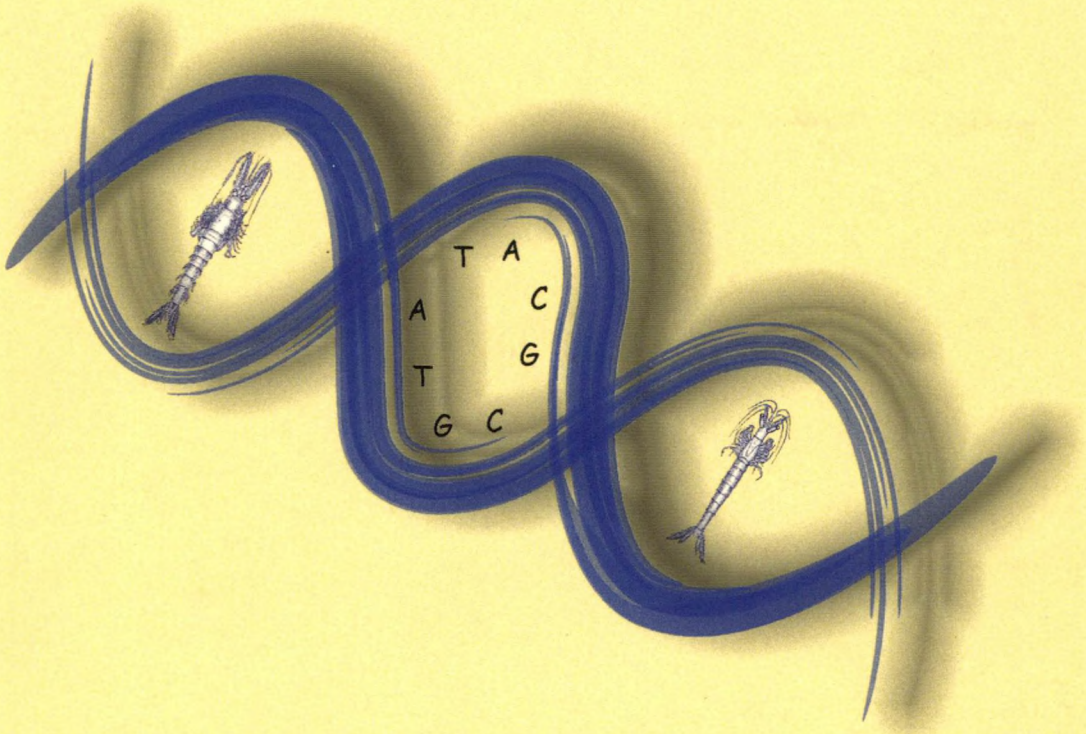
UNIVERSITEIT GENT
Faculteit Wetenschappen

Vakgroep Biologie
Krijgslaan 281-S8, B-9000 Gent, BELGIUM

Molecular diversity and population structure of two mysid taxa along European coasts

Moleculaire diversiteit en populatiestructuur van twee taxa aasgarnalen langsheen Europese kusten



Thomas Remerie

Promotor: Prof. dr. Ann Vanreusel

Academic Year 2004-2005

Rector: Prof. dr. Andreas De Leenheer
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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor in Sciences (Biology)

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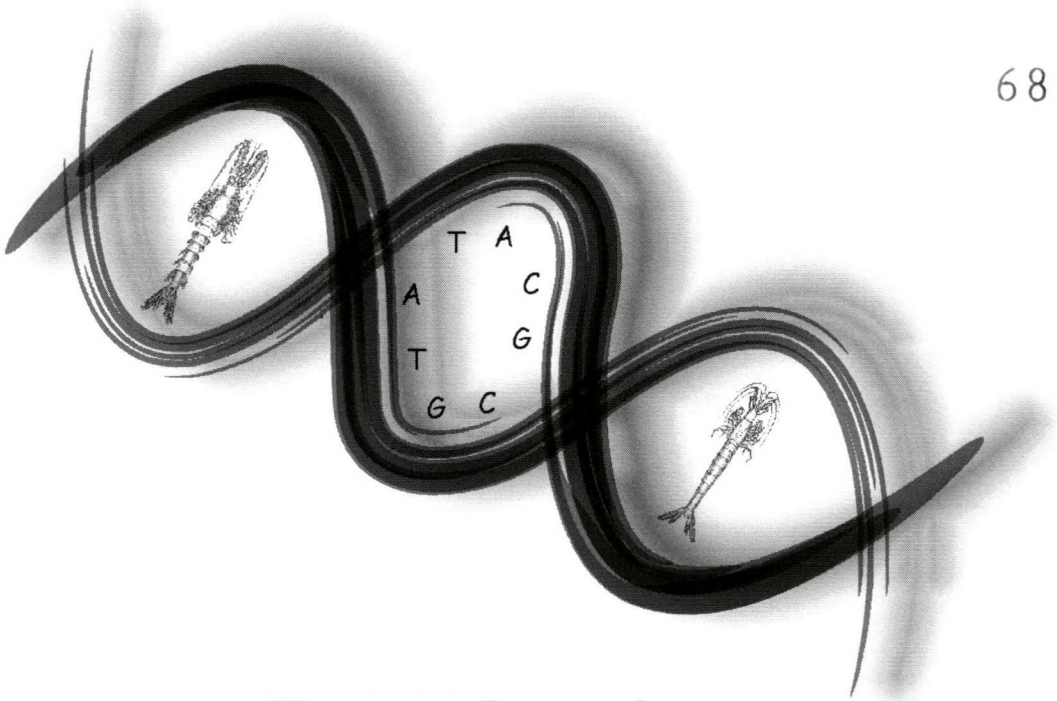

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bovenal is de zee koude soep ...
(Kamagurka)

Promotor:

Prof. Dr. Ann Vanreusel (Universiteit Gent)

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Dr. Peter Weekers (Universiteit Gent)

Prof. Dr. Wim Vyverman (Universiteit Gent)

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Finally! Eindelijk kan ik aan dat laatste stukje tekst beginnen ...
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SUMMARY

The present study deals with the molecular diversity and genetic population structure of two mysid species along the European coast. The overall aim is to provide insights in the levels of molecular genetic diversity, i.e. diversity (variation) at the level of the individual genes, within and between species of the order Mysida (Crustacea, Peracarida). A detailed understanding of the levels of genetic diversity of species is of fundamental importance since the intraspecific genetic variability provides a mechanism for populations to adapt to their ever-changing environment, and hence determines the ecological and evolutionary potential of species. Over the last decades, many marine studies have focused on the spatial patterns of genetic diversity in natural populations, providing evidence of significant genetic differentiation, even in taxa with long-life pelagic larvae and hence high dispersal potential. However, the amount of information on the genetic patterns within marine taxa with poor dispersal abilities, especially along the northeast Atlantic, remains scarce. Given the expected genetic differentiation between populations in poor dispersers and the uncovering of substantial cryptic diversity in morphological identical species in a wide variety of marine taxa, the levels of genetic diversity in poorly dispersing species may have been underestimated.

The present study focuses on mysid species within the species-rich Mysidae family, with in particular emphasis on the European species *Neomysis integer* and *Mesopodopsis slabberi*, two key species in coastal marine and estuarine ecosystems, which occur sympatrically in Northeast Atlantic estuaries. Both species have typical life history characteristics, like brooding behaviour and the absence of free-living larvae, which may result in a low dispersal potential and restricted gene flow between populations. Hence, a study of the phylogeographic patterns of both species throughout their distribution range might contribute to an understanding of molecular patterns within low dispersive marine invertebrate species. Moreover, both species have marked differences in their physiological tolerance and habitat preferences: *N. integer* is a typical brackish water species occurring in (natural) fragmented habitats (e.g. estuaries, brackish lagoons); *M. slabberi* has a broader distribution, occurring in both coastal marine and estuarine habitats, suggesting a more continuous habitat. A comparison of the genetic patterns within both species offers an opportunity to elucidate the importance of several intrinsic (i.e. biological, ecological or behavioural)

and extrinsic (i.e. physical, geological, environmental) factors on the phylogeographic structuring.

In chapter 2 a molecular phylogenetic study within the Mysidae family based on 18S ribosomal RNA sequences is presented. Two of the three subfamilies (Gastrosaccinae and Mysinae) included in this study did not appear to be monophyletic. The split of these subfamilies in different groups ('*Gastrosaccus*' and '*Anchialina*' group in case of the subfamily Gastrosaccinae; and a split of the tribe Mysini, within the subfamily Mysinae, in two groups), as suggested by the present molecular data, is also supported by several morphological differences. Hence, the 18S rRNA based phylogeny urges a taxonomic revision of the speciose Mysidae family.

Chapters 3 and 4 deal with the phylogeographic patterns of the brackish water mysid *N. integer* along the northeast Atlantic coast. First, a baseline study is presented using a limited number of DNA sequences of the mitochondrial cytochrome *b* gene, cyt *b* (Chapter 3). Subsequently a more extended analysis is performed on a total of 461 specimens from 11 sampling sites (mainly estuaries), using Single Stranded Conformation Polymorphism (SSCP) analyses on two fragments of the mitochondrial cytochrome *c* oxidase I gene, COI (Chapter 4). The results of both studies are largely concordant, showing a significant genetic differentiation throughout the distribution range of *N. integer* with a low level of intra-population variability. They corroborate the expectations of the genetic patterns observed in a low dispersal species with brackish water habitats. Different phylogeographic analyses (AMOVA, nested clade analysis, mismatch distributions) point to a complex genetic pattern shaped by the Pleistocene glaciations. The patterns clearly contradict the general expectations according to the current paleoclimatological models for terrestrial and freshwater species. These models predict that taxa inhabiting northern temperate regions were forced to southern latitudes in glacial refugia (the Iberian & Italian peninsula, Balkan region) during glacial periods because of the decreased temperatures. At the end of a glacial period the warming climate and the retreat of the glaciers led to a rapid migration of species out of the refugial areas. These subsequent waves of northward postglacial colonisation and compression of the distribution range during glacial periods generally lead to a reduction in the levels of genetic diversity compared to refugial areas. In addition, the northern populations are less structured than populations inhabiting refugial areas. In the case of *N. integer*, the phylogeographic

analyses of the mitochondrial COI gene pointed to the following contrasting results: (i) no decline in haplotype diversity is detected in formerly glaciated areas, with exception of a decrease at both the northern and southern distribution edge, (ii) the Iberian peninsula did not act as a single glacial refugium, and it seems that these southern refugial populations did not participate in the most recent postglacial range expansion after the last glacial maximum. The existence of multiple (northern) glacial refugia is suggested, probably located in the southern North Sea or English Channel, around the British Isles and in the Bay of Biscay. Moreover, both the COI and cyt *b* analyses show a clear phylogeographic discontinuity at the southern distribution edge, between the Guadalquivir and all other Atlantic populations.

The phylogeographic patterns among Atlantic and Mediterranean populations of *M. slabberi* are analysed by means of DNA sequencing of a 458 bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and a 487 bp fragment of mitochondrial 16S ribosomal RNA gene (Chapter 5). Contrary to *N. integer*, the mean levels of within-population molecular diversity are very high (mean $h = 0.807$ and $\pi = 0.0083$), a common characteristic for many marine species. A very high degree of phylogeographic structuring is apparent, and the COI and 16S phylogenies are resolved in four highly divergent, monophyletic clades (two Mediterranean and two Atlantic clades). The levels of nucleotide divergence between those clades probably exceed the intraspecific level and hence suggest the existence of various cryptic species. Along the northeast Atlantic coast *M. slabberi* shows similarities with *N. integer* in the degree of population structuring at a macrogeographic scale, which could be linked to the absence of free-living larvae in both species. However, on a smaller (meso-) geographical scale less structuring is observed between *M. slabberi* populations compared to *N. integer*, probably due to the higher continuity of available habitats. In contrast, the relatively discrete, natural fragmented estuarine and brackish habitats of *N. integer* results in more 'closed' populations, resulting in limited gene flow even at smaller geographic scales.

Chapter 6 describes the fine-scale (intra-estuarine) and temporal genetic variation, an aspect that has been very often ignored in many large-scale studies, of the brackish water mysid *N. integer* within the Westerschelde estuary. Different samples along an environmental gradient of salinity and pollution, and from different habitats (subtidal, brackish lake and harbour site) are collected in three consecutive

years (representing 9 generations) and analysed with SSCP. Within two years a small, but significant genetic differentiation is observed within the Westerschelde estuary. However, there is no evidence for temporal stability of this genetic structure. Hence, it remains unclear if this is a result of stochastic events, sampling error, or caused by the unpredictable environmental changes, typical for brackish water habitats. Furthermore, the estimates of the effective female population size of *N. integer* are 2 to 3 order of magnitude below the estimates of the census population size. This could indicate that despite their large population size, the populations of *N. integer* may be prone to rapid loss of genetic diversity.

In addition to the genetic analyses of populations of *N. integer* and *M. slabberi*, variation in 12 morphometric and two meristic characters is assessed in three populations each (Chapter 7). Multivariate analysis show clear morphometric differences (related to eye and telson morphology) between populations of both species. The morphological differentiation within *M. slabberi* is highly concordant with the available genetic data from mitochondrial loci, pointing to a large divergence between the Atlantic and Mediterranean populations. However due to some overlap of individuals between the different populations, the present morphometric analysis does not suffice to assign the different populations to a separate species status. In the case of *N. integer*, the largest divergence is observed for the Gironde population. Morphometric differences are mainly related to the eye morphology of *N. integer*. Possible interactions of this morphological character in association with environmental conditions, such as higher turbidity within the Gironde estuary, could be responsible for the observed pattern.

In conclusion, a high degree of differentiation is observed between mysid populations of both species based on several mitochondrial markers, as well as on morphometric characters. These results corroborate the expectations for species with low dispersal capacities (brood protection) and fragmented brackish habitats. The intraspecific molecular patterns show a clear phylogeographic structure, at least for *N. integer* pointing to a complex postglacial recolonisation of northern Europe from multiple glacial refugia. In the case of *M. slabberi*, the mitochondrial DNA analyses suggest to the existence of multiple cryptic species. Finally, the analyses of ribosomal 18S sequences at a higher taxonomic level, within the speciose Mysidae family, prove to be helpful in re-evaluating the morphology-based classification within this family. Based on these results the family Mysidae is in need for a taxonomical revision.

SAMENVATTING

Deze studie behandelt de moleculaire diversiteit en genetische populatiestructuur van twee soorten aasgarnalen langs de Europese kust. Het doel van de studie is inzicht te verstrekken in de graad van moleculair genetische diversiteit, d.i. diversiteit (variatie) op het niveau van individuele genen, binnen en tussen species van de orde Mysida (Crustacea, Peracarida). Deze kennis is van fundamenteel belang: genetische diversiteit zorgt er immers voor dat populaties zich kunnen aanpassen aan hun steeds veranderende omgeving, en bepaalt zo het ecologische en evolutionaire potentieel van soorten. In marien onderzoek is het aantal studies van de ruimtelijke patronen van genetische diversiteit in natuurlijke populaties gedurende de laatste 20 jaar sterk toegenomen. Vele van deze studies leverden bewijs van significante genetische differentiatie, zelfs in taxa met langlevende pelagische larven en bijgevolg een hoog dispersiepotentieel. Over genetische patronen in mariene taxa met een laag dispersiepotentieel is echter relatief weinig geweten. Rekening houdend met de te verwachten genetische differentiatie tussen populaties met een lage dispersiecapaciteit, en de recente ontdekking van een aanzienlijke hoeveelheid cryptische diversiteit in morfologisch identieke soorten bij een groot aantal mariene taxa, zijn de niveaus van genetische diversiteit in deze soorten mogelijks ten zeerste onderschat.

Deze studie handelt over aasgarnalen in de soortenrijke Mysidae familie; met nadruk op de Europese soorten *Neomysis integer* en *Mesopodopsis slabberi*, twee sleutelsoorten in kustgebonden mariene en estuariene ecosystemen. Beide soorten hebben typische karakteristieken in hun levensgeschiedenis, zoals broedgedrag en de afwezigheid van vrijlevende larven, die mogelijks resulteren in een laag dispersiepotentieel en beperkte genenflux (gene flow) tussen populaties. Het bestuderen van de fylogeografische patronen van beide soorten langsheen hun verspreidingsgebied kan bijdragen tot een inzicht in de moleculaire patronen van mariene invertebraten met een lage dispersiecapaciteit. Bovendien vertonen beide soorten duidelijke verschillen in hun fysiologische tolerantie en habitatvoorkeur: *N. integer* is een typische brakwatersoort die voorkomt in (natuurlijk) gefragmenteerde habitats (vb. estuaria, brakwaterlagunes); *M. slabberi* heeft een bredere distributie, voorkomend in zowel kustgebonden mariene als estuariene habitats, wat waarschijnlijk resulteert in een meer continu habitat. Een vergelijking van de

genetische patronen in beide soorten kan het belang van verscheidene intrinsieke (d.i. biologische, ecologische of gedragsgebonden) en extrinsieke (d.i. fysische, geologische, omgevingsgebonden) factoren op de fylogeografische structurering verhelderen.

In hoofdstuk 2 wordt een moleculaire fylogenetische studie binnen de Mysidae familie en gebaseerd op 18S ribosomale RNA sequenties voorgesteld. Twee van de drie onderzochte subfamilies vormden duidelijk geen monofyletische groep (Gastrosaccinae en Mysinae). Verder werd een opsplitsing van deze subfamilies in verschillende groepen (een '*Gastrosaccus*' en '*Anchialina*' group in het geval van de Gastrosaccinae subfamilie; en een splitsing van de tribe Mysini, binnen de Mysinae subfamilie, in twee verschillende groepen) ondersteund door verschillende morfologische kenmerken. Bijgevolg dringt de huidige 18S rRNA fylogenie aan op een taxonomische revisie van de soortenrijke Mysidae familie.

