Structure at open sea: genetics of zooplankton populations

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General introduction

Structure at open sea?

People generally perceive the open sea as a vast and rather empty space without any obvious barriers or discontinuities. Look for example at the cover of this thesis, what do you think? However, if one were to filter a small amount of this seawater and put it under a microscope, an entirely different world is revealed with a diversity of life forms of exquisite beauty. Moreover, if you were to take samples from different places, you would probably find that each sample has its own unique composition (for some artistic impressions by Pieter and Luttikhuizen see the title pages of this thesis). This is the world of plankton, the organisms that are defined as passively floating, transported by currents they are unable to withstand (Van der Spoel and Heyman 1983). They can be either of plant or animal origin, referred to as phyto- or zooplankton, respectively, and they inhabit the open waters, or pelagic realm. For us, being terrestrial creatures, it is particularly difficult to discern any barriers in such an environment.

Pelagic habitats are the largest on earth. The oceans cover about 70% of the surface of our planet to an average depth of about four kilometres. There is no biotope on land that is comparable in terms of living space. Yet, global pelagic diversity (at the species as well as the ecosystem level) is much lower compared to the terrestrial environment, though at any given place in the ocean, local species diversity may be just as high (Angel 1993, 1997). Indeed, in the pelagic realm, distributions of species and communities are usually extensive, matching patterns of large-scale circulation as characterized by the distribution of water masses, and differentiation and speciation are thought to be slow processes (e.g. Van der Spoel and Pierrot-Bults 1979; Van der Spoel and Heyman 1983). However, recent genetic studies, including the results presented in this thesis, have begun to challenge this long-standing view about the extent and origins of biodiversity in the open sea.

An influential paper published by two Japanese scientists in Nature (Miya and Nishida 1997) revealed that in populations of a tiny and highly
abundant deep-sea fish from all oceans across the globe, considerable genetic variability and localized genetic differences were present. These results showed that within this species, cryptic allopatric lineages can and have split without any obvious barriers, suggesting that oceanic biodiversity may have been seriously underestimated. Later studies have similarly suggested that, generally, genetic diversity in marine (pelagic) species is high, numerous cryptic species are present, and distribution ranges are often far more limited than expected based on their enormous dispersal potential (e.g. Norris 2000; see also introductions to Chapters 1 and 5 of this thesis).

However, the question of how differentiation and speciation processes take place at open sea is still open (e.g. Palumbi 1992, 1994). In fact, even the concept of a population is poorly conceived for pelagic organisms (e.g. Van der Spoel 1994). One reason for this is that we have only just begun looking at the genetic diversity within marine organisms, and especially within planktonic organisms. By studying the genetic trail that successful migrants leave behind, it is possible to indirectly estimate the connectivity of populations, or in other words, to determine the genetic structure. Knowing this will provide insights into the scales at which populations are demographically closed and at which they have been (or are) evolutionarily independent, and thus may evolve, for instance in response to local selection pressures. Moreover, when set into a phylogenetic context, genetic structure can reveal much about a species' demographic and biogeographic history (e.g. Grosberg and Cunningham 2001).

With this thesis, I aim to reveal genetic structure for two important zooplankton groups to define the temporal and spatial scales of pelagic populations and ultimately, to better understand how evolution takes place at open sea. To do this, I have employed two types of genetic markers. First, I used mitochondrial DNA (mtDNA) markers, which are usually haploid, maternally transmitted, and do not recombine. Hence, mtDNA provides a clonally inherited marker that traces maternal lineages, referred to as 'haplotypes'. A major advantage of this type of genetic marker is that it can be analyzed within a phylogenetic framework because ancestor-descendant relationships can be estimated. In this way, it is possible to interpret the geographic distribution of haplotypes in terms of their evolutionary relationships, or in other words, to estimate their 'phylogeography' (Avise et al. 1987). Second, I employed microsatellite markers, which are tandem repeats of 2-10 base pair nucleotide motifs, usually nuclear-encoded, and highly variable. These markers combine the advantages of high polymorphism and multiple independently segregating loci, and are generally analyzed in
terms of the spatial distribution of allele frequencies. I have concentrated on revealing the genetic structure within an important but relatively unknown group of zooplankton, namely chaetognaths, and in particular the chaetognath *Sagitta setosa*. So you may be wondering...

