Pseudo-cryptic species *Arenicola defodiens* and *Arenicola marina* (Polychaeta: Arenicolidae) in Wadden Sea, North Sea and Skagerrak: Morphological and molecular variation

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ABSTRACT

The polychaete *Arenicola defodiens,* or black lug, was recently described as a morphologically highly similar species alongside the blow lug *Arenicola marina*. So far it was only known from the British Isles. A double spawning peak was observed earlier in lugworms of the western Wadden Sea. Here, we test the hypothesis that the two spawning peaks represent the two species in sympatry. This hypothesis is refuted on the basis of both morphological and mitochondrial DNA data; both spawning peaks are attributed to *A. marina*. In spite of this, we confirm, on the basis of new collections as well as re-examination of museum collections, the presence of *A. defodiens* in the western Wadden Sea, North Sea and also the Skagerrak; its distribution is restricted to habitats which are either submerged or have short emersion times, have relatively coarse sediments and high and stable salinities. Sympatry is common. The species differ strongly in the mitochondrial DNA fragment examined; the observed 14% uncorrected minimum difference amounts to an estimated 33–63 million years since sequence divergence. The amount of intraspecific molecular variation is larger for *A. defodiens* than for *A. marina*. This is evidence to suggest that *A. marina* may have undergone more recent and/or more severe population size bottlenecks.

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1. Introduction

Lugworms *Arenicola* spp. are polychaete worms that inhabit U-shaped burrows in soft marine sediments. They form a central component of the intertidal benthic community of estuarine and coastal habitats (Beukema and De Vlas, 1979; De Vlas 1979). Already in 1898, Gamble and Ashworth (1898) reported on the existence of two varieties of lugworm, one 'littoral' and one 'laminarian'. However, this distinction was lost in subsequent literature and it was not until recently that genetic characterisations (Cadman and Nelson-Smith 1990) and detailed morphological examinations (Cadman and Nelson-Smith 1993) confirmed the species status of *Arenicola defodiens* or black lug alongside *Arenicola marina* (L.) or blow lug (Cadman and Nelson-Smith, 1993). *A. marina* is a long lived species with separate sexes that reproduce every year for multiple years (Watson et al. 1998; 2008) and the species are in general largely congruent in ecology and physiology (Cadman and Nelson-Smith 1993).

Pseudo-cryptic species are species that have been morphologically recognized as such only after other methods have unveiled their existence (Knowlton 1993; 2000). They are common within all major marine taxonomic groups (Knowlton 1993; 2000; Saez et al. 2003; Goetze 2003). With the advance of DNA sequencing methods, it has become progressively easy to detect cryptic species and hence the frequency of their discovery has rapidly increased (Knowlton 2000; Bickford et al. 2007). The complications associated with unknowingly lumping cryptic species during the study of biological systems are numerous and include: (1) difficulties in interpretation of long-term data series concerning population development, and (2) misunderstanding of apparent variation in ecology and physiology of such taxa. Here, we examine the presence of the pseudo-cryptic lugworm species pair *A. marina* and *A. defodiens* in the western Wadden Sea in order to incorporate this knowledge into future ecosystem studies.

Subtle differences between the species have been recorded for a range of aspects. The adult morphology differs in the number of subsegments of the second anterior segment, which is three in *A. marina* and two in *A. defodiens*, as well as in a number of other characters (Cadman and Nelson-Smith 1993). However, these differences are subtle enough to allow easy confusion without the help of a binocular microscope. The habitat where the species are found differs, but also overlaps: around the British Isles, *A. marina* is reported from estuaries and tidal sediments, while *A. defodiens* inhabits more exposed and also subtidal habitats (Cadman 1997). Fully grown oocytes are larger in *A. marina* (173 μm on average) than in *A. defodiens* (159 μm on average)

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(Watson et al. 1998). A difference in hormonal control of gamete maturation and spawning demonstrates disparity in physiology (Watson et al. 1998; 2008). Finally, the timing of spawning appears to be 1–2 months later in *A. defodiens* than in *A. marina* (Watson et al. 1998).

In addition to the difference in timing of spawning between the species, the blow lug *A. marina* varies markedly in reproductive timing between populations as well as between years. This counts for both spawning season timing, which may fall anywhere from early to late autumn, and for spawning season length, which ranges from a few days to 2–3 weeks (Watson et al. 2000).