Hoofdstukken 3 en 4 handelen over de fylogeografische patronen van de brakwater-aasgarnaal *N. integer* langs de noordoostelijke Atlantische kust. Eerst werd een basisstudie uitgevoerd op een beperkt aantal DNA-sequenties van het mitochondriaal cytochroom *b* gen, cyt *b* (Hoofdstuk 3). Vervolgens werd een meer gedetailleerde analyse uitgevoerd op een totaal van 461 specimens afkomstig van 11 staalnamepunten (voornamelijk estuaria), gebruik makend van Single Stranded Conformation Polymorphism (SSCP) analyses op twee fragmenten van het mitochondriaal cytochroom c oxidase I gen, COI (Hoofdstuk 4). De resultaten van beide studies kwamen in grote lijnen overeen; ze vertoonden een significante genetische differentiatie over het ganse verspreidingsgebied van *N. integer*, met een lage intra-populatie-variabiliteit. Bijgevolg stemmen deze resultaten overeen met de verwachte genetische patronen in een soort met lage dispersiecapaciteit, voorkomend in brakwaterhabitats. De verschillende fylogeografische analyses duiden op een complex genetisch patroon beïnvloed door de Pleistocene glaciaties. Deze patronen strookten niet met de algemene verwachtingen op basis van de huidige paleoklimatologische modellen voor terrestrische en zoetwater soorten. Volgens deze modellen werden taxa uit noordelijk gematigde regio's tijdens de glaciale periodes, ten gevolge van de sterk gedaalde temperaturen, verdrongen naar zuidelijke glaciale refugia (op het Iberische & Italiaans schiereiland, de Balkan regio). Op het einde van een glaciale periode zorgde de opwarming van het klimaat, en de terugtrekking van de ijskappen voor een snelle verspreiding van soorten uit de refugiale gebieden. Deze

opeenvolgende golven van noordwaartse postglaciale colonisatie en compressie van het verspreidingsgebied gedurende glaciale periodes resulteert meestal in een reductie van de genetische diversiteit in de noordelijke gebieden. Bovendien vertonen de noordelijke populaties minder genetische stucturering in vergelijking met refugiale populaties. In het geval van *N. integer* vertoonde de fylogeografie op basis van het mitochondriaal COI gen een aantal contrasterende resultaten: (i) de graad van haplotype diversiteit vertoonde geen afname in door ijstijden beïnvloede gebieden, met uitzondering van een afname aan zowel de noordelijke als zuidelijke distributierand, (ii) het Iberisch Schiereiland vormde geen enkelvoudig glaciaal refugium, en het ziet ernaar uit dat deze zuidelijke refugiale populaties niet hebben bijgedragen in de meest recente postglaciale distributie expansie na het laatste glaciaal maximum. Het bestaan van meerdere (noordelijke) glaciale refugia wordt gesuggereerd, hoogstwaarschijnlijk gesitueerd in de zuidelijke Noordzee of in het Kanaal, rond de Britse Eilanden en in de Golf van Biskaje. Daarnaast vertoonden zowel de COI als cyt b analyses een duidelijke fylogeografische breuk ter hoogte van de zuidelijke distributierand, nl. tussen de Guadalquivir en alle andere Atlantische populaties.

De fylogeografische patronen van de Atlantische en Mediterrane populaties van *M. slabberi* werden geanalyseerd met behulp van DNA-sequenering van twee fragmenten in het mitochondriale COI gen en het mitochodriale 16S ribosomale RNA gen (Hoofdstuk 5). In tegenstelling tot *N. integer* waren de gemiddelde moleculaire-diversiteitswaarden binnenin populaties erg hoog, karakteristiek voor vele mariene soorten. Een zeer hoge graad van fylogeografische structurering werd teruggevonden, en de fylogenieën op basis van de COI en 16S genen vertoonden 4 sterk gedifferentieerde, monofyletische clades (2 Atlantische en 2 Mediterrane clades). De graad van nucleotide divergentie tussen deze verschillende clades overschrijdt waarschijnlijk het intra-specifieke niveau, en bijgevolg suggeren deze patronen de aanwezigheid van verschillende cryptische soorten. Bovendien was de fylogeografische breuk waargenomen tussen de Atlantische en Mediterrane populaties één van de grootste tot nu gerapporteerd in mariene taxa. Langsheen de Atlantische kust vertoonde *M. slabberi* gelijkenissen met *N. integer* in de graad van populatie structurering over een macro-geografische schaal, welke waarschijnlijk gerelateerd is aan de afwezigheid van vrijlevende larven in beide soorten. Op een kleinere (meso-) geografische schaal werd echter minder genetische structurering waargenomen tussen

M. slabberi populaties in vergelijking met *N. integer*, dit kan waarschijnlijk gerelateerd worden aan de hogere continuïteit van beschikbare habitats voor *M. slabberi*. De relatief discrete, natuurlijk gefragmenteerde estuarine en brakwater habitats van *N. integer* daarentegen, vormen eerder gesloten populaties, wat resulteert in beperkte gene flow, zelfs over kleinere geografische schaal.

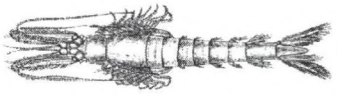
Hoofdstuk 6 beschrijft de fijnschalige (intra-estuarine) en temporele genetische variabiliteit (een aspect dat heel vaak genegeerd is in vele grootschalige moleculaire studies) van de brakwater aasgarnaal *N. integer* binnen het Westerschelde estuarium. Verschillende stations werden hiervoor bemonsterd langsheen een omgevings-gradiënt (saliniteit, pollutie) en uit verschillende habitats (subtidaal, brakwater plas, haven lokatie) in drie opeenvolgende jaren (9 generaties), deze werden geanalyseerd met SSCP. Binnen twee onderzochte jaren werd een kleine, maar significante genetische differentiatie waargenomen binnen het Westerschelde estuarium. Er was echter geen bewijs voor temporele stabiliteit van deze genetische structurering. Bijgevolg blijft het onduidelijk of de huidige patronen het resultaat zijn van stochastische processen, staalnamefouten, of veroorzaakt zijn door onvoorspelbare omgevingsveranderingen, welke typisch zijn voor brakwater habitats. Bovendien waren de schattingen van de effectieve (vrouwelijke) populatie omvang van *N. integer* twee tot drie grootte ordes kleiner dan de census populatie omvang schattingen. Dit zou erop kunnen wijzen dat ondanks hun omvangrijke populatiegrootte, de populaties van *N. integer* vatbaar zouden kunnen zijn voor een snel verlies aan genetische diversiteit.

Ter aanvulling van de genetische analyses van *N. integer* en *M. slabberi* populaties, werd de variatie in 12 morfometrische en 3 meristische kenmerken geanalyseerd in drie populaties van beide soorten (Hoofdstuk 7). Met behulp van multivariate analyses werden duidelijke morfometrische verschillen (met betrekking tot de morfologie van het telson en de ogen) aangetoond tussen de verschillende populaties. In het geval van *M. slabberi* waren de morfologische patronen overeenstemmend met de beschikbare moleculaire data van mitochondriale loci, waarbij nogmaals de grote divergentie tussen de Atlantische en Mediterrane populaties werd bevestigd. Door de overlap van een beperkt aantal individuen tussen de verschillende populaties, waren deze morfometrische patronen echter niet voldoende om de aanwezigheid van verschillende (sub)soorten te bevestigen. In het geval van *N. integer* werd de grootste divergentie waargenomen voor de Gironde

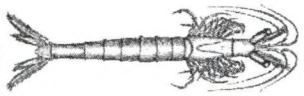
populatie. De morfometrische verschillen zijn hoofdzakelijk gerelateerd aan de morfologie van het oog bij *N. integer*. Mogelijke interacties van dit morfologisch kenmerk met verschillende omgevingsomstandigheden, zoals verhoogde turbiditeit in het Gironde estuarium, kunnen verantwoordelijk zijn voor het geobserveerde patroon.

Samenvattend, een hoge graad van differentiatie werd waargenomen tussen aasgarnaal populaties, zowel gebaseerd op een aantal mitochondriale moleculaire merkers, als op basis van morfometrische kenmerken. Deze resultaten bevestigen de algemene verwachtingen voor soorten met lage dispersie capaciteiten (broedgedrag) en gefragmenteerde brakwaterhabitats. De intraspecifieke moleculaire patronen vertoonden een duidelijk fylogeografische structuur, die voor *N. integer* het resultaat is van een complexe postglaciale colonisatie van de Noord-Europese kusten vanuit verschillende glacial refugia. In het geval van *M. slabberi* suggereren de mitochondriale DNA analyses het bestaan van verschillende cryptische soorten. Tenslotte, bleken de analyses van de ribosomale 18S sequenties binnen de soortenrijke Mysidae familie nuttig om de morfologie-gebaseerde classificatie binnen deze familie te reëvalueren. Gebaseerd op deze resultaten is een taxonomische revisie binnen de Mysidae familie noodzakelijk.

CHAPTER 1



General introduction and outline



ABSTRACT

The present study deals with the molecular diversity and genetic population structure of two mysid species along the European coast. This introductory chapter starts with a description of the concept 'molecular biodiversity'. A brief overview is presented on the different factors which shape the levels of genetic diversity and on the molecular tools used to assess molecular biodiversity. We review the main mechanisms and (contemporary, as well as historical) factors responsible for population genetic and phylogeographic structure in marine organisms. A summary on the distribution, biology and ecology of the mysid species under study is given. Finally, the main objectives and outline of this thesis are presented.

GENERAL INTRODUCTION AND BACKGROUND

What is molecular biodiversity, and how does one study it?

Biodiversity is an umbrella term encompassing many interrelated aspects (from genetics and molecular biology to community structure and habitat heterogeneity), but most commonly it refers to the full range of species on Earth (see reviews by Wilson, 1988; Féral, 2002). Given the various scales of biodiversity, it can be described at several levels: (i) genetic diversity, (ii) species diversity, (iii) ecosystem diversity, and (iv) an additional fourth level, the sea- (land)scape diversity, which integrates the type, condition, pattern, and connectivity of natural communities or ecosystems (Solbrig, 1991; NRC, 1995; Ormond *et al*, 1997). The 1992 Earth Summit in Rio de Janeiro defined biodiversity as: the variability among living organisms from all sources, including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems.

Research in the present doctoral study is focused on the molecular genetic diversity level of marine invertebrates, and more specifically of mysid species (Crustacea, Mysida). Genetic diversity can be defined as "the total variation in the amount of genetic information within and among individuals of a population, a species, an assemblage, or a community". Genes are the blueprints for life that are

passed on from generation to generation, and intraspecific genetic variation forms the raw material for evolution. It provides the foundation for diversity among species and ultimately for the diversity among ecosystems. Moreover, it determines the ecological and evolutionary potential (i.e. evolutionary adaptation to a changing environment) of species (Féral, 2002).

Genetic variation arises due to mutation (i.e. a single nucleotide change in a DNA sequence), recombination (in the case of nuclear genes) and horizontal gene transfer (viruses). Mutation is the ultimate source of all genetic variation. These changes can be neutral - having no effect - or they can result in new variants of the gene called alleles. When a new allele appears in a population, it has the potential to change the genetic make-up of successive generations. However, the probability of this change is largely dependent on the interplay of three forces: selection, random genetic drift and migration (Hartl, 2000). Natural selection, already proposed by Darwin in 1859 as the driving force of evolution, alters the frequency of alleles within a population via differential survival and reproductive success of individual organisms. Those individuals with well-adapted phenotypes (i.e. a greater "fitness") will pass a greater proportion of their genes on to the next generation. Random genetic drift refers to fluctuations in allele frequency that occur by chance, particularly in small populations, as a result of random sampling among gametes (mostly in case of a founder effect or genetic bottleneck). Finally, migration involves the exchange or transfer of genes and alleles (i.e. gene flow) in a population by the introduction (immigration) or loss (emigration) of individuals. Migration tends to have a homogenizing effect on the population structure.

The molecular tools used to study genetic diversity have experienced a large evolution during the last decades (see Jarne & Lagoda, 1996; Hoelzel, 1998; Mueller & Wolfenbarger, 1999; Féral, 2002; Morin *et al*, 2004). The advent of molecular techniques in the mid 1960's enabled evolutionary researchers to detect genetic variation in proteins, i.e. allozyme analysis (Hubby & Lewontin, 1966). However, the development of the Sanger dideoxy sequencing method in the late 1970s (Sanger *et al*, 1977) and the polymerase chain reaction (PCR) in the mid 1980s by Kary Mulis (Mulis & Faloona, 1987) induced a real methodological revolution. Since then, a dramatic progress has been made in the ability to obtain DNA sequence data, which recently gives access to virtually the entire genome of almost every organism. The relative ease with which we are now able to obtain DNA sequence data through the

development of universal primers (e.g. Kocher *et al*, 1989; Folmer *et al*, 1994) has produced a concomitant shift from typically higher level phylogenetic studies of taxa to studies that also address within-species variability. In addition, the development of high resolution mutation detection techniques, such as Single-Stranded Conformation Polymorphism (SSCP) (Orita *et al*, 1989), has produced new opportunities for researchers to efficiently screen a large number of samples without large resource investments. Hence, the applicability of such techniques in genetic diversity and population genetic studies is very high (see Sunnucks *et al*, 2000).

One of the most notable evolutions in molecular diversity studies is the development of phylogeography, a research field that deals with the processes determining the geographic distribution of genealogical lineages within and among species (Avise, 2000). Phylogeography seeks to interpret the mode by which historical processes in population demography may have left evolutionary footprints on the contemporary geographic distributions of gene-based organismal traits. This analysis and interpretation of lineage distributions usually requires extensive input from molecular genetics, population genetics, ethology, demography, evolutive biology, paleontology, geology, and historical geography (Avise, 2000). The potential usefulness and advantages of mitochondrial DNA (mtDNA) as a tool for population genetics and phylogeographic research have been extensively reviewed (see Avise, 2000; Hewitt, 1999). Approximately 70% of all phylogeographic studies conducted to date involved analysis of animal mtDNA (Avise, 2000). The higher mutation rates and smaller effective population size (about one-fourth of the nuclear DNA; Birky *et al*, 1983), because of the maternal inheritance and haploidy of the mitochondrial genome, mean that mitochondrial variants are likely to reach equilibrium more quickly and provide a better signal, as opposed to nuclear markers, for current or more recent patterns of gene flow (Moritz *et al*, 1987). The absence of recombination in the mitochondrial genome (Birky, 2001) (but see exceptions: e.g. *Mytilus galloprovincialis*, Ladoukakis & Zouros, 2001) makes them extremely useful for phylogenetic studies since a matrilineal genealogy can be reconstructed, because they are hierarchical and show clear relationships among individuals (Hewitt, 2004). In addition, supporters of the DNA-based identification of species ('DNA barcoding'; see Blaxter, 2003; Hebert *et al*, 2003a; Tautz *et al*, 2003) advocate the use of mitochondrial genes, and in particular the cytochrome *c* oxidase subunit 1 (COI),

which could serve as the core of a global bioidentification system (Hebert *et al*, 2003a, b).

Molecular phylogenetics

Traditionally, phylogenetic trees have been used to represent the historical (evolutionary) relationships of groups of organisms, often species. Classic phylogenetics dealt mainly with physical or morphological features (e.g. size, color, number of appendages, etc.). Modern phylogeny uses information extracted from genetic material, mainly DNA and protein sequences. Molecular phylogenetics can approach many problems previously considered intractable by morphologists. For instance, there are very few homologous morphological characters that can be compared among all living organisms. In contrast, a number of genes with fundamental biochemical functions are found in all species and they can be sequenced, aligned and analysed to study phylogenetic relationships even among the deepest part of the tree of life (Hillis & Dixon, 1991; Page & Holmes, 1998).

By using a comparative approach, genetic diversity can be organized into a meaningful estimation of the evolutionary relationships among lineages of organisms, i.e. a phylogeny. Reconstructing the phylogenetic relationships between gene sequences is a crucial first step towards understanding their evolution. The phylogenetic tree can therefore be thought of as the central metaphor of evolution, providing a natural and meaningful way to order data, and with an enormous amount of evolutionary information contained within its branches (Page & Holmes, 1998). However, it must be noted that a fundamental difference exists between a species tree, representing the true evolutionary pathways of a groups a species, and a gene tree, often constructed based on one gene. The gene tree and the species tree are not necessarily congruent in terms of topology or branch lengths, owing to gene duplication (resulting in paralogous genes), lineage sorting and horizontal gene transfer (Page & Charleston, 1999).

The task of molecular phylogenetics is to convert information in sequences into an evolutionary tree for those sequences. A great (and ever increasing) number of methods have been described for doing this. They can be classified into two general categories: distance methods, where the estimated genetic distance between pairs of taxa reflects the degree of relatedness (e.g. Neighbour-Joining), and discrete methods

(tree searching methods), which in contrast to distance methods operate directly on the sequences, or on functions derived from the sequences, rather than on pairwise distances (e.g. maximum parsimony, maximum likelihood and Bayesian methods). For intraspecific datasets at the population level, however, several phenomena (e.g. low sequence divergence, extant ancestral nodes, multifurcations, reticulation, large sample sizes) violate the assumptions of these 'traditional' phylogenetic reconstruction methods, leading to a poor resolution or inadequate portraits of genealogical relationships. Hence, new phylogenetic methods such as network reconstruction methods, which take into account the population evolutionary patterns, may be more appropriate (Posada & Crandall, 2001).

The genes of the nuclear ribosomal DNA (rDNA), which code for the RNA component of the ribosome, have been widely used in phylogeny reconstruction. The rDNA is a multigene family (Fig. 1.1) with nuclear copies in eukaryotes arranged in tandem arrays, in which each gene is separated from the next by regions known as spacer DNA, which varies in length and sequence among species. A single cluster consists of the rDNA genes for 18S (small subunit), 5.8S, and 28S (large subunit) rDNA molecules which are separated by internal transcribed spacers (ITS-1 and ITS-2). Adjacent clusters which have a length of about 10,000 nucleotides each are separated by external transcribed spacer regions (ETS). Different selective forces are acting on the rDNA region with as consequence varying degrees of sequence conservation across single repeat units. Therefore, each part can be employed for specific phylogenetic questions across a broad taxonomic spectrum (Hillis & Dixon, 1991). The 18S rDNA molecule is a popular phylogenetic marker for tracing relationships among distantly related taxa. It has been argued that 18S rDNA sequences cannot unambiguously resolve cladogenetic events separated by less than 40 Myr (Philippe *et al*, 1994) and that they are unsuitable for comparing taxa that diverged since the Cretaceous (Hillis & Dixon, 1991). However, 18S rDNA sequences have been used with fairly good results in phylogenetic analyses of congeneric species (see Winnepenninckx *et al*, 1998 and references therein). Hence, these studies suggest that it might also be a reliable phylogenetic marker for resolving recent divergences (Winnepenninckx *et al*, 1998). On the other hand, the faster evolving spacer regions (e.g. ITS) have been employed for population and congeneric phylogenies and have become a popular choice for phylogenetic analysis of closely related species and phylogeographic studies within species (e.g. Dahlgren *et al*, 2000;

Patti & Gambi, 2000; Rodriguez-Lanetty & Hoegh-Guldberg, 2002; Wörheide *et al*, 2002; Duran *et al*, 2004a; Schilthuizen *et al*, 2004).