**WHAT ARE CHAETOGNATHS?**

The first description of a chaetognath was that of the Dutch zoologist Martinus Slabber from 1778, which is depicted in Figure 1 and is probably a *S. setosa*. Slabber noticed a head (a) but could not distinguish any eyes, teeth, suckers, or anything of that kind. He also noted that the body was quite stiff, even during swimming movements, and recognized two bodies that looked like ovaries (b) and two thickenings in the region of its tail (c, see Fig. 1). He thought of it as a highly unusual making of a worm and described its movements as very slowly swimming along the glass wall of its container (similar to a snail), but when touched with something, shooting very rapidly like an arrow from a bow. Hence, he named these remarkable sea worms 'Sagitta' or 'arrow', from which their common name 'arrow worm' ('pijkvorm' in Dutch) is derived. Slabber also mentioned that since the day of the 10th of July 1768 when he caught this worm, he had not found any more than two individuals (see also Slabber's original description in Dutch, included in Figure 1).

Nowadays we know that chaetognaths are highly abundant organisms that occur in every marine habitat, from inshore coastal waters to the open ocean and from the surface to the bottom. We also know that they play an ecologically important role in marine foodwebs as the primary predators of copepods; their biomass has been estimated as 10-30 percent of that of copepods in the world oceans (Bone et al. 1991). A closer look at their heads (see for example Figure 1B (Chapter 1) and at the end of Chapter 5 of this thesis) reveals their true character as ferocious predators, with their impressive raptorial armature consisting of several rows of teeth and a set of grasping spines; χατη=’chaete’=bristle or spine and γναθος=’gnathos’= jaw, from which their official name 'Chaetognatha' is derived. Thuesen and co-workers discovered that a tetrodotoxin venom, an extremely potent sodium channel-blocking neurotoxin, is used by chaetognaths to paralyze their prey before ingestion (Thuesen et al. 1988; Thuesen and Kogure 1989; Thuesen 1991). This toxin is produced by bacteria. All bacterial strains that have thus far been isolated from different chaetognath's heads were *Vibrio alginolyticus*. However, the exact location and manner of accumulation of
**DERDE WAARNEEMING VAN EEN ZEE-WORM, GENAAMD SAGITTA OF PTL.**

**FIGURÉ 1.** — The first drawing and description of a chaetognath by the Dutch zoologist Martinus Slabber (1778). This figure consists of a compilation of Slabber’s third observation, that of a ‘sea-worm’ named ‘Sagitta’ or ‘arrow’, his figures 4 and 5 of Plate 6, which are drawings of probably a *Sagitta setosa*, and part of Slabber’s original description in Dutch. Slabber’s figure 4 depicts ‘Sagitta’ at its true size (which measured ~8mm in the original plate) and figure 5 shows it enlarged with (a) denoting its head, (b) showing two bodies that resembled ovaries, and (c) indicating two thickenings. In the original plate the remarkable ‘sea-worm’ was coloured in light-blue. From: *Natuurkundige verlustigingen, behelzende microscopise waarneemingen van in- en uitlandse water- en land-dieren*. J. Bosch, Haarlem. By courtesy of the Plantage Library of the University of Amsterdam.
the toxin is still unknown. Indeed, not the type of creature you would want to run into at night, especially if you were a small copepod!

Chaetognaths are an ancient phylum of bilaterally symmetrical marine animals. The oldest fossil which is probably a chaetognath is the recently reported Eognathacantha ercainella from the lower Cambrian Maotianshan Shale in South China (~520 million years old) by Chen and Huang (2002). Otherwise, the Carboniferous Paucijaculum samamithion from the Mazon Creek fauna in Illinois (United States) has been the oldest definitive fossil chaetognath (~300 million years old; Schram 1973). It seems probable though that chaetognaths evolved at a time when the body-plans of most metazoans were established, around 550 million years ago (Conway-Morris 1987).