The fact that all black lug studies to date were based on populations from British waters calls for examination of *A. defodiens* from different geographical regions. The present study was inspired primarily by the differences between the species in Britain in reproductive timing and oocyte diameter, as two spawning peaks have been reported for western Wadden Sea lugworms during the time before *A. defodiens* was recognized (Farke and Berghuis 1979; De Wilde and Berghuis 1979): an early spawning peak in September associated with fully grown oocytes of about 170–180 μm diameter and a second one in early November when fully grown oocytes were about 160–170 μm. These earlier observations led us to hypothesize that the two spawning peaks correspond to spawning of sympatric populations of *A. marina* and *A. defodiens*.

The genetic, allozyme data available for lugworms suggest that the species have been separated for quite some time and do not interbreed (Cadman and Nelson-Smith 1990). In order to estimate the timing of speciation, it is necessary to obtain DNA sequence data. Additional reasons for supplementing the allozyme data with DNA data are that allozymes may be influenced by natural selection (e.g., Powers et al. 1979; Karl and Avise 1992; Riginos et al. 2002) and originate from loci in the nuclear genome; the mitochondrial DNA gives independent information on evolutionary history. Also, a PCR-based species identification assay, which in contrast to allozymes can be run in most modern laboratories, would be helpful for identifying the species status of juvenile lugworms as well as damaged samples or samples that are otherwise difficult to identify by morphology.

The goals of the present study were four-fold: first, to examine whether the two *Arenicola* spp. spawning peaks in the western Wadden Sea correspond to the two species; second, to supplement knowledge on the genetic distinction between the species with mitochondrial DNA sequence data; third, to confirm the presence of *A. defodiens* in a wider range of European coastal marine habitats, using both morphological and molecular identification; and fourth and finally, to tentatively estimate time since speciation.

2. Materials and methods

2.1. Samples

Live *Arenicola* spp. were collected from emerged sediment at low tide by digging (with a fork), and from subtidal sediments by dredging using a box corer or by scuba diving. Tissue for DNA analysis was taken from the tail of the animals and stored at −20 ºC in pure ethanol until further processing. Tissue samples were also taken from individuals in several Dutch museum collections (Zoological Museum of Amsterdam (ZMA), the Naturalis National Museum of Natural History (RMNH) in Leiden, and the Natural History Museum (NMR) in Rotterdam) as well as a private collection (H. G. Hansson, University of Gothenburg, Sweden) and stored in the same way. Full sampling details are listed in Table 1, and a geographical overview of sampling locations is depicted in Fig. 1.

Morphological species identity was assessed under a binocular microscope on the basis of annulation patterns and gill morphology, following Cadman and Nelson-Smith (1993). They described that the number of subsegments of the second segment of the anterior end is three in *A. marina* and two in *A. defodiens*, and that the gills of the former are dendritic while those of the latter are pinnate. Animals collected alive were sedated with alcohol prior to binocular microscope examination.

Part of the samples (40 from Mok and 40 from Schorren) were examined for reproductive status and gender. The animals were dissected live under a binocular microscope and the presence of male or female sexual products in the coelomic cavity was recorded. In order to be able to assess whether early and late spawning periods in the western Wadden Sea correspond to the two different species, lugworms were collected on two tidal flats in the middle of autumn of 2006 (see Table 1), their reproductive status examined and their

Table 1
Samples of *Arenicola* spp.