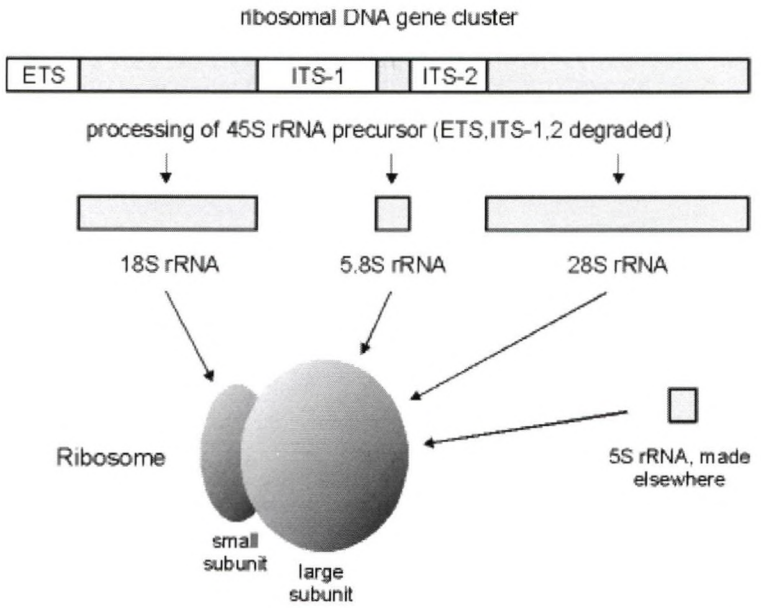


Fig. 1.1: Schematic overview of the ribosomal DNA gene cluster (ETS = external transcribed spacer, ITS = internal transcribed spacer).

Dispersal, gene flow and population genetic structure in the marine environment

A large amount of marine studies have focused on the spatial patterns of allelic frequencies in natural populations (e.g. Avise, 1994). Such an approach has been particularly useful in marine biology because the life cycle of most marine species exhibits a dispersal phase in an environment that often lacks natural boundaries. The mode of reproduction of marine species is crucial for the dispersal potential and thus for the level of genetic structuring and gene flow between geographically separated populations. Hence, it is not surprising that genetic divergence among populations of species with planktotrophic larvae and a continuous habitat is typically low, compared to species which lack a pelagic dispersal stage. A number of empirical studies indeed corroborated these expectations and lead to the general presumption that the long range of larval stages and the high fecundity of marine organisms are associated with high gene flow, and hence genetic homogeneity over vast distances (Waples, 1987; Palumbi, 1992; Ward *et al*, 1994; Palumbi, 1996; Shaklee & Bentzen, 1998). These presumptions are further strengthened by the findings that even a small amount of

gene flow between populations is usually sufficient to prevent differentiation. For example, if one migrant per generation settles and enters a local breeding population, then this small amount of genetic exchange is enough to prevent the accumulation of large genetic differences (Slatkin, 1987).

However, an increasing number of studies on marine species have highlighted instances where long-life pelagic larvae do not result in broad dispersal (Palumbi, 1997; Lessios *et al*, 1999; Luttikhuizen *et al*, 2003; Taylor & Hellberg, 2003; Ovenden *et al*, 2004). This emphasizes the importance of other factors, besides dispersal ability, in creating and maintaining population differentiation. Several factors may have a significant role, either singly or in combination, including behavioral mechanisms limiting dispersal (Beheregaray & Sunnucks, 2001), selective processes and local adaptation (Schmidt & Rand, 1999; Lemaire *et al*, 2000), complex oceanographic currents (Benzie & Williams, 1997; Palumbi *et al*, 1997; Stepien, 1999), habitat discontinuities (Johnson & Black, 1995, 1998; Riginos & Nachman, 2001) and historical barriers to gene flow (Avise, 1992; Lavery *et al*, 1996; Williams & Benzie, 1998). Last decade several studies have tried to identify, as well as unravel, the interplay between the intrinsic (i.e. biological, ecological, physiological or behavioural) and extrinsic (i.e. physical, geological, environmental) factors which influence the population structuring (see Avise, 1994, 1998). Especially the comparative assessments of the population genetic structure of sympatric (sister) species seem to provide valuable information on the effects of these intrinsic factors on the dispersal ability and the phylogeographic patterns (e.g. Dawson *et al*, 2002; McMillen-Jackson & Bert, 2003). On the other hand, broadscale studies have shown clear phylogeographical boundaries with concordant patterns in several divergent marine taxa, which are mainly attributed to extrinsic factors such as historical vicariant events (see Avise, 1994). Areas of interest, where numerous well-documented cases of strong genetic differentiation are available, seem to be located along the southeast coast of the USA, on either side of Cape Canaveral (Florida), separating the Western Atlantic and the Gulf of Mexico (reviewed in Avise, 1992, 1994). Likewise, populations of Indo-Pacific marine invertebrates and fish separated by the Indonesian Archipelago often show strong genetic differences (reviewed in McMillan & Palumbi, 1995; Palumbi, 1997; Williams & Benzie, 1998; Duda & Palumbi, 1999). Within Europe, genetic breaks have been observed in marine taxa

with an Atlantic-Mediterranean distribution (e.g. Borsa *et al*, 1997 Perdices *et al*, 2001; Bargelloni *et al*, 2003; Gysels *et al*, 2004; Roman & Palumbi, 2004).

Since most research dealing with the previously described topics has focused almost exclusively on marine taxa with moderate to high dispersal capabilities which are able to maintain genetic connectivity, genetic patterns within marine taxa with poor dispersal abilities have been little-studied to date. It is generally predicted that poor dispersal taxa exhibit even more genetic differentiation than high dispersal taxa (Waples, 1987). Several studies corroborated these expectations (e.g. Avise *et al*, 1987; Burton & Lee, 1994; Schizas, 1999). Moreover, the brooding behavior of species, which can be considered as the ultimate evolutionary stage of nonpelagic lecitotrophic development, can be responsible for some peculiarities in the pattern of genetic differentiation (e.g. see for brooding Antartic echinoids, Poulin & Féral, 1996). The brood protecting species have low dispersal capacity and hence their populations can be genetically differentiated at separating distances of a few kilometers (see Kwast *et al*, 1990; Poulin & Féral, 1994; Poulin & Féral, 1997; Ayre & Hughes, 2000; Sponer & Roy, 2002). The lack of sufficient gene flow between populations leads to gradual transformation of the isolated gene pool by random genetic drift and/or by natural selection by the local environment. Hence, the reduced gene flow associated with nondispersal larvae can induce an increase of speciation rates compared to those developing via pelagic larvae (Poulin & Féral, 1996).

The general pattern of highly geographically structured populations in poor dispersers render them more suitable in tracking biogeographical processes than more dispersive taxa (see Wilke & Davis, 2000; Gysels *et al*, 2004; Kirkendale & Meyer, 2004). Given the expected genetic differentiation in poor dispersers, together with the substantial cryptic diversity which has been uncovered by molecular analyses in a wide variety of marine taxa (reviewed by Knowlton, 1993, 2000), geographical structure and cryptic speciation within widely distributed, poorly dispersing species may have been greatly underestimated.

Impact of Quaternary climate changes on the genetic diversity

Organisms live in an environment that is not constant over time; the genetic patterns that we describe today are the result of both contemporary and historical factors (Avise *et al,* 1987). The relative significance of both factors is often difficult to distinguish based on contemporary observations. For example, genetic homogeneity among the populations of a species can be due to a recent common ancestry or contemporary gene flow. In general, species showing reduced levels of contemporary gene flow are better suited for elucidating phylogeographic patterns since these species are often composed of genetically and geographically highly structured populations (e.g. Lee, 2000; Wilke & Pfenninger, 2002; Gysels *et al*, 2004).

The Pleistocene glaciations were arguably the most significant historical event that has occurred during the evolutionary lifespan of most extant species (e.g. Bernatchez & Wilson, 1998; Taberlet *et al*, 1998; Hewitt, 2000). During the past 2.5 My, the climatic and environmental fluctuations of the Pleistocene have forced species to adjust the distributional areas according to their adaptive ability, resulting in periodical extinction-recolonisation events. Up to 20 glaciation events may have occurred during the Pleistocene (Martinson *et al*, 1987), with each glaciation spanning approximately 100,000 years, and the interglacial periods lasting 10,000 – 12,000 years (Dawson, 1992). The most recent European glaciation event ('Weichselian') reached its maximum ice coverage about 20,000 – 18,000 years ago. As shown in figure 1.1, ice sheets covered Scandinavia and most of the British Isles (Lowe & Walker, 1997) and the southern Bight of the North Sea was dry due to glacio-eustatic sea level drops (115-120 m below the present-day level) (van der Molen & de Swart, 2001). It should be noted that although the importance of the Pleistocene glaciations is stressed here, more ancient historical events that occurred during the Miocene through the Pliocene may have also played a role in shaping the pattern of genetic diversity in extant populations (e.g. within the Mediterranean region, see Box 1.1).

The impact and dramatic changes determined by the Quaternary climate events have been well documented in terrestrial and freshwater habitats of northern temperate regions (e.g. Hewitt, 1996, 2000). Comparative studies among various taxa lead to the designation of different glacial refugial areas (see Fig. 1.2) and putative northward post-glacial colonization routes (see Taberlet *et al*, 1998; Hewitt, 1999).

Much less is known about the effects of Pleistocene climate changes on marine coastal organisms. Moreover, the majority of the available marine studies have been focused on the genetic patterns of high dispersal fish or invertebrates. However, the understanding of the effects of Pleistocene climate changes on marine organisms of temperate regions could be important for the prediction of the effects of current rapid climate change driven by human activities on marine populations (Maltagliati, 2003; Cognetti & Maltagliati, 2004).

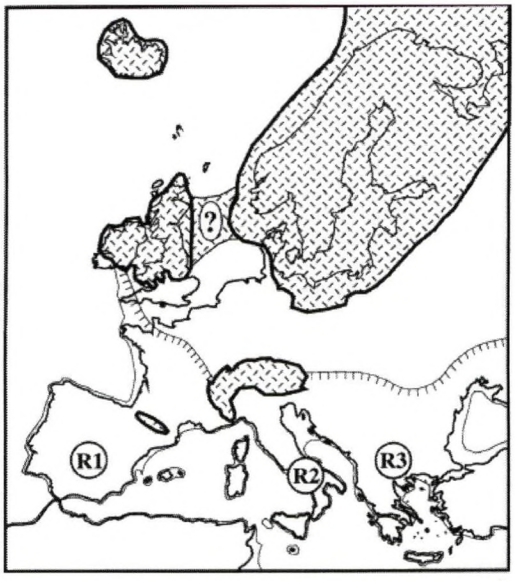


Fig. 1.2: Maximum extension of ice sheets in Europe during the last glacial maximum (20 – 18 kya) (redrawn from Frenzel *et al*, 1992). R1, R2, and R3 indicate the three main potential refugia on the Iberian, Italian Peninsula and in the Balkans, respectively. The southern limit of the permfrost is indicated by the scaled line. Lowered sea shore is shown by a thinner line at the 100 m submarine contour. Figure redrawn from Taberlet *et al*, 1998.

PLIOCENE: (5.3 - 1.8 Mya)

6 – 5.5 Mya: Messinian salinity crisis: desiccation of the Mediterranean basin and transformation into a series of hypersaline lakes with thick evaporate deposition (Krijgsman *et al*, 1999)
5.3 Mya: reflooding of the Mediterranean basin (re-establishing the Atlantic-Mediterranean connection)

PLEISTOCENE (1.8 Mya - 10 kya)

490 – 410 kya: Elsterian (glacial)
410 – 380 kya: Holsteinian (interglacial)
380 – 130 kya: Saalian (glacial)
130 - 110 kya: Eemian (interglacial)
110 – 10 kya: Weichselian (last glacial event)

→ **22 –18 kya**: Last Glacial Maximum:

- ice cover north of 50° latitude (Scandinavia & most of the British Isles) (Lowe & Walker, 1997; Benn & Evans, 1998)
- southern Bight of the North Sea was dry due to glacio-eustatic sea level drops (115-120 m below the present-day level) (van der Molen & de Swart, 2001)
- closure of the Gibraltar Straits (fragmentation of the Altantic Ocean and Mediterranean Sea)

12 kya: formation of the Baltic Ice Lake (i.e. an ice-dammed freshwater lake) in the Baltic region (Andrén *et al*, 2002)
10.3 kya: Yoldia transgression: connection between North Sea and Baltic Ice Lake (brackish water period) (Donner, 1995)
10 kya: re-establishment of the North Atlantic Current (Harland & Howe, 1995)
9.3 kya: isolation of the Baltic Sea due to the isostatic rebound of southern Sweden (Benn & Evans, 1998), freshwater period (Ancylus Lake)
8 kya: opening of the Danish Straits with colonisation of the Baltic Sea (Björck, 1995), inflow of salt water, with gradually decline of salinity since then.
7.5 kya: the present connection between the southern North Sea and the Atlantic Ocean was formed through the English Channel (landbridge between England and France disappeared)
1.7 – 1.55 ka BP: Younger Drias ('Little Ice Age'), a short cold (glacial?) period.

Box 1.1: Time scale of the major palaeogeographical events during the late Miocene, Pliocene & Pleistocene relevant for European marine taxa.

Mysid taxonomy, biology, ecology and distribution

Mysids (Crustacea, Peracarida, Mysida) are relatively small (the majority between 5 – 25 mm) shrimp-like animals that occur in vast numbers in various aquatic habitats all over the world, including freshwater, groundwater, brackish, estuarine, coastal and oceanic habitats (Tattersall & Tattersall, 1951; Mauchline, 1980). They are often referred to as 'opossum shrimp' due to the presence of a ventral marsupium in female mysids, and in which the entire larval development takes place. The order Mysida currently comprises 1053 species and 165 genera (see NeMys database, http://intramar.ugent.be/nemys, Deprez *et al*, 2004).

The present study focuses on species within the Mysidae, the most speciose family (157 genera, 1004 species) within the order Mysida. Based on the geographical distribution, ecological significance and specific habitat requirements, two species were selected for detailed phylogeographic and population genetic research: *Neomysis integer* and *Mesopodopsis slabberi*. On morphological grounds, both species are placed in the same subfamily and same tribe (Table 1.1). The next two paragraphs summarize the available information on their distribution, biology and ecology.

Phylum Arthropoda
Subphylum Crustacea
Class Malacostraca
Subclass Eumalacostraca
Superorder Peracarida
Order Mysida
Family Mysidae
Subfamily Mysinae
Tribe Mysini
Mesopodopsis slabberi (van Beneden, 1861)
Neomysis integer (Leach, 1814)

Table 1.1: Systematic position of *Mesopodopsis slabberi* and *Neomysis integer* within the phylum Arthropoda (after Müller, 1993).

Biology of Neomysis integer Leach, 1814

Fig. 1.3: *Neomysis integer*, adult female

The genus *Neomysis* Czerniavsky consists of more than twenty species, of which only two representatives inhabit the northern Atlantic: *N. americana* (NW Atlantic) and *N. integer* (NE Atlantic; Fig. 1.3) (see NeMys database, http://intramar.ugent.be/nemys, Deprez *et al*, 2004). Morphological characteristics of *N. integer* are the subtriangular, elongated telson without a cleft and the pointed distal end of the antennal scale (Tattersall & Tattersall, 1951).

N. integer is one of the most common mysids around the coasts of Europe. Its distribution ranges from the Baltic Sea to the north African coasts of Morocco; it is absent from the Mediterranean Sea (Fig. 1.4). It is a euryhaline and eurytherme species (see Box 1.2), which typically dominates the hyperbenthic communities of the brackish part of estuaries (Tattersall & Tattersall, 1951; Mees *et al*, 1995; Cunha *et al*, 1999). It is also common in the Baltic Sea, in various brackish habitats such as small ponds, sea loughs and lagoons (Parker, 1979 and references therein), and in freshwater bodies which in recent geological history were connected to the sea (Bremer & Vijverberg, 1982). Occasionaly, *N. integer* is observed in fully marine conditions, especially during winter months when the floodwater discharge from estuaries is higher (Beyst *et al*, 2001).

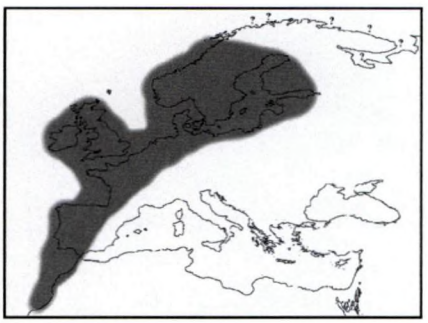


Fig. 1.4: Distribution of *Neomysis integer*

N. integer is a typical omnivorous species which mainly utilizes mesozooplankton (e.g. the calanoid copepod *Eurytemora affinis*, cladocera of the genus *Bosmina*, rotifers of the genus *Keratella* and *Brachionus*), and macrophytal detritus and amorphous material originating from suspended sediment flocs (Fockedey & Mees, 1999), and an important prey for demersal and pelagic fish (e.g. *Pomatoschistus minutus*, *P. lozanoi*, *Trisopterus luscus*, *Merlangius merlangus*, *Pleuronectes flesus*, *P. platessa*, *Clupea harengus*, *Sprattus sprattus*, *Dicentrarchus labrax*, *Anguilla anguilla*) and larger epibenthic crustaceans (e.g. *Crangon crangon*)

(Mauchline, 1980; Hostens & Mees, 1999; Hostens, 2003; Maes *et al*, 2003). As such it is believed to be a key species in estuarine ecosystems. The life history and growth of *N. integer* are well studied in the Westerschelde estuary. Reproduction starts in early spring (when water temperature > 10°C) and lasts until late September/October. This results in three generations per year (spring, summer and overwinter generation) (Mees *et al*, 1994). However, at lower latitudes the life cycle can be more complex, with breeding almost continuous throughout the year (Sorbe, 1981).