Ever since their discovery by Slabber, who mentioned that he had no idea in which group to place the astonishing sea-worms (see title page of this thesis), chaetognaths have remained one of the most isolated phyla in the Animal Kingdom. Even Darwin (1844) spoke of them as 'remarkable for the obscurity of their affinities'. Indeed, the monophyletic character of the group has not been questioned, as chaetognaths have several morphological features that set them apart from all other animals, including a multilayered epithelium, a unique structure of the fins, a retractile hood, and a 'corona ciliata' of unknown function (Bone et al. 1991; Nielsen 2001). However, there has been much discussion about the phylogenetic position of chaetognaths, both in relation to other animal phyla as well as the classification within the phylum itself. One reason for this is that the highly conserved body-plan of chaetognaths leaves very few morphological characters to use for a reliable classification. Compare, for instance, the morphological diversity of chaetognaths from all extant genera in Figure 2, representing the full range of diversity in the phylum, with much more morphologically diverse groups such as arthropods, molluscs, or vertebrates.

Based on morphological and embryological features, chaetognaths have been suggested to be related to almost every metazoan phylum imaginable, including protostomes (e.g. nematodes, molluscs, arthropods, and rotifers) and deuterostomes (e.g. echinoderms and vertebrates; reviewed in Bone et al. 1991; Nielsen 2001). Within the phylum, the morphological conservatism has led to considerable disagreement about the classification as well. Moreover, it is very likely that this conservatism hides numerous cryptic species (Pierrot-Bults 1995). At present, the consensus is that the phylum comprises two orders, the Phragmophora and Aphragmophora. The first order includes three benthic genera and two planktonic genera com-
Figure 2. — An overview of the phylum Chaetognatha with examples of all extant genera and species groups within the genus Sagitta (not drawn to scale, after Bieri 1991 and from Pierrot-Bults 1995).
prising about 40 recorded species. The second order contains the majority of chaetognath species (about 80, all of which are planktonic; Pierrot-Bults 1995). Within this order, the genus *Sagitta*, including almost half of the currently described species, is generally considered to consist of several subgroups of species that are more similar to each other than to other species or species groups within the genus. This has led some authors to raise the genus *Sagitta* to family level (Sagittidae), raising groups within it to the level of genus (Tokioka 1965a, 1965b; Bieri 1991). However, even these studies do not agree about the number and composition of genera within the Sagittidae and this classification has not been widely accepted (Bone *et al.* 1991). In this thesis, therefore, I have not subdivided the genus *Sagitta*. From all of this, it should be clear that this enigmatic group of animals is particularly well-suited to study from a genetic perspective. Molecular analyses of their diversity may resolve relationships with other phyla as well as within the phylum itself, and may uncover morphologically cryptic species.

**Nuclear genomes of chaetognaths**

The first genetic studies of chaetognaths examined variation within their nuclear genomes. Thuesen *et al.* (1993) studied population genetic variation of allozymes (products of alternative alleles of a particular enzyme locus that have different electrophoretic mobilities) within the chaetognaths *Sagitta elegans* and *Eukrohnia hamata* from waters off Japan. This study has remained the only study on chaetognaths with a population genetic focus until the results presented in this thesis. Generally, Thuesen *et al.* (1993) found low levels of genetic variability and little evidence of population genetic structuring.