Location	Coordinates	Date	Emersion	Morphological identification		Molecular identification		Sediment (M)
				A. mar.	A. def.	A. mar.	A. def.	
1. Terneuzen, NL	51°20.746′N3°47.742′E	9 May 2008	High intertidal	18	–	6	–	Sandy mud (162 μm[a])
2. Delta, NL		4 July 1915	Intertidal	2	–	–	–	
3. North Sea, NL		1905–1993	Washed ashore / subtidal	2	26[b,c]	–	1	
4. Huisduinen, NL	52°56.875′N4°43.040′E	17 Apr. and 8 Jul. 2007	Low intertidal	4	1	4	1	Sand
5. Malzwin, NL	52°58.918′N4°54.188′E	2, 16 Apr. 2007 and 7 Feb. 2008	Never	2	1	2	–	Sand (240 μm[d])
6. Wadden Sea, NL		1937–1994	Intertidal	17[c]	–	–	–	
7. Molenrak, NL	53°0.955′N4°41.755′E	22 Apr. 2008	Never	–	1	–	1	Sand
8. Mok, NL	53°00.472′N4°45.652′E	3 Oct. 2006	High intertidal	40	–	6	–	Mud
9. Texel beach, NL	53°6.715′N4°45.865′E	24 Feb. 2008	Washed ashore	–	1	–	1	
10. Schorren, NL	53°7.005′N4°54.045′E	3 Oct. 2006	High intertidal	40	–	6	–	Mud
11. Eierlandse Gat, NL	53°8.453′N4°58.310′E	1 Dec. 2006	Low intertidal	1	7	1	7	Sandy mud
12. Slufter, NL	53°8.470′N4°47.858′E	11 Jun. 2007	Mid intertidal	–	–	8	–	Sandy mud
13. Saltö, SE	58°52.380′N11°8.811′E	29 May 2007	Occasionally (very shallow)	24[e]	–	6[e]	–	Mud
14. Lindholmen, SE	58°53.444′N11°7.533′E	18 and 20 May 2007	Occasionally (very shallow)	22[c]	–	6[c]	–	Sandy mud
15. Koster Islands, SE		Jul. 1994	Never (10 m deep)	–	1	–	–	

In italics: summary of samples obtained from museum or private collections. For detailed list of museum samples see Supplementary Materials. 'NL' = the Netherlands; 'SE' = Sweden; M = median grain size. Emersion times are from Zwarts et al. (2004) or given in general categories.

  [a] Provided by S. Santos, NIOZ (unpublished data from 2009).
  [b] Includes three individuals with deviating annulation patterns (twice 2-2-3-4 and once 2-2-2-4).
  [c] Includes one individual with annulation pattern cf. *A. defodiens* (2-2-4-4) and overall *A. marina*-like morphology.
  [d] Own unpublished data.
  [e] Includes two individuals with annulation pattern cf. *A. defodiens* (2-2-4-4) and overall *A. marina*-like morphology.
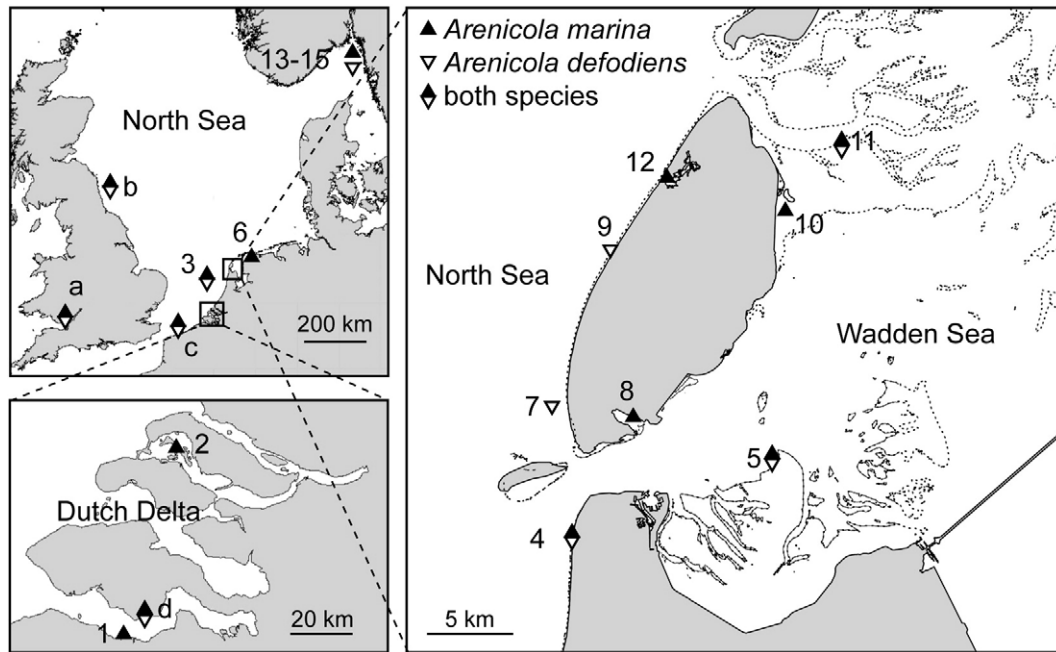
Fig. 1. Distribution of *Arenicola defodiens* and *A. marina* identified by molecular and morphological methods in this study. Open triangle = *A. defodiens*; closed triangle = *A. marina*. Numbers identify samples and correspond to those in Table 1. Also included is an overview of known *A. defodiens* distribution to date: a) South Wales (Cadman 1997); b) Northumberland, UK (Watson et al. 1998); c) Belgian North Sea coast and the Somme estuary, France (Müller 2004; F. Kerckhof pers. comm.); and d) Westerschelde, the Netherlands (Sistermans et al. 2006). Dotted line in right panel indicates lower low water spring or −110 cm Dutch Ordnance Level (NAP).