Ecophysiological tolerances, temperature and salinity effects on post-marsupial growth and embryogenic development of *N. integer* have gained increasing interest during last years, especially since *N. integer* has been proposed as a toxicological test species for estuarine systems (Roast *et al,* 1998; Verslycke 2004). Under laboratory conditions its temperature tolerance ranges from 0 to 30°C (Arndt & Jansen, 1986) and it tolerates salinities of 0.5 to 40 psu (Vlasblom & Elgershuizen, 1977; Barnes, 1994; Roast *et al*, 2001). Lab experiments have shown that *N. integer* is distinctly euryplastic regarding temperature and salinity. It is described as thermophobic with optimal resistance to salinities higher and lower than its isosmotic point (16-19 psu) in the lower temperature ranges (Arndt & Jansen, 1986). *N. integer* seems to be extremely tolerant to very large, short-term salinity fluctuations between 1 and 30 psu, showing no distinguishable behavioural changes when exposed to such large variations in salinity (Moffat & Jones, 1992; Roast *et al*, 1998). An extremely efficient osmoregulatory physiology (hyper-hypo-osmoregulator) (McLusky & Heard, 1971) that attains osmotic balance within 2 h of exposure to a change in salinity (Moffat, 1996) is a necessary adaptation for life in the variable environment of the upper estuarine regions (Roast *et al*, 1999). The upper tolerance limits of temperature and salinity for *N. integer* range between 20-25°C and 25-30 psu, with a substantially increase in mortality at higher salinity and temperature values (Kuhlman, 1984). Field observations corroborate these trends; the southern distribution range (Guadalquivir estuary) corresponds to an average summer water temperature of 29°C (Drake *et al*, 2002). In well-oxygenated western European estuaries the maximum abundance is located at around 5 psu (Mees *et al*, 1995), and it is generally rare in waters of more than 20 psu (Tattersall & Tattersall, 1951; Vlasblom & Elgershuizen, 1977).

N. integer has a sigmoid growth pattern, which can be described by the von Bertalanffy growth model (Winkler & Greve, 2002; Fockedey *et al*, submitted). *N. integer* growing at 15 psu has the shortest intermoult period and yields the largest

animals (and hence the largest fecundity; Mees *et al*, 1994), those growing at 30 psu the smallest; this is independent of temperature. Growth at 15°C and at 15 psu results in mysids with a larger standard length in comparison with other temperatures (Winkler & Greve, 2002; Fockedey *et al*, submitted). The generation time of *N. integer* is 70 d at 15°C. Fertilized eggs are released from the marsupium after 19 d incubation as post-larvae, and after 9 to 10 moults maturity (mean length = 8 mm) is reached in 50 d. At 10°C maturity occurs after 15 to 16 moults and at an age of 110 d (Winkler & Greve, 2002). Experiments on the effect of temperature and salinity on the marsupial growth and embryogenic development in *N. integer* have shown that the highest survival (60%) and hatching (40%) of the embryos falls within a salinity range of 14 – 17 psu, under temperatures below 15°C (Fockedey *et al*, in preparation).

Neomysis integer Leach, 1814	
Adult size	10 - 17 mm
Distribution	NE Atlantic
Habitat	Brackish water, estuarine, marshes & brackish lagoons hyperbenthic swarming behaviour in relation to tidal flow
Physiology	euryhaline (0.5 to >25 psu, optimum: 2 - 5* psu), eurythermal (< 20°C)
Feeding	omnivorous (mainly mesozooplankton & detritus)
Reproduction	brooder
Breeding season	April to late September (in Westerschelde estuary)
Generation time	3 - 6 months (shorter at lower latitudes)
Fecundity	10 - 80 embryos per brood (related to size[1], salinity & temperature[2])
# chromosomes	$n = 34$[3]

Box 1.2: Ecological, distributional and biological characteristics of *Neomysis integer* (* optimum in the Westerschelde estuary around 8 psu, see Mees *et al*, 1995). [1]Mees *et al*, 1994; [2]Mauchline, 1973; [3]Salemaa,1986.

In order to retain its position within the estuarine environment and avoid a seaward transport, *N. integer* has developed some behavioural adaptations such as alterations in swimming activity at different tidal phases. Hough & Naylor (1992) reported that *N. integer* could maintain its position in a tidally-mixed estuary by increasing its swimming activity on the flood tide to counter seaward displacement on the ebb tide. Further experiments on the swimming behaviour of *N. integer* in relation to tidal flow have shown that it can tolerate current velocities of 6 and 9 cm s^{-1}, a few could swim at speeds of up to 27cm s^{-1}, but this was not sustainable for more than a

few seconds (Roast *et al*, 1998). This corresponds with field observations, where mysids were found consistently in slower moving water (<15 cm s^{-1}), such as in the lee of rocks and macroalgal clumps, and were absent in faster flowing water (>20 cm s^{-1}) (Roast *et al*, 1998; Lawrie *et al*, 1999). Moreover, at higher velocities, *N. integer* can utilise the substratum and the bottom boundary layer, where flow is reduced, in an attempt to prevent displacement (Roast *et al*, 1998)

Little is known about the actual dispersal potential of *N. integer*. Apart from evidence of migration over small geographic scales, such as vertical diurnal migrations, tidal migration and seasonal migrations within an estuary (Mauchline, 1980; Hough & Naylor, 1992; Mees *et al*, 1993b), nothing is known about the dispersal capacities of *N. integer* over larger scales, e.g. between neighbouring estuaries, or even over larger distances. However, since *N. integer* lives in discrete brackish water habitats and lacks a planktonic dispersal stage, it has been assumed that its dispersal potential ranges from 100 – 1000m and that long-range dispersal events are probably rare (Mauchline, 1980).

Biology of Mesopodopsis slabberi van Beneden, 1861

The mysid *Mesopodopsis slabberi* has typical morphological characteristics and hence can be easily distinguished: it has a very slender and delicate, transparent body (length: 11-15 mm) and its eyestalks are exceptionally long (twice as long as the diameter of the carapace in the gastric region) (Fig. 1.5). The telson of this mysid species lacks an apical cleft. Instead, the telson ends terminally in two lateral and one larger median lobe, armed with spines (see descriptions in Tattersall & Tattersall, 1951 and Wittmann, 1992). In the field this mysid shows an astonishing agility and is often hard to recognize due to its complete transparency. However, its presence can be betrayed by its very black eyes (Tattersall & Tattersall, 1951).

Fig. 1.5: *Mesopodopsis slabberi*, adult female

The genus *Mesopodopsis* Czerniavsky is found in a wide geographical area extending from the East Atlantic, Mediterranean, the Black Sea, the Indian Ocean and Australian waters in temperate to tropical zones. The taxonomy of this genus, and of the species *M. slabberi* in particular, has long been a matter of controversy. The most

recent revision by Wittmann dates back to 1992. Based on morphogeographic variations this genus can be split in two groups: (i) the Euro-African species (with a spine below the statocyst): *M. slabberi* van Beneden, 1861, *M. aegyptia* Wittmann, 1992, *M. tropicalis* Wittmann, 1992, *M. wooldridgei* Wittmann, 1992, *M. africana* O.S. Tattersall, 1952 and (ii) the Indo-Australasian species (without a spine below the statocyst): *M. orientalis* W.M. Tattersall, 1908 and *M. zeylanica* Nouvel, 1954.

M. slabberi is widely distributed along the European coasts (Fig. 1.6), ranging from the western Baltic, northeast Atlantic to the entire Mediterranean, Marmara, Black, and Azov Seas (Tattersall & Tattersall, 1951; Mauchline 1980; Wittmann 1992). It tolerates a wide range of salinities (1.3 – 43 psu) and is therefore dominantly observed in the surf zone hyperbenthos of temperate beaches (Beyst *et al*, 2001), coastal zones (Dewicke *et al*, 2003) as well as in estuaries where it lives sympatrically with *N. integer* (Gomoiu, 1978; Greenwood *et al*, 1989; Moffat & Jones, 1993; Mees *et al*, 1995). As an omnivore feeding on phytoplankton, zooplankton and detritus (Tattersall & Tattersall, 1951; Wittmann, 1992), and as a prey for numerous species of fish (e.g. *Sprattus sprattus*, *Clupea harengus, Pomatoschistus microps*, *P. minitus, P. lozanoi*, *Stizostedion lucioperca*, *Anguilla anguilla, Belone belone*) (Greenwood *et al*, 1989; Hostens & Mees, 1999; Maes *et al*, 2003), *M. slabberi* is believed to be an important part of the food web in these ecosystems and is likely a key species regarding trophic interactions (Azeiteiro *et al*, 1999).

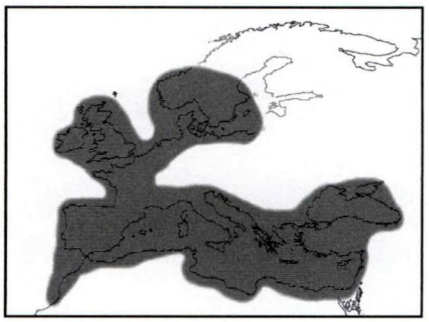


Fig. 1.6: Distribution of *Mesopodopsis slabberi*

As opposed to *N. integer*, the ecophysiological tolerances of *M. slabberi* and the responses of postmarsupial growth in relation to temperature and salinity are less well studied. According to its large geographical range, the temperature tolerance of *M. slabberi* should be broad with winter temperatures in the field ranging from 0°C in the Western Baltic to 15°C in the Eastern Mediterranean Sea and summer temperatures varying from 26°C (Western Baltic) to 30°C (East Mediterranean). *M. slabberi* is a euryhaline species, tolerating salinities between 1.3 and 43 psu (Tattersall & Tattersall 1951). Adults seem to tolerate the full range of salinities, but ovigerous females are more euryhaline than males, with the highest tolerance within

the range of 10.5 – 24.5 psu (Greenwood *et al*, 1989). Juveniles seem to survive less well than adults in low saline waters and hence occur slightly down-river of adults (Bhattacharya, 1982; Greenwood *et al*, 1989). Embryos of *M. slabberi* take from 9 to 16 days to complete development at 15°C and brood mortality of *M. slabberi* was estimated as 23% (Greenwood *et al*, 1989). The Mediterranean populations of *M. slabberi* seem to have a reduced fecundity (~5 embryos per brood, Delgado *et* al, 1997) in comparison to the British populations (maximum 25 – 29 embryos per brood, Greenwood *et al*, 1989; Moffat, 1996).

Extensive seasonal migrations have been observed for *M. slabberi*, mainly triggered by reduced temperatures. During the cold months it is virtually absent from the estuary and in the surf zone, while it seems to occur in the adjacent shallow subtidal, suggesting a migration towards deeper waters to avoid low temperatures (Mees *et al*, 1993b; Beyst *et al*, 2001; Dewicke *et al*, 2003). In early spring *M. slabberi* enters again the surfzone and the marine part of the estuary, and in summer it migrates into the brackish reaches of the estuary (Mees *et al*, 1993b; Beyst *et al*, 2001). Such seasonal onshore/offshore migrations may also have underlying salinity-related reproductive significance (Greenwood *et al*, 1989). Diurnal migratory movements are also characteristic for *M. slabberi*. During daytime it is typically hyperbenthic, gathering in large and dense swarms or schools close to the substrate. During night or in turbid waters it becomes planktonic and disperses between bottom and surface waters (Wittman, 1992). Similar to *N. integer*, little is known about the actual dispersal potential of this mysid. *M. slabberi* may have restricted dispersal capacities due to its brooding behaviour, but on the other hand, the more continuous distribution of available habitats and the larger physiological tolerance, as compared to *N. integer*, may enhance the connectivity between populations.

	***Mesopodopsis slabberi*** van Beneden, 1861
Adult size	5 - 11 mm
Distribution	NE Atlantic, Mediterranean & Black Sea
Habitat	Coastal marine (depths < 30m), surfzone beaches, estuarine, marshes & brackish lagoons hyperbenthic & pelagic extensive swarming behaviour
Physiology	euryhaline (1.3 – 43 psu) , eurythermal (0 - 30°C)
Feeding	omnivorous (phyto- & mesozooplankton, detritus)
Reproduction	brooder
Breeding season	April to late September (all year round in Mediterranean)
Generation time	3 - 6 months (shorter at lower latitudes)
Fecundity	5-25 embryos per brood (related to size & temperature[1])
# chromosomes	$n = 22$[2]

Box 1.3: Ecological, distributional and biological characteristics of *Mesopodopsis slabberi*. [1]Mauchline, 1973; [2]Mauchline, 1980.

OBJECTIVES AND THESIS OUTLINE

The **overall aim of this study** is to provide insights in the levels of **molecular genetic diversity within and between species of the order Mysida** (Crustacea, Peracarida). The few attempts to study phylogenetic relations, as well as the absence of molecular DNA studies within this species-rich order, indicate that the evolutionary relationships at different taxonomical levels (families, subfamilies, tribes, genera and species) may not be fully understood.

The two mysid species (*Neomysis integer* and *Mesopodopsis slabberi*) selected for this doctoral study show large similarities in their ecological significance, geographical distribution (at least along the NE Atlantic coasts) and dispersal potential (brooders, absence of pelagic larvae), but they also have marked differences such as in their physiological tolerance and habitat preferences (fragmented brackish habitat vs. more continuous coastal marine, estuarine habitat). Hence, a study of the phylogeographic patterns of both species within Europe could not only contribute to the understanding of molecular patterns within low dispersive marine invertebrate species, a comparison of the obtained genetic patterns within both species could also give insights on the influence of the different intrinsic and extrinsic factors (see General introduction) on the population genetic structure. Therefore we studied and compared the phylogeographic patterns of the mysids *N. integer* and *M. slabberi* using mitochondrial DNA analyses from (mostly estuarine) population samples of both species throughout their distribution range (Chapters 3, 4 & 5). In addition, the fine-scale (intra-estuarine) and temporal genetic variation of the brackish water mysid *N. integer* within the Westerschelde estuary (Chapter 6), as well as the morphometric variation in populations of both mysids (Chapter 7) were assessed.

In **Chapter 2** the result of a molecular phylogenetic analysis within the Mysidae, the largest family within the order Mysida, based on nuclear 18S ribosomal RNA sequences is presented. The aim of this study was to offer complementary information (based on 18S sequences) on the phylogenetic relations in order to identify the evolutionary relationships within this speciose family and to verify if the current morphology-based accepted systematic knowledge is supported by genetic evidence. This chapter has been published as *Remerie T., Bulckaen B., Calderon J., Deprez T., Mees J., Vanfleteren J., Vanreusel A., Vierstraete A., Vincx M., Wittmann K.J., Wooldridge T. (2004). Phylogenetic relationships within the Mysidae*

(Crustacea, Peracarida, Mysida) based on nuclear 18S ribosomal RNA sequences. Molecular Phylogenetics and Evolution 32, 770 - 777.

Both **Chapters 3 and 4** deal with the distribution of genetic variation throughout the whole distribution range of the mysid *Neomysis integer*. In **Chapter 3**, a baseline study is presented using a limited number of DNA sequences of the mitochondrial cytochrome *b* (cyt *b*) gene. The aims of this study were: (1) to give insights in the patterns of genetic structure within a low dispersal mysid; and (2) to interpret the observed patterns in function of the Pleistocene glaciations. This chapter has been submitted for publication in *Vie et Milieu* as *Thomas Remerie, Els Gysels, Andy Vierstraete, Jacques Vanfleteren and Ann Vanreusel (submitted). Evidence of genetic differentiation of the brackish water mysid Neomysis integer (Crustacea, Mysida) concordant with Pleistocene glaciations.*

In **Chapter 4** a thorough phylogeographic study of the brackish water mysid *N. integer* along the northeast Atlantic coasts is presented. As molecular techniques, a combination of the Single Stranded Conformation Polymorphism (SSCP) technique with DNA sequencing was used in order to detect variation within a fragment of the cytochrome *c* oxidase subunit 1 (COI) gene. These techniques provide an efficient, fast and relatively cheap way to analyse a large number of samples with a relatively high mutation detection resolution (up to 99% for 200 – 300bp fragments) (Sunnucks, 2000). The change from cyt *b* to COI as molecular marker was done for comparative reasons with the *Mesopodopsis slabberi* data set (chapter 5), since the amplification of the cyt *b* gene consistently failed or gave dubious results for this species. In particular, within this study we wanted to (1) assess and compare the levels of genetic diversity throughout the distribution range of *N. integer*, with specific emphasis on the latitudinal trends, (2) reconstruct the most likely historical processes that led to the current distribution of mitochondrial haplotypes, and (3) estimate the levels of genetic exchange that currently take place among European populations of *N. integer*. The results of this study were also put in the light of other marine, as well as terrestrial and freshwater phylogeographic studies in Europe. This chapter has been submitted for publication in *Molecular Ecology* as *T. Remerie, A. Vierstraete, D. Peelaers, J. R. Vanfleteren, A. Vanreusel (submitted). Patterns of genetic diversity, contemporary gene flow and postglacial colonisation history of a low dispersal mysid, Neomysis integer (Crustacea, Mysida), along the northeast Atlantic coasts.*

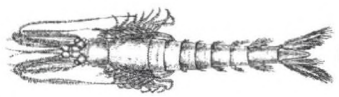
The patterns of genetic differentiation and diversity of Atlantic and Mediterranean populations of the mysid *Mesopodopsis slabberi* are discussed in **Chapter 5**. By means of DNA sequence analysis of the mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rRNA (16S) genes, the geographic patterns of genetic variation were examined at different spatial scales, i.e. at a mesogeographic scale (50-400 km), at a macrogeographic scale within the Atlantic and Mediterranean basin (> 1000s km) and across the Atlantic-Mediterranean biogeographic boundary. In addition, the patterns of Atlantic-Mediterranean differentiation were compared with those of other marine species. This manuscript is submitted as *Thomas Remerie, Tine Bourgois, Kimberly Murray, Danny Peelaers, Andy Vierstraete, Jacques Vanfleteren & Ann Vanreusel (submitted). Phylogeographic patterns within the mysid Mesopodopsis slabberi (Crustacea, Mysida): evidence for high molecular diversity and cryptic speciation* to *Marine Biology*.

Chapter 6 focuses on the fine-scale (intra-estuarine) and temporal genetic variation of the brackish water mysid *N. integer* within the Westerschelde estuary. In order to test for intra-estuarine differentiation, different samples along an environmental gradient (salinity, pollution) and from different habitats (subtidal, brackish lake, harbour site) were analysed with the SSCP technique. The temporal stability of the genetic structure was assessed by analysing samples over 3 consecutive years (9 generations). This fine-scale and temporal approach may be important in assessing the role of microevolutionary processes in producing genetic divergence among populations. This manuscript is submitted as *Thomas Remerie, Danny Peelaers, Andy Vierstraete, Jacques Vanfleteren & Ann Vanreusel (submitted). Patterns of genetic diversity of the brackish water mysid Neomysis integer (Crustacea, Mysida) within the Westerschelde estuary: panmictic population or local differentiation in a highly variable environment?* to *Estuarine, Coastal and Shelf Sciences*.

