London Series B – Biological Sciences* **353:** 2039–2061.

Dahlgren TG, Åkesson B, Schander C, Halanych KM, Sundberg P. 2001. Molecular phylogeny of the model annelid *Ophryotrocha*. *Biological Bulletin* **201:** 193–203.

Dahlgren TG, Glover AG, Baco A, Smith CR. 2004. Fauna of whale falls: systematics and ecology of a new polychaete (Annelida : Chrysopetalidae) from the deep Pacific Ocean. *Deep-Sea Research Part I – Oceanographic Research Papers* **51:** 1873–1887.

Dahlgren TG, Lundberg J, Pleijel F, Sundberg P. 2000. Morphological and molecular evidence of the phylogeny of Nereidiform polychaetes (Annelida). *Journal of Zoological Systematics and Evolutionary Research* **38:** 249–253.

Dahlgren TG, Pleijel F. 1995. On the generic allocation of *Chrysopetalum caecum* Langerhans, 1880 (Polychaeta, Chrysopetalidae). *Mitteilungen aus dem Hamburgischen Zoologischen Museum und Institut* **92:** 159–173.

Dahlgren TG, Wiklund H, Källström B, Lundälv T, Smith CR, Glover AG. 2006. A shallow-water whale-fall experiment in the north Atlantic. *Cahiers de Biologie Marine* **47:** 385–389.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3:** 294–299.

Fujikura K, Fujiwara Y, Kawato M. 2006. A new species of *Osedax* (Annelida: Siboglinidae) associated with whale carcasses off Kyushu, Japan. *Zoological Science* **23:** 733–740.

Glasby CJ. 1993. Family revision and cladistic analysis of the Nereidoidea (Polychaeta: Phyllodocida). *Invertebrate Taxonomy* **7:** 1551–1573.

Glover AG, Goetze E, Dahlgren TG, Smith CR. 2005a. Morphology, reproductive biology and genetic structure of the whale-fall and hydrothermal vent specialist, *Bathykurila guaymasensis* Pettibone, 1989 (Annelida: Polynoidae). *Marine Ecology – an Evolutionary Perspective* **26:** 223–234.

Glover AG, Källström B, Smith CR, Dahlgren TG. 2005b. World-wide whale worms? A new species of *Osedax* from the shallow north Atlantic. *Proceedings of the Royal Society B – Biological Sciences* **272:** 2587–2592.

Holmer M, Kristensen E. 1996. Seasonality of sulfate reduction and pore water solutes in a marine fish farm sediment: the importance of temperature and sedimentary organic matter. *Biogeochemistry* **32:** 15–39.

Jones WJ, Won YJ, Maas PAY, Smith PJ, Lutz RA, Vrijenhoek RC. 2006. Evolution of habitat use by deep-sea mussels. *Marine Biology* **148:** 841–851.

Kiseleva MI. 1959. Distribution of larvae of polychaete worms in the plankton of the Black Sea. *Trudy Sevastopol Biologicheskoi Stantsii* **12:** 160–167.

Kiseleva MI. 1992. New genus and species of the family Chrysopetalidae (Polychaeta) from the Black Sea. *Zoologiceskij Zurnal* **72:** 128–132.

Knowlton N, Weigt LA, Solorzano LA, Mills DK, Bermingham E. 1993. Divergence in proteins, mitochondrial-DNA, and reproductive compatibility across the Isthmus of Panama. *Science* **260:** 1629–1632.

Lê HLV, Lecointre G, Perasso R. 1993. A 28S rRNA based phylogeny of the gnathostomes: first steps in the analysis of conflict and congruence with morphologically based cladograms. *Molecular Phylogenetics and Evolution* **2:** 31–51.

Medlin L, Elwood HJ, Stickel S, Sogin ML. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* **71:** 491–499.

Nygren A, Sundberg P. 2003. Phylogeny and evolution of reproductive modes in Autolytinae (Syllidae, Annelida). *Molecular Phylogenetics and Evolution* **29:** 235–249.

Nylander JAA. 2004. *MrModeltest v2*. Program distributed by the author. Uppsala: Uppsala University, Evolutionary Biology Centre.

Palumbi SR. 1996. Nucleic acid II: the polymerase chain reaction. In: Hillis DM, Moritz G, Mable BK, eds. *Molecular systematics*. Sunderland, MA: Sinauer Associates, 205–247.

Perkins T. 1985. *Chrysopetalum, Bhawania* and two new genera of Chrysopetalidae (Polychaeta), principally from Florida. *Proceedings of the Biological Society of Washington* **98:** 856–915.

Pettibone MH. 1993. Polynoid polychaetes associated with a whale skeleton in the bathyal Santa Catalina Basin. *Proceedings of the Biological Society of Washington* **106:** 678–688.

Pleijel F, Dahlgren TG. 1998. Position and delineation of Chrysopetalidae and Hesionidae (Annelida, Polychaeta, Phyllodocida). *Cladistics* **14:** 129–150.

Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Rouse GW, Goffredi SK, Vrijenhoek RC. 2004. *Osedax*: bone-eating marine worms with dwarf males. *Science* **305:** 668–671.

Rubinoff D, Cameron S, Will K. 2006. Are plant DNA barcodes a search for the Holy Grail? *Trends in Ecology & Evolution* **21:** 1–2.

Sergeeva NG, Zaika VE, Kiseleva MI. 1997. Life cycle and ecological demands of larval and adult *Vigtorniella zaikai* Kiseleva 1992 (Chrysopetalidae) in the Black Sea (Abstract). *Bulletin of Marine Science* **60:** 622–623.

Smith CR, Baco AR. 2003. Ecology of whale falls at the deep-sea floor. *Oceanography and Marine Biology* **41:** 311–354.

Smith CR, Kukert H, Wheatcroft RA, Jumars PA, Deming JW. 1989. Vent fauna on whale remains. *Nature* **341:** 27–28.

Swofford DL. 2002. *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*, Version 4. Sunderland, MA: Sinauer Associates.

Teske A, Brinkhoff T, Muyzer G, Moser DP, Rethmeier J, Jannasch HW. 2000. Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. *Applied and Environmental Microbiology* **66:** 3125–3133.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25:** 4876–4882.

Turbeville JM, Field KG, Raff RA. 1992. Phylogenetic position of phylum Nemertini, inferred from 18S-ribosomal-RNA sequences – molecular data as a test of morphological character homology. *Molecular Biology and Evolution* **9:** 235–249.

Van Dover CL, German CR, Speer KG, Parson LM, Vrijenhoek RC. 2002. Evolution and biogeography of deep-sea vent and seep invertebrates. *Science* **295:** 1253–1257.

Watson C. 2001. New genus and species of Chrysopetalidae (Polychaeta) from hydrothermal vents (south-western Pacific). *The Beagle, Records of the Museums and Art Galleries of the Northern Territory* **17:** 57–66.

Watson Russell C. 2000. Family Chrysopetalidae. In: Beesley PL, Ross GB, Glasby CJ, eds. *Polychaetes and allies: the southern synthesis*. Melbourne: CSIRO Publishing, 121–125.