DNA compared. Those still containing gametes were considered as belonging to the late spawning group and those already empty to the early spawning group (*cf.* De Wilde and Berghuis 1979).

2.2. Molecular analysis

DNA was extracted using the GenElute Mammalian Genomic DNA kit (Sigma) following the manufacturer's protocol. A portion of the cytochrome-*c*-oxidase I gene was amplified using the primers LCO1490 and HCO2198 (Folmer et al. 1994). DNA sequencing was carried out on an ABI3730xl genetic analyzer in either forward or both directions. Sequence data were obtained for a total of 56 individuals. Sequences were aligned by eye using the program BioEdit (Hall 1999) and trimmed to a final length of 637 bp.

2.3. Data analysis

A minimum spanning network was created (using the software Arlequin; Excoffier et al. 2005) for the haplotypes detected. Molecular diversity was compared between species on the basis of nucleotide diversity (Tajima 1983). Mismatch distributions were compared between species to gauge their demographical population histories, comparing observed distributions with those expected under 1) a model of constant population size; and 2) a model of sudden population size expansion. The latter was performed using the program DnaSP (Rozas et al. 2003).

Time since speciation was estimated based on a molecular clock of 0.4% sequence divergence per million years as estimated by Chevaldonné et al. (2002), who used the K2P (Kimura-two-parameter; Kimura 1980) model of sequence evolution. For comparability, the K2P model was applied here, also. The net between group distance, which subtracts average within-group distance from average between group distance and can thus be used for estimating group splitting times (Nei 1987) was used as a measure of the amount of divergence since speciation.

3. Results

3.1. Early versus late spawning groups

Early and late spawning *Arenicola* spp. as recorded by De Wilde and Berghuis (1979) were detected on both tidal flats visited on 3 October 2006, as evidenced by the presence of both spent and non-spent individuals (Fig. 2). The fraction of early spawners differed significantly between the tidal flats: 57% at Mok and 78% at Schorren ($P = 0.0027$, Fisher's exact test). Morphological examination of all 80 individuals suggested that all belonged to the species *A. marina*. DNA sequencing of the COI gene of twelve of these individuals (three spent, two females and one male sequenced for both tidal flats) confirmed that both early and late spawners are *A. marina*.

3.2. Molecular characterization

DNA sequence data for the 637 bp portion of the mitochondrial COI gene were obtained for 56 individuals (Genbank accession numbers
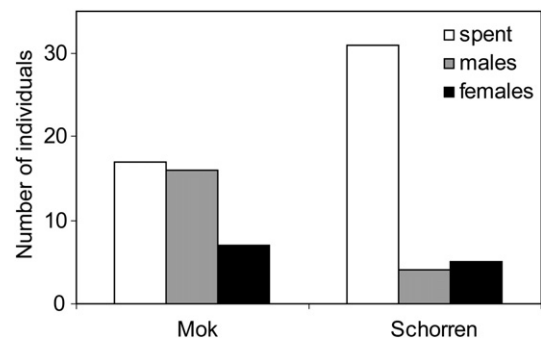

Fig. 2. Reproductive status of *Arenicola* spp. in mid autumn on two western Wadden Sea tidal flats 'Mok' ($N = 40$) and 'Schorren' ($N = 40$). 'Spent' are individuals without gametes; 'males' are individuals containing spermatocytes; 'females' are individuals containing oocytes.