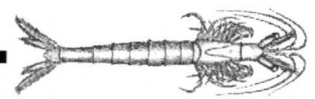
The relation between the morphometric differentiation of populations of both *N. integer* and *M. slabberi* and the patterns of genetic differentiation within both species obtained in the previous chapters, are the scope of **Chapter 7**. For this purpose, three population samples of each species were examined morphologically by measuring several morphometric and meristic characters. The patterns and the extent of morphometric variation were analysed with multivariate methods. This chapter has been submitted for publication in *Hydrobiologia* as *Thomas Remerie, Tine Bourgois*

& Ann Vanreusel (submitted). Morphological differentiation between geographically separated populations of Neomysis integer and Mesopodopsis slabberi (Crustacea, Mysida).

CHAPTER II



Phylogenetic relationships within the Mysidae (Crustacea, Peracarida, Mysida) based on nuclear 18S ribosomal RNA sequences



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ABSTRACT

Species of the order Mysida (Crustacea, Peracarida) are shrimp-like animals that occur in vast numbers in coastal regions of the world. The order Mysida comprises 1,053 species and 165 genera. The present study covers 25 species of the well-defined Mysidae, the most speciose family within the order Mysida. 18S rRNA sequence analysis confirms that the subfamily Siriellinae is monophyletic. On the other hand the subfamily Gastrosaccinae is paraphyletic and the subfamily Mysinae, represented in this study by the tribes Mysini and Leptomysini, consistently resolves into three independent clades and hence is clearly not monophyletic. The tribe Mysini is not monophyletic either, and forms two clades of which one appears to be closely related to the Leptomysini. Our results are concordant with a number of morphological differences urging a taxonomic revision of the Mysidae.

INTRODUCTION

Mysid phylogeny is poorly understood and few attempts were made over the last decades to revise the earlier established systematic relationships between higher taxonomic levels within the Mysida. These attempts dealt with the status of orders and suborders within the superorder Peracarida (De Jong & Casanova, 1997; Spears & Abele, 1997; Jarman *et al*, 2000; De Jong-Moreau & Casanova, 2001; Martin & Davis, 2001; Richter & Scholtz, 2001; Casanova *et al,* 2002). These studies gave more insight in the evolutionary link between the formerly accepted suborders Lophogastrida and Mysida within the order Mysidacea, which now can be considered different orders while the "old" Mysidacea disappears. However this ongoing debate does not discuss the status of lower taxonomic levels within the order Mysida (families, subfamilies, tribes and genera). The latest systematic overviews, not based on a phylogenetic approach, date back to 1977 and 1993 (Mauchline, 1977; Müller, 1993), indicating the lack of novel morphological evidence since the early years of mysid systematics. Some recent efforts to study mysid phylogenetics were based on the foregut morphology (Kobusch, 1998), and statolith composition (Ariani *et al*, 1993; Wittmann *et al*, 1993). The development of molecular techniques and their application in recent phylogenetic research provides a useful tool to verify if the current morphology-based accepted systematic knowledge is supported by genetic evidence. DNA sequencing indeed could offer complementary information on phylogenetic relations in order to identify evolutionary relationships among morphologically similar taxa within the Mysida, as done for many other invertebrate and particularly crustacean taxa (e.g. Abele, 1991; Abele *et al*, 1992; Spears & Abele, 1997; Palumbi & Benzie, 1991; Giribet *et al*, 2001; Braga *et al*, 1999). To our knowledge no phylogenetic study of the order Mysida has been published so far using both molecular and morphological data.

In the present study 25 species from 19 genera of the largest family within the Mysida, the Mysidae, were analysed based on 18S rRNA sequence data. The selected species represent a worldwide coverage of the three most important subfamilies in terms of numbers of species and /or genera i.e. the Siriellinae, the Gastrosaccinae and the Mysinae. This is particularly true for the large subfamily Mysinae (sensu Müller, 1993) that comprises 91% of the genera and 80% of all species classified within the Mysidae. No members of the subfamilies Boreomysinae (1 genus),

Rhopalophtalminae (1 genus) or Mysidellinae (3 genera) were included. However the selected species should already provide a basis for beginning to infer the molecular phylogeny of the family Mysidae. Indeed, the present data analysis provides a tool to test the morphology-based classification of the Mysidae. The large subfamily Mysinae, which contains many genera and species compared to other subfamilies, can be questioned as a natural group. A molecular approach can supply additional evidence for, or reject the monophyletic character of the Mysinae, which are represented here by five genera of the tribe Leptomysini and nine genera belonging to the Mysini. It is of particular interest to test the relationships between these tribes, in order to validate their phylogenetic strength. We show that both molecular and morphological evidence urges a taxonomic revision of the family Mysidae.

MATERIALS & METHODS

A total of 25 mysid species were analysed (Table 2.1) in addition to four outgroup species from other crustacean taxa. All samples were stored in ethanol (70 – 95%) at 4°C. Genomic DNA was extracted using a modified CTAB protocol (Kocher *et al*, 1989). Mysid tissue was crushed using a beadbeater and afterwards incubated for a minimum of 3 hours at 60°C in 500 µl CTAB buffer with 6 µl proteinase K (1 mg 100 μl^{-1}). After an overnight incubation at 37°C the DNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1 PH 8) and chloroform:isoamylalcohol (24:1). Finally, DNA was precipitated with isopropanol and rehydrated in 25 µl water. Small aliquots of extracted nucleic acids were used as template for polymerase chain reaction amplification (PCR). The 18S ribosomal gene (1990 bp) was amplified using the 5'-EM (5'-TYC CTG GTT GAT YYT GCC AG-3') and 3'-EM (5'-TGA TCC TTC CGC AGG TTC ACC T-3') primers (Weekers *et al*, 1994). Cycle conditions were 95°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min for 35 cycles. PCR amplification products were sequenced using a Perkin Elmer ABI Prism 377 automated DNA sequencer. PCR product was treated with shrimp alkaline phosphatase (1 U/µl, Amersham E70092Y) and exonuclease I (20 U/µl, Epicentre Technologies X40505K) for 15 minutes at 37 °C, followed by 15 minutes at 80 °C to inactivate enzymes. This material was then used for cycle sequencing without any further purification, using the ABI Prism BigDye Terminator Cycle Sequencing kit. The sequencing conditions were 30 sec at 96 °C, 15 sec at 50 °C and 4 min at 60 °C for 27 cycles. Cycle sequence products were precipitated by adding 25 µl of 95 % ethanol and 1 µl 3 M sodium acetate, pH 4.6 to each cycle sequencing reaction (10 µl). The samples were placed at –20 °C for 15 minutes and centrifuged at 14,000 rpm for 15 minutes. After precipitation, an additional wash of the pellet was performed with 125 µl of 70 % ethanol and centrifuged at 14,000 rpm for 5 minutes. The pellet was dried in a Speedvac concentrator, redissolved in loading buffer and run on a 48 cm 4.25 % acrylamide:bisacrylamide (29:1) gel. All sequences have been submitted to EMBL (accession numbers: AJ566084-AJ566109).

Four 18S ribosomal RNA sequences of the more or less closely related crustaceans *Diastylis* sp. (Peracarida, Cumacea), *Euphausia pacifica* (Eucarida, Euphausiacea), *Squilla empusa* (Hoplocarida, Stomatopoda) and *Nebalia* sp. (Leptostraca, Nebaliida) were obtained from GenBank and used as outgroups in the

analysis. All sequences were aligned with ClustalX (Version 1.74, Thompson *et al*, 1997) using the default settings (pairwise alignment parameters: Slow-Accurate pairwise alignment method, Gap opening penalty= 15.00, Gap extension penalty= 6.66, IUB DNA weight matrix; and multiple alignment parameters: Gap opening penalty= 15.00, Gap extension penalty= 6.66, Delay divergent sequences= 30%, DNA transition weight= 0.50), followed by limited manual editing to improve inferences of positional homology. Parsimony analysis was performed using PAUP 4.0b10 (Swofford, 2001) with the following heuristic search settings: 100,000 random taxon addition replicates followed by tree-bisection-reconnection (TBR) branch swapping. Nodal support was assessed by calculating bootstrap values (Felsenstein, 1985) from 1,000 bootstrap replicates obtained by heuristic search with 10 random sequence addition replicates each. In addition, taxon jackknifing was performed to assess the effects of taxon sampling on the tree resolution (Lanyon, 1985). In this analysis, individual taxa were sequentially removed and the resulting data set of n-1 taxa was analyzed using parsimony with 1,000 random addition replicates. All Jackknife generated trees were evaluated manually by comparing the nodes in each consensus tree with those in the bootstrapped parsimony tree generated by the full data set.

The likelihood ratio test in MODELTEST 3.06 (Posada & Crandall, 1998) was used to determine the model of DNA evolution that best fitted the dataset. Based on this test, the general time-reversible substitution model with a discrete gamma correction for among site variation and corrected for invariable sites (GTR + G + I model) (Rodriguez *et al*, 1990) was chosen for maximum likelihood analysis. ML was performed using the heuristic search option with TBR branch swapping, MulTrees option in effect, no steepest descent, rearrangements limited to 10,000 and with 50 random sequence addition replicates. Bootstrap values were determined from 100 bootstrap replicates obtained by heuristic search with 10 random sequence addition replicates each.

Table 2.1: List of the different species used in this study with indication of the systematic position, geographic origin and GenBank sequence accession numbers.

Order	Family	Subfamily	Tribe	Species	Geographic origin	EMBL Accession No.
Mysida	Mysidae	Siriellinae		*Siriella armata* (Milne-Edwards, 1837)	coast of Apulia , Adriatic Sea, Italy	AJ566105
				Siriella clausii G.O. Sars, 1877	coast of Apulia , Adriatic Sea, Italy	AJ566107
				Siriella jaltensis Czerniavsky, 1868	coast of Apulia , Adriatic Sea, Italy	AJ566106
		Gastrosaccinae		*Anchialina agilis* (G.O. Sars, 1877)	Belgian continental shelf, Belgium	AJ566089
				Archaeomysis japonica Hanamura, Jo & Murano,1996	Otsuchi bay, Japan	AJ566084
				Archaeomysis kokuboi II, 1964	Otsuchi bay, Japan	AJ566085
				Bowmaniella sp.	Valdivia beach, Guayas province, Ecuador	AJ566086
				Gastrosaccus psammodytes Tattersall, 1958	Algoa bay, South Africa	AJ566087
				Gastrosaccus spinifer (Goës, 1863)	Westerschelde, The Netherlands	AJ566088
		Mysinae	Leptomysini	*Americamysis bahia* Molenock, 1969	West coast USA	AJ566095
				Leptomysis lingvura adriatica (G.O.Sars, 1866)	Pilone estuary, Adriatic Sea, Italy	AJ566098
				Leptomysis lingvura lingvura (G.O.Sars, 1866)	Belgian continental shelf, Belgium	AJ566099
				Metamysidopsis sp.	Valdivia beach, Guayas province, Ecuador	AJ566096
				Mysidopsis sp.	Valdivia beach, Guayas province, Ecuador	AJ566094
				Mysidopsis gibbosa (G.O. Sars, 1864)	Belgian continental shelf, Belgium	AJ566097
			Mysini	*Acanthomysis longicornis* (Milne-Edwards, 1837)	Westerschelde, The Netherlands	AJ566093
				Diamysis mesohalobia mesohalobia Ariani & Wittmann, 2000	coast of Apulia , Adriatic Sea, Italy	AJ566100
				Hemimysis anomala Sars, 1907	Danube river, Austria (orig. Caspian Lake)	AJ566104
				Holmesimysis costata Holmes, 1910	West coast USA	AJ566090
				Limnomysis benedeni Czerniavsky, 1882	Danube River, Austria	AJ566101
				Neomysis integer (Leach, 1814)	Westerschelde, The Netherlands	AJ566091
				Paramesopodopsis rufa Fenton, 1985	Taroona beach, Tasmania	AJ566108
				Praunus flexuosus (Müller, 1776)	Westerschelde, The Netherlands	AJ566102
				Schistomysis kervillei (Sars, 1885)	Belgian continental shelf, Belgium	AJ566103
				Schistomysis spiritus (Norman, 1860)	Voordelta, The Netherlands	AJ566109
Euphausiacea	Euphausiidae			*Euphausia pacifica* Hansen, 1911	N.A./from EMBL database	AY141010
Cumacea	Diastylidae			*Diastylis* sp.	N.A./from EMBL database	Z22519
Leptostraca	Nebaliidae			*Nebalia* sp.	N.A./from EMBL database	L81945
Stomatopoda	Squillidae			*Squilla empusa* Smith, 1958	N.A./from EMBL database	L81946

RESULTS

Sequence data and alignment

A total of 25 different mysid species were sequenced, the length of the mysid 18S rRNA gene varies between 1,788 bp (*Schistomysis spiritus*) and 1,811 bp (*Archaeomysis japonica*). GC content varies between 46.6% (*Acanthomysis longicornis*) and 49.8% (*Anchialina agilis*), and has an average of 48.6%. The block of aligned 18S rRNA sequences contains 1,889 positions; 1,175 (62.2%) characters are constant, 439 (23.2%) are parsimony non-informative and 275 (14.6%) are parsimony informative. No obvious large expansion segments are observed within the aligned 18S sequences.

Parsimony analysis

The parsimony (MP) analysis with heuristic search generated three most parsimonious trees of 2,192 steps (consistency index= 0.5132, retention index= 0.5266, rescaled consistency index= 0.2703) that had some topological changes. The strict consensus MP tree is shown in Fig. 2.1. The subfamily Gastrosaccinae is resolved as a paraphyletic group, while the Siriellinae are resolved as a well-defined monophyletic clade supported by high bootstrap values (100%) (Fig. 2.1). The relationships within the subfamily Gastrosaccinae are less clear, two most parsimonious trees suggests that *Bowmaniella* sp. is more closely related to the genus *Archaeomysis* than to *Gastrosaccus*, while the other tree suggest the opposite (trees not shown). The analysis also shows that the subfamily Mysinae, represented by the tribes Mysini and Leptomysini, is polyphyletic. One group of species belonging to the tribe Mysini (Mysini-A-group) forms a monophyletic clade that is closely related to the subfamily Siriellinae (Fig. 2.1). The MP analysis fails to resolve the two species of the genus *Schistomysis* as sister taxa. The three other species of this tribe (*Neomysis integer*, *Holmesimysis costata* and *Acanthomysis longicornis*) form a clade (Mysini-B-group) closely related to the species of the tribe Leptomysini (Fig. 2.1). It should also be noted that the genus *Mysidopsis* is resolved as a paraphyletic taxon by the MP analysis. Few trees obtained from the parsimony analysis with taxon jackknifing displayed deviations from the strict consensus MP tree. In particular the exclusion of

the ingroup species *Gastrosaccus psammodytes*, *Bowmaniella* sp. and *Anchialina agilis* and the outgroup species *Squilla empusa* caused changes in the position of Gastrosaccinae and Siriellinae and the relationships within the Mysini-A clade.

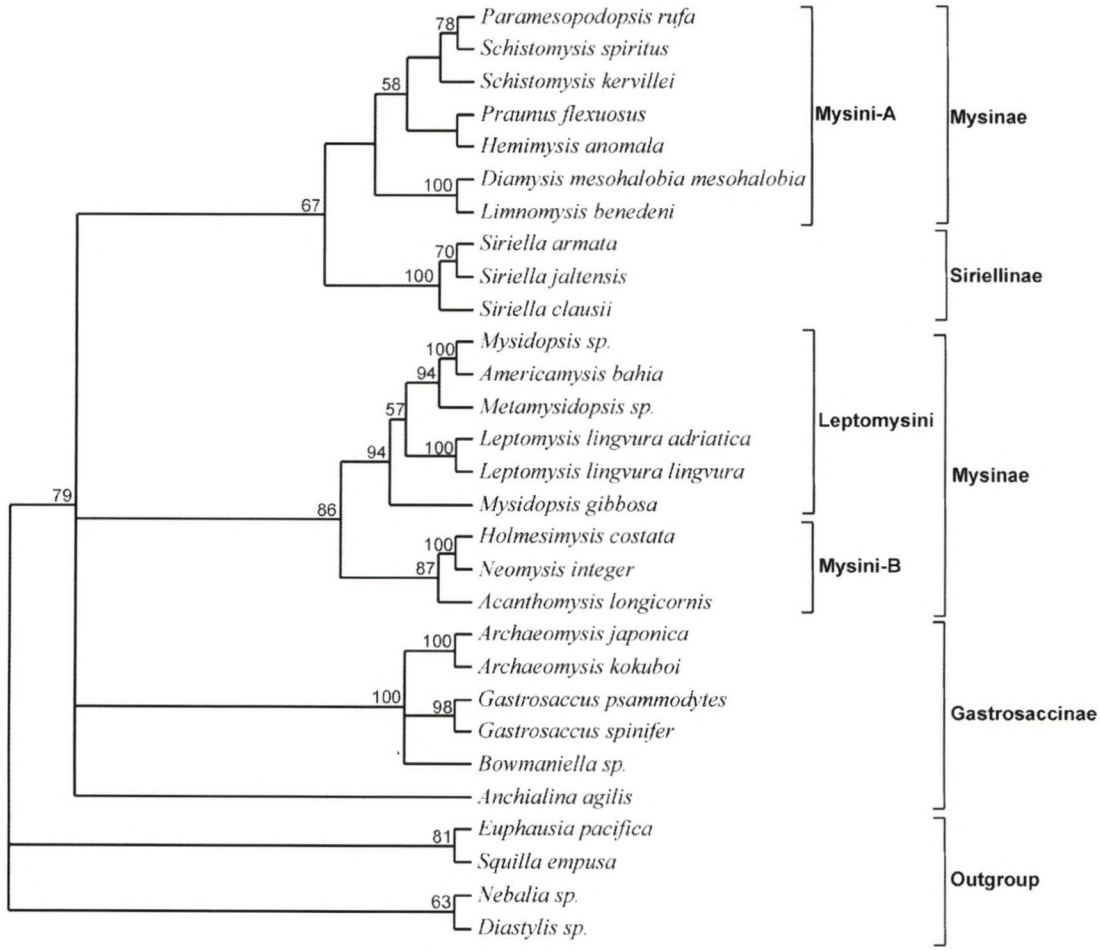

Fig. 2.1: Strict consensus Maximum Parsimony tree of 2192 steps obtained after 100,000 replicates (CI= 0.5132, RI= 0.5266, RC= 0.2703). The numbers along the branches indicate MP bootstrap support, only bootstrap values higher than 50% are shown.