Later studies concentrated on DNA sequences of the small (18S) and large (28S) subunits of ribosomal genes (rDNA) encoded in the nuclear genome, mostly to resolve the phylogenetic affinities of the Chaetognatha. These studies consistently suggested that chaetognaths are not deuterostomes (Telford and Holland 1993; Wada and Satoh 1994; Halanych 1996, 2004; Giribet *et al.* 2000; Mallat and Winchell 2002). However, chaetognath sequences were always extremely divergent from those of other metazoans and highly skewed in base composition, leading most authors to conclude that their phylogenetic position remained uncertain because of probable artefacts from 'long branch attraction' (grouping of lineages not because of shared characters, but because of degree of divergence from other lineages in the analysis). One study aimed to resolve phylogenetic relationships
within the phylum. This study examined 28S rDNA sequences of 18 species collected from across the globe by different chaetognath specialists (Telford and Holland 1997). An unexpected result was the presence of two distinct classes of 28S rDNA genes which probably arose by a gene duplication event in a common ancestor of extant chaetognaths. Preliminary molecular species phylogenies support the Aphragmophora-Phagmophora division, and also provide evidence for some of the groupings within the genus *Sagitta* (viz. ‘Solidosagitta’, ‘Parasagitta’, and ‘Pseudosagitta’). Furthermore, the authors proposed that relationships within the Aphragmophora are uncertain, reflecting rapid, recent radiation during chaetognath evolution.

In 1986 Catherine Thiriot and Serge Dallot, working at the marine station in Villefranche-sur-Mer (France), studied the chromosomes of some Mediterranean chaetognaths, the results of which were never published (but see Kapp 1991 for one of their photographs). The generous gift of two photographs and a summary of their results by C. Thiriot allows me to present those data here. A diploid chromosome number of $2n=18$ and a haploid chromosome number of $n=9$ were observed in three species, namely *Sagitta enfleta* (Fig. 3A), *S. setosa* (Fig. 3B), and *S. bipunctata*. Although compared to other metazoan groups this number is quite low, this does not imply that chaetognaths have a small nuclear genome. No sex chromosomes, or any other chromosomes that looked unusual, were observed in karyotypes from any of the three chaetognaths (C. Thiriot, personal communication).
In the summer of 2004, three papers were published presenting the first DNA sequences of chaetognath mitochondrial genomes. One of these papers is presented in Chapter 2 of this thesis and concentrates on variation within a single mitochondrial gene to reveal phylogeographic patterns in the planktonic chaetognath *S. setosa*. The other two papers presented whole mitochondrial genome sequences of two closely related benthic chaetognaths, *Paraspadella gotoi* (Helfenbein et al. 2004) and *Spadella cephaloptera* (Papillon et al. 2004), aiming to shed light on the phylogenetic position of the Chaetognatha within the Animal Kingdom. These two studies showed that chaetognaths have highly unusual mitochondrial genomes. They are the smallest yet known from any animal, containing only 14 and 13 genes in *Paraspadella gotoi* and *Spadella cephaloptera*, respectively (Fig. 4), instead of the usual 37 (Boore 1999). In both genomes two protein-coding genes, ATP synthetase subunit 6 (*atp6*) and subunit 8 (*atp8*), were lacking. The absence of *atp6* has never been reported before for any other animal mtDNA. Furthermore, 21 and all of the usual 22 tRNA genes were reported missing from the genomes of *Paraspadella gotoi* and *Spadella cephaloptera*, respectively. These tRNA encoding genes must be located somewhere in chaetognath cells in order to transport amino-acids for protein synthesis. They may be located elsewhere in the mitochondria (possibly encoded by a
second DNA molecule, which is unlikely) or somewhere in the nucleus (Helfenbein et al. 2004). Gene order and the arrangement of genes on the two strands differed between these closely related species, preventing any further generalizations.

Regarding the phylogenetic position of the Chaetognatha, the two mitochondrial data sets support the identification of chaetognaths as protostomes and thus corroborate earlier results based on 18S and 28S rDNA sequences. However, the exact position of chaetognaths relative to the protostomes remains uncertain (see Fig. 5; Telford 2004). Helfenbein et al. (2004) suggested that chaetognaths are the sistergroup of protostomes based on parsimony analysis of amino acid sequences from eight mitochondrial encoded proteins. By contrast, analyses by Papillon et al. (2004) placed chaetognaths within the protostomes, associated with a subgroup that includes molluscs and annelids. However, these authors admitted that their data did not provide strong evidence for this and only concluded that chaetognaths are protostomes. To come back to my original question: what
are chaetognaths? It is clear that this is not so easily answered and will likely remain an active area of research for many years to come.