GQ487319–GQ487325). The sequences formed two distinct groups, which conformed to the morphological species identification, although morphological identification was more difficult in some cases due to some degree of variation in the diagnostic characters (see Section 3.3). In this way, morphological and molecular data were compared for 45 *A. marina* and 11 *A. defodiens* originating from a range of locations in the Westerschelde, North Sea, Wadden Sea and Skagerrak (Fig. 1).

Amplification and sequencing of the fragment in museum specimens were tested for nine individuals, stored on ethanol between 1905 and 1994. This was successful only in one case: an *A. defodiens* washed ashore and collected from the North Sea beach at Hoek van Holland in 1993 (see Supplementary Material).

Among the total of 56 individuals sequenced, seven haplotypes were detected. The nature of the molecular variation is summarized in Table 2. While the majority of all substitutions (97) were silent substitutions, two replacement substitutions were found: one within *A. marina* (valine to phenylalanine) and one between the species (alanine to serine). The minimum spanning network (Fig. 3) shows that variability in *A. marina* consists of a common haplotype with two rarer variants. In *A. defodiens*, in contrast, four more or less common haplotypes were found, one of which differs by four substitutions from the rest. The smallest distance between the taxa is 89 differences (Fig. 3), which amounts to 14% sequence difference.

The amount of molecular variation within *A. marina* was found to be remarkably limited and smaller than in *A. defodiens*. This can be seen both from the nucleotide diversity π, which amounts to 0.00014 for *A. marina* versus 0.00400 for *A. defodiens* (Table 2), and by eye from the minimum spanning network (Fig. 3).

Mismatch distributions also differed between the species: in *A. marina*, most pairwise comparisons are identical, corresponding to a unimodal distribution, while in *A. defodiens* the mismatch distribution is bimodal (Fig. 4). Neither a model of population growth nor of stable population size could be rejected in either species, as indicated by non-significance of the R2 criterium of Ramos-Onsins and Rozas (2002). However, this test is rather conservative (Ramos-Onsins and Rozas 2002), and R2 was larger in *A. defodiens* (R2 = 0.203; theta/theta0 = 1.234 and tau = 1.312) than in *A. marina* (R2 = 0.103; theta/theta0 = 0.000 and tau = 0.089), which is consistent with a more stable and/or older population size history in the former.

3.3. Morphological variability

One of the characters used to distinguish *A. marina* from *A. defodiens* is the pattern of annulation of the first head segments (Cadman and
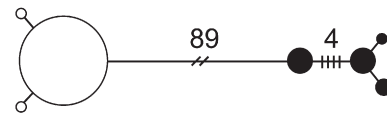

Fig. 3. Minimum spanning network for 637 basepair fragment of the mitochondrial cytochrome-*c*-oxidase I gene in *Arenicola marina* (in white) and *A. defodiens* (in black). Each circle represents a haplotype; size of circle is representative of frequency of observation. Distance between haplotypes is one substitution unless otherwise indicated.

Nelson-Smith 1993). In *A. marina* the first, anterior, segment has two annuli, the second one three, and the following ones all have four annuli (2-3-4-4). In *A. defodiens* the anterior segment has two annuli, the second also has two, and the following ones four (2-2-4-4). In preserved material this is by far the easiest characteristic to verify. In most specimens, the annulation corresponded with the other external characteristics of the animals. Five samples (see Table 1 and Supplementary Material) stood out in this respect.

First, a sample from the North Sea beach near Katwijk (NMR 9910-00303) consisted of a total of seven specimens, all with the external appearance of *A. defodiens*, i.e. first two segments with 2 annuli. In two of these specimens the third segment bore three annuli (2-2-3-4), and in one specimen this segment bore only two annuli (2-2-2-4). In all seven specimens the fourth and other segments bore four annuli.

Second, a sample from the North Sea off Texel (RMNH An 2070) consisted of a total of three specimens with external *A. defodiens* appearance, two with the regular 2-2-4-4 sequence of annuli, while one had only two annuli in the third segment (2-2-2-4), similar as described above for the Katwijk sample.