Maximum likelihood analysis

Maximum likelihood (ML) analysis was performed using the GTR + *G* + *I* model of molecular evolution with following values: substitution rates *R*= (1.1617, 2.2699, 1.4924, 0.646 and 4.569), proportion of invariable sites= 0.3798 and gamma shape parameter, α= 0.4756. The most likely tree had a -lnL = 12,677.09 and is shown in Fig 6.2. The subfamilies Siriellinae and Gastrosaccinae are each monophyletic, the latter only with 68% bootstrap support (Fig. 2.2). Interestingly, the

Gastrosaccinae are now shown as a sister group to all other subfamilies. Also the ML tree confirms the morphology-based grouping of the genera within the subfamily Gastrosaccinae: *Bowmaniella* sp. is more closely related to the genus *Archaeomysis* than to *Gastrosaccus*. The polyphyly of the tribe Mysini within the subfamily Mysinae is indicated by the ML tree, with the split of the tribe Mysini in two different clades (Mysini-A and Mysini-B) as proposed by the MP analysis being confirmed by ML. The tribe Leptomysini is also resolved by ML as a monophyletic clade, and again the genus *Mysidopsis* is shown as a paraphyletic taxon. ML, unlike MP, supports the monophyly of the genus *Schistomysis*.

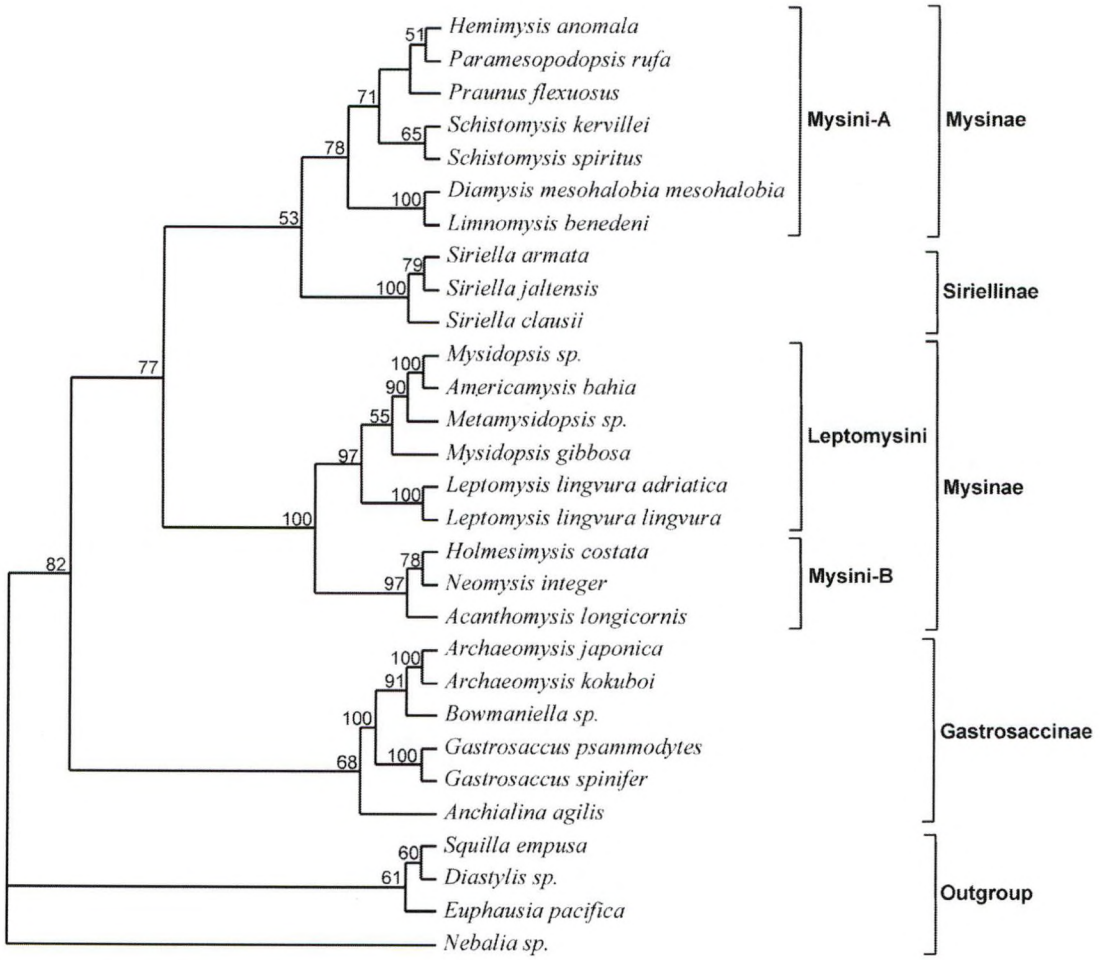

Fig. 2.2: Heuristic Maximum Likelihood tree based on the GTR + *G* + *I* model of sequence evolution and with -lnL = 12,677.09. The parameters were: nucleotide frequencies: A=0.2488, C=0.2171, G=0.2701, T=0.264; substitution rates *R*= (1.1617, 2.2699, 1.4924, 0.646 and 4.569); proportion of invariable sites= 0.3798 and gamma shape parameter, α= 0.4756. The numbers along the branches indicate ML bootstrap support, only bootstrap values higher than 50% are shown.

DISCUSSION

The family Mysidae is divided into six subfamilies of which only three were represented in this study: Siriellinae, Gastrosaccinae and Mysinae. In terms of numbers of species and genera these three subfamilies can be considered as the most important groups of the family, although the omission of the other three subfamilies (Boreomysinae, Rhopalophthalmidae and Mysidellinae) lowers the value of the analysis in terms of general conclusions on phylogenetic relationships within the whole family.

According to the different methods (MP and ML) applied here to reconstruct phylogenetic relationships, the subfamily Siriellinae can be considered as a monophyletic clade. Some typical morphological characteristics support the monophyly of this group: the exopod of the uropod is devided into two segments, the mandibular molar process is reduced, the marsupium consists of three oostegites and males of almost every species have the typically spirally coiled pseudobranchiae at the pleopods (morphological data was taken from the NeMys database, http://intramar.ugent.be/nemys, see also Deprez *et al*, 2004)

The paraphyly of the Gastrosaccinae is caused by the deviant placement of *Anchialina agilis*. The group formed by members of *Bowmaniella, Archaeomysis* and *Gastrosaccus* can be considered as a well-defined monophyletic group. Morphologically this group of species (the "*Gastrosaccus*–group") indeed displays several differences with members of the genus *Anchialina*. Common characteristics for the whole subfamily are the presence of a spine on the antennal scale (which is setose all around), the typical shape of the telson (with a cleft, armed with spines, without setae), and the presence of spine on the labrum (absent in all other Mysinae species). Considering the combination of these characteristics taxonomists grouped the *Anchialina* species within the Gastrosaccinae subfamily although there are morphological differences, mainly in pleopod structures. Within the genus *Anchialina* the first pair of thoracopods bears a strongly developed claw on the dactylus, uniramous female pleopods are present and the third pair of the male pleopods has an only slightly elongated exopod. In the "*Gastrosaccus*-group" at least the first pair of female pleopods are uniramous and in members of *Archaeomysis* and *Bowmaniella* also the second to the fifth pair are biramous. This may be an argument why in two of the three most parsimonious trees (Fig. 2.1, MP tree #2&3) and in the ML analysis

(Fig. 2.2) *Bowmaniella* sp. is closer related to *Archaeomysis* than to *Gastrosaccus*. Members of *Anchialina* posses an uniramous first male pleopod while all male pleopods are biramous in the "*Gastrosaccus*-group".

Morphological evidence strongly suggests that the genus *Gastrosaccus* is the sister group to the genera *Bowmaniella* and *Archaeomysis*, which is partly supported by our molecular analysis (ML analysis). Biramy is considered to be more ancestral then uniramy (e.g. Wilson, 1989). By this criterion *Bowmaniella* and *Archaeomysis* are assumed to be more closely related to the ancestral form, while members of *Gastrosaccus* are more derived. Based on these morphological characteristics we can also classify the members of the subfamily Gastrosaccinae not included in this study either in the "*Anchialina*-group" (e.g. *Pseudanchialina* Hansen, 1910 and *Paranchialina* Hansen, 1910 species) or in the "*Gastrosaccus*-group" (e.g. *Haplostylus* Kossmann, 1880 and *Iiella* Bacescu, 1968 species). Already in 1882 Czerniavsky erroneously created the "divisio Anchialidae" (= tribe Anchialini in current terminology; this taxon was rejected by subsequent authors) based on the morphological characteristics that diverge the *Anchialina* species from the "true" Gastrosaccinae. A more profound study that would include more species might provide additional evidence for the creation of two monophyletic subfamilies as also indicated by our molecular analysis.

The subfamily Mysinae, represented in this study by the tribes Mysini and Leptomysini, consistently resolves into three clades (Leptomysini: 1 clade; Mysini: 2 clades) and hence is clearly not monophyletic. This subfamily was originally split into different tribes based on morphological characteristics (Hansen, 1910; Tattersall, 1955; Ii, 1964; Bacescu & Iliffe, 1986). Only two of the six tribes (Leptomysini with 31 genera and Mysini with 52 genera) are represented in our analysis. The subfamily Mysinae comprises the largest number of species (806) and genera (143) of the entire family Mysidae (157 genera, 1004 species) and even of the order Mysida (165 genera and 1053 species). The division into different tribes permitted structuring of this large subfamily, but the taxonomic value is doubtful – as reflected in our analysis.

Relationships within the Mysini are much less straightforward, since two clades are resolved in the analyses. One group includes *Praunus flexuosus, Hemimysis anomala, Schistomysis kervillei, S. spiritus, Limnomysis benedeni, Diamysis mesohalobia mesohalobia* and *Paramesopodopsis rufa* (Mysini-A-group). The other group includes the species *Neomysis integer, Holmesimysis costata* and *Acanthomysis*

longicornis (Mysini-B-group) and appears to be more closely related to the Leptomysini than to the Mysini-A-group. This is confirmed by the topology of all tree construction methods. The Mysini are usually differentiated based on the following morphological characteristics: the second male pleopod is rudimentary and uniramous, and the fourth male pleopod is elongated and mostly modified. The uniramous character of the second male pleopod constitutes the most important difference between the tribes Mysini and Leptomysini. Morphological indications for the splitting of the Mysini in two separate clades can be found in the exopod on the third male pleopod which is reduced in the Mysini-B-group whereas in the Mysini-A-group this structure is either slightly or well developed, and a cleft in telson is present. The Mysini-B-group seems to correspond to the definition of the tribe Mysini by Hansen (1910): the exopod of the male third pleopod has one or two segments, and mostly an entire telson. The genera *Acanthomysis*, *Neomysis* and *Holmesimysis* display a very similar appearance, causing their pooling under a single generic name, *Neomysis* (Zimmer, 1915) in the past.

The Mysini-A-group comprises three species (*H. anomala, D. mesohalobia mesohalobia, L. benedeni*) that have calcareous (as the mineral vaterite) statoliths. The remaining four species of this group (*S. kervillei, S. spiritus, P. flexuosus* and *P. rufa*) precipitate fluorite, as do the great majority of Mysidae. Although weakened by the absence of some essential taxa (e.g., *Mysis*, *Paramysis*) the present molecular analysis is in keeping with the conclusion of Ariani *et al* (1993) that within the Mysini both calcareous and fluorite statoliths originate from common ancestors. These ancestors had the ability or predisposition to form calcareous statoliths, favouring a phylogenetically rapid shift of statolith mineral composition from fluorite to vaterite. The actual distribution of the mineral types (vaterite versus fluorite) seems to be paraphyletic with respect to the true phylogeny (i.e., mineral type represents analogy, not homology). The grouping of the closely related genera of Mysini in a '*Diamysis* group' (*Diamysis, Limnomysis* and *Antromysis* Creaser, 1936) and the '*Paramysis* group' (*Paramysis* Czerniavsky, 1882; *Katamysis,* Sars, 1877 and *Schistomysis*) based on features of antennal scale and male pleopods as suggested by Ariani *et al* (1993) is also confirmed by our molecular analysis. However, more detailed molecular and morphological analyses covering members of the other tribes are needed to reach a more detailed and correct view of the genealogy of the different clades within the Mysidae.

The tree topology for the Leptomysini is nearly identical in all analyses. Morphological evidence suggests that *Mysidopsis* sensu Sars (1864) is more closely related to *Leptomysis*. The genera *Metamysidopsis*, *Brasilomysis* Bacescu, 1968 and *Americamysis* were more recently created and in many cases are synonymous with *Mysidopsis* species (e.g. *Americamysis almyra* was formerly known as *Mysidopsis almyra* Bowman, 1964; *Metamysidopsis munda* was formerly known as *Mysidopsis munda* Zimmer, 1918). However, even after later revisions the genus *Mysidopsis* sensu Price seems to remain a paraphyletic mixture of species (Price *et al*, 1994). This is consistent with our results and indicates that this genus is taxonomically not well defined and needs to be profoundly revised.

Based on molecular and morphological arguments we can conclude that the subfamily Siriellinae is a well-defined taxonomic unit. On the other hand the subfamily Gastrosaccinae is found to be paraphyletic and a split in two monophyletic subfamilies (the "*Gastrosaccus*-group" and the "*Anchialina*-group") should be considered. The third subfamily present in this study, Mysinae, represented here by the tribes Mysini and Leptomysini, is clearly not monophyletic. A revision of the Mysini is suggested in order to tune taxonomy to phylogenetic relationships based on morphological and molecular data. On the other hand the tribe Leptomysini appears to be a well-defined taxonomical unit, although a revision of the genus *Mysidopsis* and its related genera (e.g. *Metamysidopsis*, *Americamysis*) is needed. Obviously, future research should include more genes and more species, since the selection of taxa has a large and unpredictable effect on phylogeny (Lecointre *et al*, 1993). First, a sufficient number of representatives of the subfamilies Boreomysinae, Rhopalophthalmidae and Mysidellinae, not included here, should be analysed to evaluate the taxonomic rigidity of the Mysidae. Second, species belonging to all existing tribes within the subfamily Mysinae must be included to assess the value of these taxonomical units as well as their relations.

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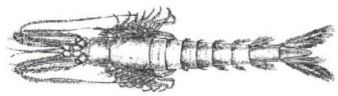
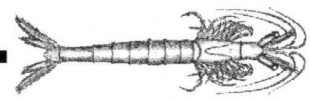
CHAPTER III



Evidence of genetic differentiation of the brackish water mysid *Neomysis integer* (Crustacea, Mysida) concordant with Pleistocene glaciations. Pilot study



Submitted for publication as:

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ABSTRACT

The genealogical relationships and distribution of molecular variation of the mysid *Neomysis integer* was examined throughout most of its geographical range, in order to interpret phylogeographic patterns. *N. integer* (Leach, 1814) is a common hyperbenthic species that typically dominates the brackish part of estuaries and occurs along the northeastern Atlantic from the Baltic Sea to Morocco. As a pilot study, nine samples, comprising 45 individuals, were collected across the species' range of distribution, and sequenced using a segment of 390 base pairs of the mitochondrial cytochrome *b* gene. A clear geographic structuring was found with one common haplotype occurring in most samples, while two samples (the Guadalquivir and Gironde estuary) consist solely of unique variants. At the southern distribution range a remarkable genetic break was observed between the Guadalquivir population and all other samples. These findings are discussed in the perspective of the presence of glacial refugia and postglacial recolonisation routes of low-dispersal organisms along the northeastern Atlantic coasts.

INTRODUCTION

The population genetic structure of a species tends to be determined by current population dynamics like contemporary gene flow, as well as by historical patterns of gene flow shaped by past climate events (Avise *et al,* 1987). Climate oscillations during the Pleistocene were responsible for a series of contractions and expansions of species ranges all over the world, particularly as documented for terrestrial species of northern temperate regions (Hewitt, 1996, 2000). Decreased temperatures in these regions during ice ages pushed the geographical distribution of many species to southern regions (Hewitt, 2000). At the end of the last glacial maximum (18 kya) the warming climate and the retreat of the glaciers led to the rapid migration of species out of refugial areas as they spread into previously unavailable or unsuitable habitats. Evidence and effects of these contractions and expansions on the genome have been reported for several marine species (e.g. Dawson *et al*, 2001; Edmands, 2001; Wares, 2002). Although the number of phylogeographic studies along the northeastern Atlantic coasts is growing, most studies have focused on postglacial colonisation routes of fish like anadromous salmonids (Verspoor *et al*, 1999; Consuegra *et al*, 2002), highly vagile mackerel (Nesbø *et al,* 1999), flounder (Borsa *et al,* 1997a) and small demersal gobies (Gysels *et al,* 2004). Phylogeographic information of marine invertebrates along the northeastern Atlantic remains scarce and is mostly focused on species with high dispersal capacities like bivalves (e.g. Nikula & Väinölä, 2003; Luttikhuizen *et al*, 2003), gastropods (Wilke & Davis, 2000), krill (Zane *et al*, 2000). Despite the importance of these studies in gaining knowledge of the molecular diversity and population genetic structuring, it is quite possible that the signatures of the Pleistocene glaciations have been erased in high gene flow species. On the other hand, species with restricted levels of gene flow are often composed of genetically and geographically highly structured populations, which in general are shaped by past palaeoclimatological events. Consequently, the present study may provide insights in the impact of Pleistocene glaciations on coastal populations of species with limited dispersal capacity.

Neomysis integer (Leach, 1814) is one of the most common mysids around the northeastern Atlantic coasts (from the Baltic Sea to Morocco). It is a hyperbenthic, euryhaline and eurythermic species, typically occurring in high numbers in estuarine, brackish water environments (Tattersall & Tattersall, 1951; Mauchline, 1971a).