This thesis - framework and outline
To frame the central question of this thesis - "what are the scales of genetic structuring for zooplankton populations?", we can consider what might be the relevant barriers to dispersal for a planktonic organism. This assumes that geographic separation, or allopatry (Mayr 1963), is the predominate influence on pelagic gene flow (i.e. the movement of genes into, or through, a population by interbreeding, or by migration and interbreeding; Ridley 1993). I use this as a first and central hypothesis in this work, and consider the different signals expected from extant or historical separation. Possible contemporary barriers include, for instance, continental landmasses, tropical waters dividing (disjunct) cold-water populations in Northern and Southern hemispheres, strong and persistent hydrographic features such as fronts, gyral currents, or regions of strong upwelling. Alternatively, historical vicariance events during which entire populations are broken into large subgroups have been hypothesized to play a key role in the evolution of pelagic species (e.g. Van der Spoel et al. 1990; Angel 1997).

Vicariance events are usually associated with either tectonic phenomena or climatic changes and occur on geological time scales. A well-known example of a major tectonic event is the elevation of the Isthmus of Panama which produced progressive barriers to dispersal for deep to shallow to all marine taxa as the landbridge emerged from approximately twelve to three million years ago (Knowlton and Weight 1998). The Pleistocene climatic oscillations resulting in alternating cycles of glaciation and deglaciation with approximate periodicities of between 100,000 and 19,000 years are an example of climatic changes that were shown to have greatly affected the evolution of terrestrial organisms (e.g. Hewitt 2000). However, we still know very little about the relative importance of vicariance events on the evolution and current distributions of marine organisms, especially those living in open waters.

The chaetognath Sagitta setosa provides an ideal study species to examine the impact of both contemporary and historical barriers on differentiation processes in the pelagic realm. First, because it is a holoplanktonic animal, never leaving the open water column during its life time, if there are contemporary barriers across seemingly continuous habitat they are likely to be revealed in S. setosa genetic structuring. Second, because it is a
neritic species, *i.e.* it occurs in waters above continental shelf areas only, it is likely to be particularly responsive to climate induced changes in sea levels. Furthermore, sharp changes are documented in pelagic communities across shelf breaks of continental margins despite present day exchange of water across these 'boundaries'. Apparently, the depth of the sea floor is 'felt' in the overlying water column, preventing neritic species from moving into oceanic waters and *vice versa*. Third, as is often the case for neritic species, the reported distribution pattern of *S. setosa* is disjunct, *i.e.* populations from the NE Atlantic, Mediterranean Sea, and Black Sea are not geographically continuous (see Fig. 1, Chapter 2 of this thesis). This raises the question ‘are holoplanktonic organisms really just drifting with currents they are unable to withstand?’ And if so, the next question is ‘how then are their distribution patterns maintained?’. Fourth, phenotypic differences are observed in *S. setosa*; specimens from the Mediterranean Sea are generally smallest, from the Black Sea largest, and from the North East (NE) Atlantic intermediate in size (examples are shown in Fig. 6). It could be that these differences result from either phenotypic plasticity, *i.e.* without a genetic basis, or *S. setosa* populations may be genetically unconnected leaving the possibility open for adaptive evolution. Fifth, *S. setosa* provides an example of an hypothesized 'glacial relict' in the Mediterranean Sea, *i.e.* a species that supposedly colonized Mediterranean waters during Pleistocene glacial and has since remained. Furthermore, *S. setosa* populations in the Mediterranean and Black Sea basins offer a unique opportunity to study the impact of a series of tectonic events opening and closing sea-ways, resulting in episodes of desiccation and (catastrophic) re-flooding, and fluctuating levels of salinity. Summarizing, applying genetic tools to reveal the quantity and patterns of genetic diversity within *S. setosa* may reveal a great deal about the contemporary and historical barriers that are, or have been, important in a pelagic organism.