Third, a sample from a relatively high intertidal flat near Den Helder (Balgzand, 't Kuitje) (collection ZMA) consisted of three
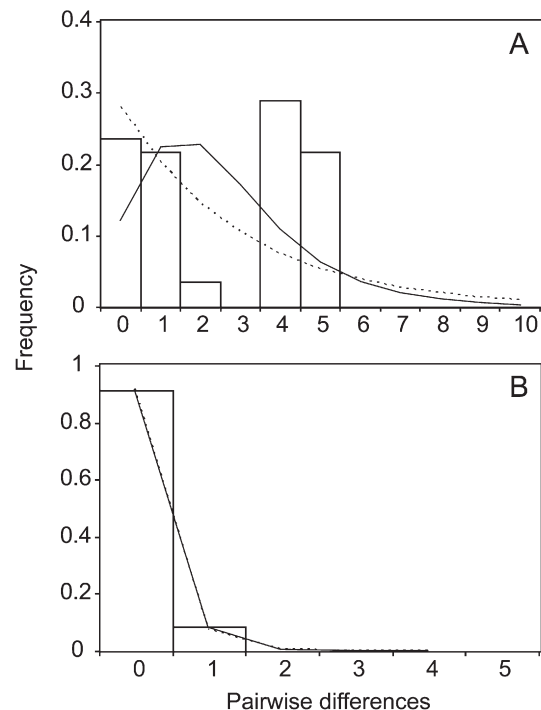
Table 2
Summary of molecular characteristics in 637 basepair fragment of mitochondrial gene cytochrome-*c*-oxidase 1 in *Arenicola marina* and *A. defodiens*.

	A. marina	*A. defodiens*	Interspecific
No. of sequenced individuals	45	11	
No. of haplotypes	3	4	
No. of polymorphic sites	2	6	89
No. of transitions	0	6	50
No. of transversions	2	0	39
No. of silent substitutions	1	6	49
No. of replacement substitutions	1[a]	0	1[b]
C:T:A:G(%)	23:33:28:16	26:30:26:18	
Haplotypes (no. of observed)	h01(43)	h04(4)	
	h02(1)	h05(4)	
	h03(1)	h06(1)	
		h07(2)	
Nucleotide diversity π	0.000140	0.003996	
(SD)	(0.000284)	(0.002620)	

'SD' = standard deviation.
[a] Nucleotide position 349, substitution from Valine to Phenylalanine.
[b] Nucleotide position 58, substitution from Alanine to Serine.

Fig. 4. Mismatch distributions for A) *Arenicola defodiens* and B) *A. marina*. Pairwise difference indicates the number of different nucleotides between a pair of DNA sequences; distribution is among all possible pairwise comparisons within a set of sequences. Bars indicate observed values, lines signify expected distributions under models of constant population size (dashed line) and sudden population expansion (solid line) according to models referred to in main text.

specimens with overall external appearance of *A. marina*. Two of them had the characteristic annulation pattern of *A. marina* (2-3-4-4), while the third specimen bore the *A. defodiens* pattern (2-2-4-4). Molecular identification of the latter specimen was not possible due to its poorly conserved DNA.

Finally, two samples from western Sweden included, in low frequency, three individuals with *A. defodiens* annulation (2-2-4-4) but with an overall appearance of *A. marina*, similar to the individual from Den Helder mentioned above. This was the case for two individuals out of 22 at Saltö and for one out of 21 at Lindholmen, both shallow water sites. Molecular identification of these three individuals confirmed their species status as *A. marina*.

3.4. Occurrence of A. defodiens

Using both the morphological and molecular data sets, the occurrence of *A. defodiens* in several Dutch coastal waters was confirmed, mainly in the North Sea (Table 1). Notably, the species is rare in the western Wadden Sea, with only two observations in the more exposed and marine parts of the area (Fig. 1). The species is found in locations where the sediment is relatively course and emersion times are limited (Table 1). These are also areas where salinity is higher and more stable than in areas where *A. marina* is found exclusively.

Examination of museum collections demonstrated that specimens that wash up on the shore after storms are primarily *A. defodiens*; 23 out of a total of 25 museum specimens were identified as *A. defodiens* (Table 1 and Appendix A). Presumably, strong wave action can dislodge them.

The presence of both species in the Swedish Skagerrak area was also confirmed, with *A. marina* inhabiting shallow, episodically exposed sediments (exposure is regulated by air pressure and wind regime rather than tide), while the single *A. defodiens* known from the region was collected at 10 m depth (Table 1).