Several recently published studies indicate *N. integer* as the dominant species in the brackish part of West European estuaries, both in terms of densities and biomass (Mees *et al,* 1995; Cunha *et al,* 1999). It is an omnivorous species which mainly utilizes mesozooplankton and detritus carbon pools (Fockedey & Mees, 1999), and an important prey for demersal and pelagic fish and larger epibenthic crustaceans (Mauchline, 1980; Hostens & Mees 1999). As such it is believed to be a key species in the ecosystems of the brackish part of estuaries. Like most mysids, *N. integer* is a brooder. Females possess a brood pouch (marsupium) attached to the bases of the pereiopods in which the eggs hatch and the young develop until they can survive individually. Little is known about the dispersal capacities of *N. integer*, since studies provide evidence for migration (tidal, diel, seasonal) only over small geographic scales (10 km), but not for greater distances (Mauchline, 1980; Mees *et al*, 1993b). Since *N. integer* lives in discrete brackish water habitats and lacks a planktonic dispersal stage, it has been assumed that long-range dispersal events are probably rare (Mauchline, 1980). If this hypothesis is true this should result in low rates of current gene flow. These putative low dispersal features render *N. integer* useful to study phylogeographic patterns along the northeast Atlantic coasts, and may clarify the impact of climate oscillations on the patterns of genetic variation in marine species.

Nothing is known about the genetic diversity in mysids on a macrogeographic scale along the northeastern Atlantic coast. Using mtDNA sequences we address the following issues: firstly, since the dispersal abilities of *N. integer* are probably very limited, the patterns of genetic variation that were created during the establishment of its current distribution should have been preserved. This will probably produce geographically highly structured populations. Secondly, haplotype distribution should reflect postglacial recolonisation routes and possible glacial refugia for *N. integer*. The present distribution range of *N. integer* is necessarily the result of a northward range expansion after deglaciation, since northern Europe was unsuitable as habitat during glacial periods (see Fig. 3.1 for a reconstruction of the sea-level during the last glacial maximum). We expect that *N. integer* survived the Pleistocene glaciations in a glacial refugium along the Iberian and north African coasts, as proposed for other marine species such as salmon and brown trout (García-Marín *et al,* 1999; Consuegra *et al,* 2002). However, the possible existence of small northern refugia, located in ice-free habitats in the English Channel and southern North Sea, as proposed for salmonids (García-Marín *et al,* 1999; Verspoor *et al,* 1999), gobies (Gysels *et al,*

2004) and snails (Wilke & Davis, 2000) cannot be excluded. If so, the signature of a postglacial recolonisation event from multiple refugia may also be observed in the phylogeographic pattern of *N. integer*.

MATERIALS AND METHODS

Sampling

Specimens of *Neomysis integer* were collected from 9 European estuaries (Fig. 3.1). Samples from each estuary were collected with a hand net or a hyperbenthic sledge (mesh size 1 mm). After collection, the samples were stored in ethanol (70 – 95%) or acetone at 4°C.

DNA extraction, PCR and Sequencing

DNA was extracted using a modified CTAB protocol (Kocher *et al,* 1989). Mysid tissue was crushed using a beadbeater and afterwards incubated for minimum 3 hours at 60°C in 500 µl CTAB (2% (w/v) CTAB, 1.4M NaCl, 0.2% (v/v) mercaptoethanol, 20 mM EDTA, 100 mM Tris/HCl pH 8) with 6 µl proteinase K (1 mg 100 μl^{-1}). After an overnight incubation at 37°C, the DNA was purified with phenol/chloroform/isoamylalcohol (25:24:1 PH 8) and chloroform:isoamylalcohol (24:1). Finally, DNA was isopropanol-precipitated and rehydrated in 25 µl water. Small aliquots of extracted nucleic acids (1 µl) were used as template for polymerase chain reaction amplification (PCR). The conditions for the cyt *b* amplifications were: 10 x PCR buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2mM dNTP, 1 µM forward and reverse primer and 1.25 units Taq polymerase. Cytochrome *b* amplifications used the universal molluscan primers 151F (5'-TGTGGRGCNACYGTWATYACTAA-3') and 270R (5'-AANAGGAARTAYC AYTCNGG YTG-3') (Merritt *et al.*, 1998). The following thermocycle profile was used: denaturation of template DNA at 94°C for 2 min, followed by a stepdown PCR of 4 cycles (30s at 94°C, annealing at 53°C for 90 s, extension at 72°C for 90 s) with a decrease in annealing temperature of 1°C for each cycle, followed by 40 cycles of 30 s at 94°C, 90 s at 49°C and 2 min at 72°C, followed by a final extension of 5 min at 72°C. A small aliquot (5 µl) of each amplification was loaded on a 1 % agarose gel, stained with ethidium bromide, and

visualized under UV light. PCR products were purified with exonuclease I (10 U μl^{-1}; Amersham) and shrimp alkaline phosphatase (1 U μl^{-1}; Amersham). Purified products were cycle sequenced using BigDye Terminator Mix (PE Applied Biosystems) and following conditions: 25 cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Cycle sequencing products were electrophoresed on a Perkin-Elmer ABI Prism 377 DNA sequencer. After trimming both ends of the sequences we obtained a fragment of 390 bp.

Data analysis

Alignment of the data was produced with the Clustal X program (Version 1.74, Thompson *et al,* 1997). When needed, the alignment was manually corrected with the program GeneDoc Version 2.6 (Nicholas & Nicholas, 1997). A parsimony network between the haplotypes was constructed to visualize evolution among haplotypes, haplotype frequency and geographical representation with the program TCS (version 1.13, Clement *et al*, 2000). This method estimates an unrooted tree and provides a 95% plausible set for all haplotype connections within the unrooted tree. Overall levels of molecular diversity (nucleotide and haplotype diversity, π and h respectively) were calculated using ARLEQUIN 2.0 (Schneider *et al*, 2000). An analysis of molecular variance (AMOVA) was used to examine hierarchical population structure (Excoffier *et al*, 1992). This method was used to partition the genetic variance into components of within population, among individuals, and among population differences. In addition an AMOVA was performed on different groups of samples in order to detect further significant geographic group structure. All analyses were performed using 10,000 permutations with the ARLEQUIN 2.0 software (Schneider *et al*, 2000).

RESULTS

A total of 8 haplotypes was observed among the 45 individuals analysed from 9 different locations along the northeastern Atlantic. Sixteen variable positions (4.1%) between the different haplotypes were recorded, including 9 (2.3%) parsimony-informative characters. All the polymorphisms were due to single nucleotide changes, and all but three of them were transitions. Most nucleotide changes were silent mutations, corresponding to transitions at the 3rd codon position. Four nucleotide changes involved 2nd and 1st codon positions, producing amino acid changes (Table 3.1). Considering that mutations on the 2nd codon are extremely rare, we re-sequenced these putative haplotypes. However, this yielded the same results, confirming that these are genuine haplotypes and not a laboratory artefact. Pair-wise DNA differences between haplotypes ranged from 0.26% (a single substitution) to 2.82% nucleotide divergence (11 base substitutions). The overall haplotype diversity (h) was 0.665 and nucleotide diversity (π) amounted to 0.00679.

Table 3.1: Variable nucleotide positions of the 8 cyt *b* haplotypes (A – H) observed in *Neomysis integer* with indication of the EMBL Accession numbers of the haplotypes, parsimonious sites (P) and the codon positions. aa1 and aa2 indicate the amino acid changes in both haplotypes after mutation (amino acid codes: T=Threonine, A=Alanine, M=Methionine, V=Valine, G=Glycine, P=Proline, S=Serine).

Haplotype	4	8	52	58	73	76	199	229	255	256	307	316	325	357	362	367	EMBL N°
A	A	A	G	G	C	G	T	C	C	A	C	A	T	T	C	G	AJ549186
B	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	AJ549187
C	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	AJ549188
D	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	AJ549189
E	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	AJ549190
F	.	.	C	A	T	.	C	T	.	.	T	.	C	.	.	A	AJ549191
G	.	.	C	A	T	.	C	T	T	.	T	.	C	.	.	A	AJ549192
H	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	AJ549193
			P	P	P	P	P	P			P		P			P	
codon pos.	3	1	3	3	3	3	3	3	2	3	3	3	3	2	1	3	
aa1		T							T					V	P		
aa2		A							M					G	S		

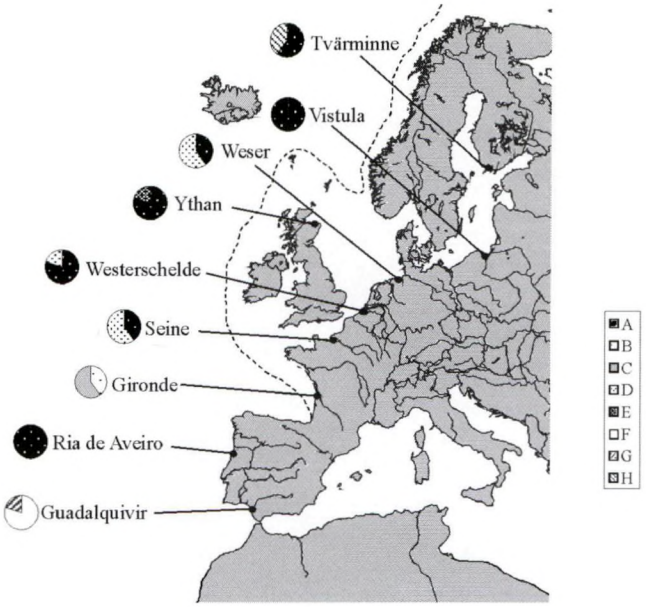

Fig. 3.1: Geographic location of sampling sites and relative frequency of the different haplotypes at each location. Dashed line indicates the shoreline during the last glacial maximum (18 kya) (redrawn from Frenzel *et al,* 1992).

The geographic distribution of the 8 haplotypes is shown in Table 3.2 and Figure 3.1. The most common haplotype A (n = 25), present in more than half of the analysed specimens, was observed at all locations except in the Gironde and the Guadalquivir, the most southern location. The second most frequent haplotype D was observed at 3 different locations: Seine, Westerschelde and Weser. Of the 8 haplotypes identified, 2 were singletons (E and G, i.e. represented by only one individual). Four of the 9 locations studied possessed unique haplotypes (Tvärminne, Ythan, Gironde and Guadalquivir) and in two of them (Gironde and Guadalquivir) solely unique haplotypes were found.

Table 3.2: Distribution of the cyt *b* haplotypes among the 9 locations studied, with indication of the sample size, total number of specimens analysed and number of haplotypes found in each location.

Location	Sample size	A	B	C	D	E	F	G	H	# haplo.
Tvärminne	5	3	0	0	0	0	0	0	2	2
Vistula	5	5	0	0	0	0	0	0	0	1
Weser	5	2	0	0	3	0	0	0	0	2
Westerschelde	5	4	0	0	1	0	0	0	0	2
Ythan	5	4	0	0	0	1	0	0	0	2
Seine	5	2	0	0	3	0	0	0	0	2
Gironde	5	0	2	3	0	0	0	0	0	2
Ria de Aveiro	5	5	0	0	0	0	0	0	0	1
Guadalquivir	5	0	0	0	0	0	4	1	0	2
TOTAL	45	25	2	3	7	1	4	1	2	

The evolutionary history of the observed haplotypes was determined by constructing a statistical parsimony network (Fig. 3.2). The most frequent haplotype A is located in the center of the network and all other haplotypes, except two (F and G), are connected by one or two mutation steps. This suggests that haplotype A is the ancestral haplotype from which the haplotypes B, C, D, E and H have more or less recently, radiated. Haplotypes F and G, unique in the Guadalquivir sample, are 8-10 mutation steps separated from all other haplotypes, which corresponds with a significantly large phylogeographic break.

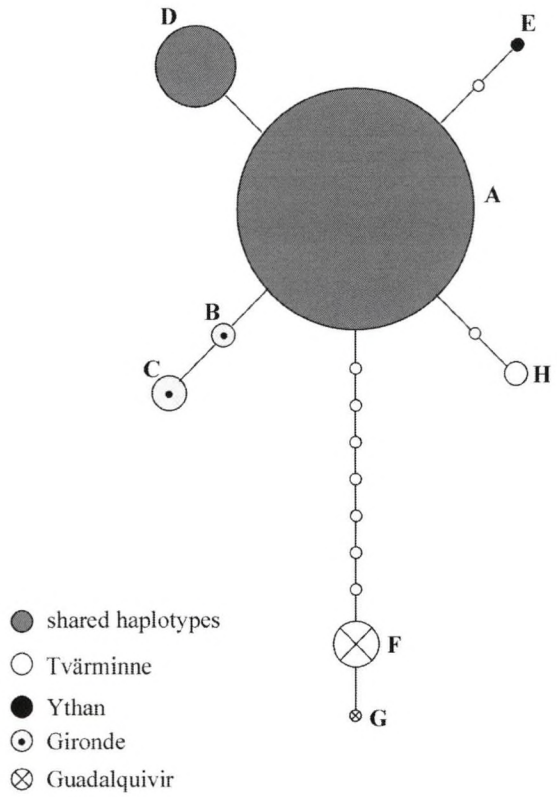

Fig. 3.2: Statistical parsimony network among cyt *b* haplotypes found in *Neomysis integer*. Branches connecting circles represent mutation steps and the small open circles indicate missing haplotypes. The area of each circle is representative of the frequency with which the haplotypes occurred in the total sample. Circles are shaded according to their geographic occurrence.

The results of the AMOVA analyses are summarized in Table 3.3. For the analysis conducted on all samples, most of the variation (78.42%, $P<0.001$) is found among the populations. The overall fixation index (Φ_{ST}) amounts to 0.7842, which points to a high genetic structuring. However, much of the apparent among-population structure is due to the inclusion of the very divergent haplotypes of the Guadalquivir

sample. If this sample is excluded from the AMOVA analysis, the among-population component decreases by nearly half, to 43.58 %, with a corresponding increase in the within-population component. However the null hypothesis of panmixia could still be rejected, with a Φ_{ST} value of 0.4358 ($P<0.001$). In order to assess whether any significant geographical structuring of samples could be detected, we performed an AMOVA dividing samples in three groups: (1) a 'northern group' with the Baltic samples (Tvärminne and Vistula) and the Scottish Ythan sample, (2) the southern North Sea and the English Channel samples (Seine, Weser and Westerschelde) and (3) the samples south of the English Channel (Gironde and Ria de Aveiro). The Guadalquivir sample was excluded to avoid distortion of the results because of its uniqueness. A significant amount of between-group variation was found (Φ_{CT}=0.1969, P=0.011). When grouping the samples in two groups, a northern group (Tvärminne, Vistula, Ythan, Weser, Westerschelde and Seine) and a southern group (Gironde and Ria de Aveiro), the among group variance component was slightly lower, albeit not significant (Φ_{CT}=0.1862, P=0.073).

Table 3.3: Results of the AMOVA analyses of mtDNA haplotype variation without and with geographic structuring of the samples. Note that in the AMOVA's with geographic structuring the Guadalquivir sample was excluded from the analyses. Sampling site abbreviations: TV, Tvärminne; VI, Vistula; YTH, Ythan; WE, Weser; WS, Westerschelde; SEI, Seine; GI, Gironde; RdA, Ria de Aveiro.

Analysis	Source of variation	% variation	Fixation indices	*P*
All samples				
	Among populations	78.42	Φ_{ST} = 0.7842	< 0.001
	Within populations	21.58		
Without Guadalquivir sample				
	Among populations	43.58	Φ_{ST} = 0.4358	< 0.001
	Within populations	56.42		
Two groups (TV, VI, YTH, WE, WS, SEI) (GI, RdA)				
	Among groups	18.62	Φ_{CT} = 0.1862	0.073
	Among populations within groups	30.96	Φ_{SC} = 0.3805	< 0.001
	Within populations	50.42	Φ_{ST} = 0.4958	< 0.001
Three groups (TV, VI, YTH) (WE, WS, SEI) (GI, RdA)				
	Among groups	19.69	Φ_{CT} = 0.1969	0.011
	Among populations within groups	26.67	Φ_{SC} = 0.3320	0.002
	Within populations	53.64	Φ_{ST} = 0.4635	< 0.001

DISCUSSION

The samples of *Neomysis integer* along the Atlantic coast show a clear geographic mtDNA structure with the following striking patterns: (1) the complete distinctness of the Guadalquivir sample, (2) the occurrence of one dominant haplotype (A) that is common to the Baltic Sea, the North Sea, the English Channel and the Portuguese estuary Ria de Aveiro, and (3) the apparent isolation of the Gironde sample, which consisted solely of 2 unique haplotypes which are closely related to the most frequent haplotype A. The high degree of differentiation between the Guadalquivir and all the other samples points to a large phylogeographic break in the area. The Gironde population has probably been isolated too, but to a lesser extent.

The ubiquitous distribution of the most common haplotype A along the sampled range, with exception of the Gironde and Guadalquivir samples, does not contradicts with our initial hypothesis about a postglacial northward range expansion from the proposed refugium along the coast of the Iberian peninsula. The interior position of haplotype A in the statistical pasimony network suggests this may be an ancestral haplotype for the northern group, from which the others radiated. Hence, it is not unlikely that haplotype A survived in the Iberian glacial refugium, as proposed for a variety of other marine and anadromous marine species, and spread out northward after deglaciation (Garcia-Marin *et al*, 1999; Consuegra *et al*, 2002).

However, our data indicates also that the Iberian Peninsula may not have acted as the only glacial refugium from where northern areas were colonized after the last glaciation. For example, the second most common haplotype D, which was not observed along the Portuguese coast, seems to be restricted to the English Channel and the Southern Bight of the North Sea (Seine, Westerschelde and Weser samples) and was found in a relatively high frequency in these samples (46%). A possible explanation for this distribution could be the existence of a 'northern' glacial refugium, possibly located in the Southern Bight of the North Sea. Since *N. integer* is a euryhaline and eurythermic species, it should be capable of surviving in ice-free shallow areas such as a large glacial lake that has existed in the southern North Sea during the Elsterian glaciation (late Middle Pleistocene, 450-420 kya). This ice-dammed lake had massive proportions; the Thames, Rhine, Meuse, Scheldt and possibly the Ems all discharged into it, and it remained unglaciated (Benn & Evans, 1998; Gibbard 1988). During the consecutive interglaciations and glaciations (Saalian

and Weichselian) the southern North Sea floor has been repeatedly submerged and emerged and there have been a series of estuarine like environments, at shifting locations where *N. integer* could have survived (Cohen, *pers com*), provided it was able to withstand the less than hospitable conditions that must have reigned in the area by then.

An alternative hypothesis would be that the postglacial recolonisation of northern European areas took place from a single highly polymorphic refugial population when sea level rose. However, detailed analysis of the mitochondrial COI gene, and comprising a larger amount of samples, are largely congruent with the present study and may support the hypothesis of recolonisation from multiple refugia (see Chapter 4). Our data are also in line with those for fish and invertebrates pointing to a glacial refugium in the English Channel or the Southern Bight of the North Sea (e.g. polychaetes: Breton *et al,* 2003; snails: Wilke & Davis, 2000; bivalves Luttikhuizen *et al,* 2003; sand and common goby: Gysels 2003, 2004; salmon: Verspoor *et al,* 1999 and brown trout: Garcia-Marin *et al,* 1999). Since the dominating haplotype observed within the Baltic Sea was the most common Atlantic haplotype A, an invasion by this haplotype from the North Sea after opening of the Danish Straits 8000 year ago (Björck, 1995) can be suggested.