This thesis consists of five main chapters presenting the data collected during my PhD research programme. Since each of the chapters resulted from a collaborative effort, I have used the term 'we' in the next section to refer to the different co-authors of each of the chapters.

**Chapter 1** starts by reviewing and comparing the quantitative morphological variation described for *S. setosa* from the NE Atlantic, Mediterranean, and Black Sea, and then continues to compare this variation with morphological differences that have been reported for two closely related, but disputed, taxa. As morphological characters turned out to be
too variable within populations and largely overlapping between populations, we regard these unsuitable to answer questions about the degree of isolation between *S. setosa* populations and thus about their evolutionary status.

Hence, in Chapter 2, mitochondrial DNA sequences are presented that tested whether geographically disjunct populations of *S. setosa* are also genetically disjunct, whether cryptic species are present, and which biogeographic scenario best explains the distribution pattern of this species. As sequences were highly variable and divergent from each other, we analyzed these by taking a phylogenetic approach (using maximum likelihood and Bayesian techniques). Finally, geographic distributions of samples were mapped onto the resulting phylogeny to reveal the species' phylogeography. This was then used to test hypotheses about contemporary barriers to gene flow as well as hypothesized vicariance events resulting from Ice Ages and major tectonic events in the European seas.

Revealing barriers within a planktonic organism is one thing, to show that similar barriers occur in other, unrelated, planktonic taxa as well, is the contribution of Chapter 3 to this thesis. If marine conditions are the primary factor determining dispersal, then planktonic species with similar biogeographic distributions are expected to have shared historical and contemporary patterns of gene flow between populations. The ideal comparative taxa are the copepods *Calanus helgolandicus*, occurring in the NE Atlantic and Mediterranean Sea, and *C. euxinus* from the Black Sea. These closely related species have a similar distribution pattern to *S. setosa*, are probably an important component of *S. setosa*’s diet, and phenotypic differences have been reported that are reminiscent of those observed for *S. setosa* (see Fleminger and Hulsemann 1987). Thus, in this chapter we explore the phylogeography of these copepods, using mitochondrial DNA markers and a coalescent approach to differentiate between contemporary and historical levels of gene flow between populations from the three European basins, and compare the results with those obtained for *S. setosa*.

In Chapter 4 we make use of the fact that mitochondrial sequence data contain information about a species demographic history, often termed a 'genetic signature'. In this chapter, mitochondrial sequences from two highly abundant chaetognaths in the NE Atlantic, *S. elegans* and *S. setosa*, are compared (see Fig. 6 to compare the morphology of the two species). Though the two species are phylogenetically and ecologically quite similar, *S. elegans* has a much wider distribution pattern than *S. setosa*. We contrast these two species' genetic signatures from NE Atlantic populations in order
Figure 6. — Morphological diversity in formalin-fixed samples of *Sagitta setosa* from the NE Atlantic, Mediterranean Sea, and Black Sea (first eight pictures) compared with 'closely related' species *S. friderici* and *S. elegans*, both from the NE
Atlantic (last four pictures). Scale bars are indicated in each picture and correspond to 1 mm. Photographs by J. van Arkel.
to test the prediction that the neritic *S. setosa* has been more vulnerable to population size reductions resulting from climatic fluctuations compared to the more widely distributed *S. elegans*.

Chapter 5 focuses on the question of what (finer) spatial and temporal scales reveal structure among *S. setosa* populations. To be able to detect subtle levels of differentiation, we used a much greater sampling intensity compared to the other chapters in this thesis and developed four nuclear-encoded microsatellite loci. In this chapter, we first test whether phylogeographic patterns are concordant between mitochondrial and nuclear markers. This would indicate that partitions in gene trees accurately demonstrate real barriers to dispersal, mostly at a between-basin scale. Then, we characterize the genetic structure within each of the three basins to test whether contemporary hydrographical features (such as surface current patterns) are a good predictor of the level of population structuring expected within each of these basins.

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