3.5. Molecular clock

Net K2P (Kimura-two-parameter) genetic distance between *A. marina* and *A. defodiens* was 0.16337. Using a rate of substitution of 0.2% per million years, as calibrated by Chevaldonné et al. (2002) for polychaetes, we arrive at a time since speciation of 40.8 million years ago. The range of substitution rates found by Chevaldonné et al. (2002) is 0.13–0.25% and this yields a range of divergence times of 32.7–62.8 million years ago.

4. Discussion

4.1. Two spawning peaks

The two spawning peaks observed earlier in *Arenicola* spp. in the western Wadden Sea (Farke and Berghuis 1979; De Wilde and Berghuis 1979) could not be attributed to the two species but solely to *A. marina*. This seems to mean that *A. marina* has either two separate spawning moments or a very broad spawning time window in these populations. This adds to the pattern of large interpopulational variability in this taxon with respect to reproduction (Watson et al. 2000); some populations spawn over a shorter time period than others, some spawn earlier in the season than others, and the present study suggests that two spawning peaks within a year within a population may also occur. The latter possibility raises the question whether the different spawning peaks might represent different developmental stages. Alternatively, population subdivision may be present within *A. marina sensu stricto*, as the early and late spawners might be reproductively isolated.

4.2. Black lug in north European coastal waters

Distribution of *A. marina* and *A. defodiens* in Wadden Sea and North Sea coastal waters broadly follows the patterns observed in British waters (Cadman 1997; Watson et al. 1998). The distribution of *A. defodiens* is restricted to high-energy low intertidal and subtidal marine habitats. The distribution of *A. marina* in Dutch waters can be characterized as estuarine and is restricted to predominantly intertidal and sheltered sediments. The difference in habitat between the species, however, is relative, so that sympatric occurrence is quite common (see Fig. 1). Locations where *A. marina* occurs alone are areas with lower and more fluctuating salinities, finer sediments and longer emersion times (Table 1). Sympatry is seen in areas where salinity is high and stable, emersion times are smaller and wave action is larger.

Monitoring surveys mention the occurrence of *A. defodiens* in the Westerschelde (Sistermans et al. 2006), and along the North Sea coasts of Belgium (F. Kerckhof, pers. comm.) and the north of France (Müller 2004; F. Kerckhof, pers. comm.). The Westerschelde *A. defodiens* were collected at the 'Plaat van Baarland', on the edge of an intertidal mudflat; a site where median grain size equaled 100–150 µm (Sistermans et al. 2006), *i.e.*, not particularly coarse. Perhaps not surprisingly, then, it occurred there sympatrically with *A. marina*. The Belgian and French observations of *A. defodiens* were from sandy beaches (F. Kerckhof, pers. comm.).

In the Skagerrak, where near-shore sediment exposure is wind and air pressure driven (unpredictable) rather than driven by the lunar cycle (predictable), *A. marina* was found in shallow water and *A. defodiens* in deeper water (10 m).

4.3. Molecular variation

In this study genetic variation was found to be higher in *A. defodiens* than in *A. marina*. Comparing this to the allozyme data presented earlier (Cadman and Nelson-Smith 1990) it can be seen that allozyme variation, too, seems to be higher overall in *A. defodiens*: average gene diversity (Nei 1973) equals 0.23 for *A. defodiens* versus 0.17 for *A. marina* (calculated from Table 1 in Cadman and Nelson-Smith 1990, for Jersey Marine samples only and excluding the monomorphic locus superoxide dismutase). Such a difference may be caused by a difference in mutation rate, population size or population history. Population size is not likely to explain the observations; if anything, sensus population size appears to be larger in *A. marina* than in *A. defodiens*, which would predict the opposite pattern of genetic variability. Since there is also no obvious reason why mutation rate should be higher in *A. defodiens*, the observed lower level of molecular variation in *A. marina* is likely the result of population history. Historical events that can cause this kind of difference are (1) recent or recurring population bottlenecks in *A. marina*, as indicated by the mismatch analysis (Fig. 4), and (2) a history of alternating phases of isolation and reunion in *A. defodiens*. Although one might additionally hypothesize that *A. marina* originated recently from *A. defodiens*, this is not corroborated by the fact that speciation apparently occurred a very long time ago (see below).

Lower levels of genetic variation in a near-shore species than in a close relative with a more offshore distribution have been observed before for the arrow worm species *Sagitta setosa* and *S. elegans* in the North Sea (Peijnenburg et al. 2005). Peijnenburg et al. suggest that the near-shore *S. setosa* was more severely impacted by the Pleistocene ice ages than *S. elegans*. A similar scenario may explain the observations for *Arenicola* spp.; tidal habitats may have been under stronger influence of glaciation than subtidal habitats, leading to stronger population bottlenecks in the near-shore than in the offshore populations, and opportunities for seeking refuge by moving to deeper waters may have been hampered by competition effects with related taxa.

The fact that population genetic structure between Dutch and Swedish waters for *A. marina* was not observed further corroborates a scenario of recolonisation for this taxon following the retreat of the Pleistocene glaciations. Further examination of this scenario awaits a more thorough analysis of geographic population genetic structure for the two lugworm species.

The molecular clock estimation presented in this paper must be viewed with caution. Many problems are associated with such estimates, including possible non-neutrality of mitochondrial DNA, the need to sample multiple loci and rate heterogeneity among taxonomic lineages (Ballard and Whitlock 2004; Pulquerio and Nichols 2007). The estimated time of divergence, 41 (range 33–63) million years ago (MYA), falls within the Eocene epoch (34–55 MYA). The Eocene is the middle epoch of the Paleogene period, a warm, largely ice-free period, during which the modern mammals originated and radiated. The time estimate obtained for the *Arenicola* species pair is thus very old for such a strong morphological similarity. It implies that either both species have remained very similar in appearance, or evolved in parallel. Morphological stasis in extreme environments is one of the ways in which cryptic species originate; there are only a limited number of ways in which an organism can adapt to a harsh environment (Nevo 2001; Bickford et al. 2007). Alternatively, the molecular clock used (0.4% sequence divergence per million years; Chevaldonné et al. 2002) is too slow. If instead we use what is seen as a fast clock for the same gene, the Crustacean COI molecular clock (1.4% divergence per million years; Knowlton and Weigt 1998), we arrive at 11.7 MYA, which is still extremely old; for example, it is millions of years before the onset of the Pleistocene ice ages 1.8 MYA.

4.4. Morphological variation

The present study uncovered more morphological variation in the main diagnostic character (annulation pattern) of *A. defodiens* than observed by Cadman and Nelson-Smith (1993). While 89% of the black lug individuals possessed the standard pattern, *i.e.* i 2 ii 2 iii 4 iv 4 v ... xviii 4 ixx (following notation by Wells 1957) or 2-2-4-4 in short, we encountered 5.4% with a 2-2-3-4 and 5.4% with a 2-2-2-4 pattern. In *A. marina*, we found 2.3% individuals with annulation patterns deviating from the standard pattern, *i.e.* i 2 ii 3 iii 4 iv 4 v ... xviii 4 ixx (following notation by Wells 1957) or 2-3-4-4 in short; all cases of deviation lacked the third annulus on the second segment and thus possessed the (*A. defodiens*-like) 2-2-4-4 pattern. This frequency of absent third annulus in *A. marina* is very similar to that observed by Cadman and Nelson-Smith (1993), which was 2.2%. In conclusion, the possibility of mistaken identification on the basis of annulation patterns alone is not negligible. The combined use of annulation pattern and a molecular identification method would give more reliable results. The large interspecific differences in the mitochondrial DNA fragment presented here can form the basis for a time- and cost-efficient molecular identification method on the basis of restriction enzyme assay of the PCR product.

In conclusion, the morphologically highly similar polychaetes *A. marina* and *A. defodiens* parasympatrically occupy different but overlapping habitats throughout northern European estuaries and shallow offshore sediments. The double spawning peak observed earlier in the western Wadden Sea does not represent the two species but, instead, *A. marina* alone, adding to the large variability in spawning behaviour known for this taxon. Genetic biodiversity is higher in *A. defodiens* than in *A. marina*, which is tentatively suggested here to result from a history of more severe population size reductions in the latter than in the former. Speciation most likely occurred long before the Pleistocene ice ages and the taxa probably (re)colonised European habitats independently before or during that time.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.seares.2009.09.001.

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