The absence of the most common haplotype A and the exclusive presence of unique haplotypes in the Guadalquivir sample point to a complete isolation of this population for a considerable period of time. The genetic distances between the Guadalquivir haplotypes and all other Atlantic haplotypes (ranging from 0.021 to 0.032 using the Kimura 2-parameter model) seem to fall within the range of intraspecific variation when compared with genetic divergence values observed among other invertebrate taxa of different taxonomic levels (Rocha-Olivares *et al,* 2001) and among vertebrate taxa (Johns & Avise, 1998). When using a general mutation rate for crustacean mtDNA COI ranging from 1.4 to 2.6% of nucleotide divergence per million years (Knowlton *et al,* 1993; Patarnello *et al,* 1996), then the time of divergence of the Guadalquivir population corresponds to an estimated 0.78 – 1.23 million years ago (early Pleistocene). Due to the limited literature available on the Pleistocene palaeogeography of the Guadalquivir basin, we have no direct evidence for a historical change in coastal topography that could have caused a long-term isolation of the Guadalquivir *N. integer* population. Bearing in mind the limited sample size, the significant geographic structuring of genetic variation in the

mitochondrial cyt *b* gene of *N. integer* supports the expectation that low-dispersal species are highly structured genetically. Populations of species with brooding behaviour tend to be more differentiated than those with a planktonic dispersal stage (e.g. Breton *et al,* 2003; Wares, 2001). In addition to these biological restrictions to dispersal, the estuarine habitat of *N. integer* may also form a barrier to gene flow, since estuaries represent spatially discrete habitats that are isolated from each other by barriers to dispersal or physiological tolerance (Bilton *et al,* 2002). Local, genetically differentiated populations of typical brackish species have been demonstrated, which sometimes may lead to cryptic species complexes (see examples in Cognetti and Maltagliati, 2000 and Bilton *et al,* 2002). However, in order to draw more firm conclusions regarding the amount of gene flow, a much larger sample size is needed. Preliminary analyses of larger samples (see Chapter 4) confirm the apparent limited degree of gene flow between populations of *N. integer* along the Atlantic coasts of Europe. Likewise intriguing, albeit less pronounced, is the genetic differentiation of the Gironde sample. Despite the fact that two unique haplotypes were observed, their closer relationship to the other Atlantic haplotypes results in a reduction of the among population variation component in the AMOVA analysis. The low sample size doesn't allow us to make further inferences and hence additional analyses are needed to find out if the Gironde sample is part of a separate glacial refugial population.

CONCLUSIONS

Despite the fact that this study is based on a low number of individuals, which may result in an inaccurate measurement of molecular diversity values, an interesting population genetic differentiation along the distribution range of *Neomysis integer* was observed, with a remarkable genetic break between the Guadalquivir population and all other samples. The distribution of the haplotypes is concordant with a northward recolonisation from a southern glacial refugium. The presence of a haplotype which was so far only found in the English Channel and the North Sea may suggest an additional glacial refugium in this area. Alternatively, a single recolonisation event from a highly polymorphic population may offer an alternative explanation. Obviously, future research of other loci and more individuals per sampling site is needed to reach a more detailed view of the genetic structuring and the possible postglacial recolonisation routes of the mysid *N. integer*.

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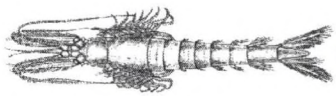
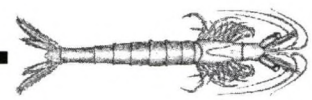
CHAPTER IV



Patterns of genetic diversity, contemporary gene flow and postglacial colonisation history of a low dispersal mysid, *Neomysis integer* (Crustacea, Mysida), along the northeast Atlantic coasts



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ABSTRACT

The brackish water mysid, *Neomysis integer*, is one of the most common mysids along the coasts of the northeast Atlantic, with a distribution that extends from the Baltic Sea to Morocco. It typically dominates the hyperbenthic communities of estuaries and brackish water environments where it is believed to play a key role in these ecosystems. In the present study the phylogeographic patterns of this low dispersal mysid were examined throughout its distribution range. A total of 461 specimens from 11 sampling sites were analysed by means of single stranded conformation polymorphism (SSCP) combined with sequence analysis of a 447 bp fragment of the mitochondrial cytochrome *c* oxidase I (COI) gene. The present study corroborates the expectations of the genetic patterns observed in a low dispersal species with estuarine habitats. A large heterogeneity was observed between the analysed populations (global $\Phi_{ST} = 0.787$), as evidenced by the disparate distribution of the COI haplotypes. All populations north of the English Channel shared several common haplotypes, while the southern samples consisted solely of unique haplotypes. Moreover, a clear genetic break (2.4% sequence divergence) occurred between the southernmost Guadalquivir population and all other Atlantic populations. Phylogeographic analysis revealed a complex pattern pointing to the existence of multiple glacial refugia and suggested multiple past expansion events possibly predating the last glacial maximum. The levels of genetic diversity were relatively uniform throughout the distribution range, with exception of a decline at the northern and southern edge of distribution.

INTRODUCTION

As opposed to terrestrial and freshwater studies, only recently there has been a growing interest in phylogeographic studies of marine taxa in Europe (e.g. Wilke & Pfenninger, 2002; Coyer *et al*, 2003; Luttikhuizen *et al*, 2003; Gysels *et al*, 2003, 2004; Olsen *et al*, 2004). Environmental perturbations and the transformation of the northern European geography during the Pleistocene glaciations are thought to have had a major impact on the phylogeographic patterns in extant species with range compression and expansion in function of glacial events (Avise *et al*, 1998; Taberlet *et al*, 1998; Hewitt, 2000). In addition to these historical changes, current population dynamics (like contemporary gene flow), which are related to specific life-history traits (e.g. dispersal capacity, existence or absence of pelagic larvae), might also affect the distribution of genetic variation. Both historical and contemporary factors have their own specific effect and they can either counteract each other and erase historical patterns of genetic diversity (e.g. in highly vagile species), or act in similar directions, hereby making it possible to detect the historical processes that lead to the present genetic patterns (e.g. in species with restricted dispersal capacities).

Data on terrestrial and freshwater biota provide convincing evidence that the southern European regions, in particular the Iberian, Italian peninsula and Balkan region served as refugia during glacial events, harbouring the greatest amount of genetic diversity (Taberlet *et al*, 1998; Hewitt, 1999). The postglacial range expansion of limited and/or genetically homogenous numbers of colonists, possibly in combination with bottlenecks, is probably the cause of the usually lower degree of diversity at higher latitudes (Hewitt, 1996, 2000, 2001; Ibrahim *et al*, 1996). However, the picture in marine species is less clear. Indeed, some marine taxa including gobies, copepods and seaweed (Edmands, 2001; Coyer *et al*, 2003; Gysels *et al*, 2004) show a correlation between higher latitude and reduced diversity, while others fail to show the expected decline in variation in more northern areas (Consuegra *et al*, 2002; Marko, 2004; Olsen *et al*, 2004). In addition, contemporaneous populations of several species in refugial regions (e.g. the Iberian peninsula) might be impoverished due to the post-glacial warming starting from about 11 500 years BP. This event may have constituted a strong selective force for refugial populations resulting in a (southward) decline in genetic diversity (Dahlgren *et al*, 2000; Consuegra *et al*, 2002; Coyer *et al*, 2003).

A second analogy with terrestrial studies could be expected in the location of glacial refugia. Several studies indicate that the present distribution of molecular variation in western European taxa can be explained by a northward dispersal from a southern Iberian refugium (see Taberlet *et al*, 1998; Hewitt, 1999). Although this pattern has been confirmed by several marine taxa along the northeastern Atlantic (Garcia-Marin *et al*, 1999; Consuegra *et al*, 2002), other studies provide evidence for additional 'northern' glacial refugia (e.g. Verspoor *et al*, 1999; Breton *et al*, 2003; Coyer *et al*, 2003; Luttikhuizen *et al*, 2003; Gysels *et al*, 2004). Hence, populations of these species should have survived range compression during glacial periods and post-glacial expansions in unglaciated areas. Since the comparison of phylogeographic patterns between different species in the same geographic region is a potential powerful tool to evaluate alternative biogeographical scenarios (e.g. post-glacial colonization routes) or the location of glacial refugia (Avise, 2000), phylogeographic studies of species with restricted dispersal capacities could be very valuable.

In the present study we examined the phylogeographic structure of the brackish water mysid, *Neomysis integer* (Crustacea, Mysida) along the northeast Atlantic coasts. *N. integer* (Leach, 1814) is one of the most common mysids around the northeastern Atlantic coasts (from the Baltic Sea to Morocco) and it is believed to be a key species in the marine ecosystems of these regions (Mees *et al*, 1994; Mees *et al*, 1995; Fockedey & Mees, 1999; Hostens & Mees, 1999). It is a euryhaline and eurythermic species that dominates the hyperbenthic fauna of estuarine, brackish water environments in western European estuaries (Mees *et al*, 1995; Cunha *et al*, 1999). Apart from evidence of migration over small geographic scales, as vertical dial migrations, tidal migration and seasonal migrations within an estuary (Mauchline, 1980; Mees *et al*, 1993b), nothing is known about the dispersal capacities of *N. integer* over larger scales, e.g. between neighbouring estuaries, or even over larger distances. However, its specific life history traits might suggest that long-range dispersal events are probably rare. Like most mysids *N. integer* is a brooder, and hence lacks a planktonic dispersal stage so that the actual dispersal should only take place through the movement of juveniles or adults. In addition, *N. integer* lives in discrete brackish water habitats and is rarely encountered in offshore or coastal waters (Mauchline, 1971a). An earlier study on the genetic differentiation of *N. integer* throughout its distribution range based on DNA sequences of the mitochondrial

cytochrome *b* gene supports the hypothesis of limited gene flow resulting in the genetic differentiation of populations (see Chapter 3). Most striking in that study was the genetic break at the southern distribution range of *N. integer*, pointing to the existence of multiple glacial refugia. Although these analyses were based on a very limited number of samples, the results strongly suggested that *N. integer* is a promising candidate for elucidating the phylogeographic patterns of low dispersal marine invertebrates along the NE Atlantic coasts.

Therefore the present study was designed to explore in greater detail the phylogeographic structure and patterns of molecular diversity and contemporary gene flow throughout the whole distribution range of *N. integer* by analyzing 447 bp of the mitochondrial cytochrome *c* oxidase I gene (COI) of a more extended number of specimens (30-60 individuals per sample) from 11 samples with single stranded conformation polymorphism (SSCP) analysis (Orita *et al*, 1989; Sunnucks *et al*, 2000) combined with DNA sequencing. In particular, we wanted to (1) assess and compare the levels of genetic diversity throughout the distribution range of *N. integer*, with specific emphasis on the latitudinal trends, (2) reconstruct the most likely historical processes that led to the current distribution of mitochondrial haplotypes, and (3) estimate the levels of genetic exchange that currently takes place among European populations of *N. integer*. The genetic structure of *N. integer* was examined by using a progression of phylogenetic, demographic and population genetic analyses of mtDNA sequence data. Such an approach has proven to be useful in elucidating not only geographic structure, but also the evolutionary history producing that structure (Althoff & Pellmyr, 2002).

MATERIALS AND METHODS

Sampling

A total of 461 specimens were collected from 11 locations comprising eight estuaries, one coastal site (Tvärminne), one low salinity lagoon (Kilkeran Lake) and one estuary-coastal lagoon system (Ria de Aveiro) (Fig. 4.1). This sampling scheme covers most of the current distribution range of *Neomysis integer*. Samples from each site were collected with a hand net or a hyperbenthic sledge (mesh size 1 mm) and collections were made between 1999 and 2001. After collection, the samples were stored in ethanol (70 – 95%) or acetone (Fukatsu, 1999) at 4°C. *N. americana* specimens were collected from the Damariscotta River (Maine, USA).

Fig. 4.1: Geographic location of the sampling sites of *Neomysis integer*. Shaded area represents the distribution range of *N. integer*. For details on the sampling locations and abbreviations see Table 4.1.

DNA extraction, PCR, single-stranded conformation analysis and sequencing

DNA was extracted using a modified CTAB protocol (Kocher *et al*, 1989). Mysid tissue of single individuals was crushed using a beadbeater and afterwards incubated for minimum 3 h at 60°C in 500 µl CTAB buffer (2% (w/v) CTAB, 1.4M NaCl, 0.2% (v/v) mercaptoethanol, 20 mM EDTA, 100 mM Tris/HCl pH 8) with 6 µl proteïnase K (1 mg 100 μl^{-1}). After an overnight incubation at 37°C, the DNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1 PH 8) and chloroform:isoamylalcohol (24:1). Finally, DNA was isopropanol-precipitated and rehydrated in 25µl water. A 651 bp fragment of the cytochrome *c* oxidase subunit I gene (COI) was amplified using the universal primers LCO1490 and HCO2198 (Folmer *et al*, 1994). The conditions for the COI amplifications were: 10 x PCR

buffer with $(NH_4)SO_4$ included (MBI Fermentas), 2 mM $MgCl_2$, 0.2mM dNTP, 1 μM forward and reverse primer and 1.25 units Taq polymerase. The following thermocycle profile was used: denaturation of template DNA at 94°C for 2 min, followed by a stepdown PCR (annealing temperature decrease of 1°C per cycle) of 4 cycles (30s at 94°C, annealing at 59°C for 50 s, extension at 72°C for 90 s), followed by 40 cycles of 30 s at 94°C, 50 s at 55°C and 2 min at 72°C, followed by a final extension of 5 min at 72°C. PCR products were purified with exonuclease I (10 U μl^{-1} ; Amersham) and shrimp alkaline phosphatase (1 U μl^{-1}; Amersham). Purified products were cycle sequenced using BigDye Terminator Mix (PE Applied Biosystems) and the following conditions: 25 cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Cycle sequencing products were electrophoresed on a Perkin-Elmer ABI Prism 377 DNA sequencer.

For the single-strand conformation polymorphism (SSCP) analysis (Orita *et al*, 1989) two sets of internal primers were designed within the 651bp COI fragment, generating two COI fragments of size < 250 bp. This was done to ensure a high mutation detection resolution of the SSCP technique within the COI fragment, as the sensitivity of SSCP is generally inversely proportional to the size of the fragment; e.g. single base pair differences are resolved 99% of the time for 100-300 bp fragment, while > 80% for 400 bp ones (see Sunnucks *et al*, 2000). The position of both fragments within the COI gene was chosen based on the variability observed in a small pilot study of 10 COI sequences of 651 bp from different sampling sites. The amplification of the two COI fragments (COI-1 and COI-2, 215 bp and 232 bp respectively) used the primer sequences: LCO1490 (Folmer *et al*, 1994) and COIR3 (5'-GAG GGA AAG CTA TAT CTG GAG C-3'), COIF2 (5'-TTT AGC AGG GGC TTC CTC TA-3') and HCO2198 (Folmer *et al*, 1994). Conditions for the PCR were as previously described, but with an annealing temperature of 56°C instead of 55°C. SSCP analysis were performed using 0.5 mm thick nondenaturing polyacrylamide gels (250 x 110 mm). The ideal running conditions for the SSCP analysis of both COI fragments were assessed by using different conditions (electrophoresis temperature and gel composition) and comparing the banding patterns of all gels. An electrophoresis at a constant power of 8 W at 5°C for the COI-1 fragment and at 12°C for COI-2 for 3.5 h using polyacrylamide gels with T = 12.5% and C = 2% proved to give the best resolution. Bands were visualized with a DNA silver staining kit (Amersham Biosciences) and scored by their relative mobility. Samples showing

mobility differences were sequenced in both directions under the previously described conditions. At least two replicates of each haplotype were sequenced, with the exception of haplotypes only found in one individual.

Sequence alignment and phylogenetic analysis

In the further phylogenetic and phylogeographic analysis the DNA sequences of both COI fragments screened with SSCP (COI-1 and COI-2) were combined, producing a COI fragment of 447 bp, in order to enhance the resolution of the different statistical methods. Alignment of the sequence data was produced with the Clustal X program (Version 1.74, Thompson *et al*, 1997). When needed, the alignment was manually corrected with the program GeneDoc (Version 2.6, Nicholas & Nicholas, 1997). Phylogenetic relationships between the haplotype sequences were investigated by building a tree with the neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) algorithm in PAUP 4.0b8 (Swofford, 1998). The likelihood ratio test in MODELTEST 3.06 (Posada & Crandall, 1998) was employed to determine the appropriate substitution model of DNA evolution that best fitted the dataset. Sequences of the mysids *Neomysis americana* (AJ852562), *Mysis relicta* (AY529027), *Tenagomysis australis* (AF052394), *Paramysis kroyeri* (AY529036), *Pseudomma* sp. (AY624281) and the euphausiids *Euphausia superba* (AF177182) and *Meganyctiphanes norvegicus* (AF177191) were added to root the phylogenetic tree. Bootstrap values were calculated after 1,000 replications for NJ and MP and 100 replications for the ML analysis. In addition, a network between the haplotypes was constructed to visualize evolution among haplotypes, haplotype frequency and geographical representation. This haplotype network was created using a parsimony criterion in the program TCS (version 1.13, Clement *et al*, 2000).

Population and phylogeographic analysis

Nucleotide diversity (π, the mean number of differences between all pairs of haplotypes) and haplotype diversity (h, the probability that two randomly chosen haplotypes are different in a sample) and its standard deviation (SD) were calculated for each population using the program ARLEQUIN 2.0 (Schneider *et al*, 2000). An exact test of population differentiation based on haplotype frequencies (Raymond & Rousset, 1995) was used to test the null hypothesis of random distribution of the observed haplotypes with respect to sampling location. These analyses where performed using 10 000 randomizations with the ARLEQUIN 2.0 program (Schneider *et al*, 2000).

The geographical differentiation of haplotypes was quantified using a hierarchical analysis of variance (AMOVA, Excoffier *et al.* 1992) using ARLEQUIN 2.0 program (Schneider *et al*, 2000). The significance of variance components and Φ-statistic analogues was tested by multiple (1000) random permutations. Pairwise Φ_{ST} values were calculated based on Tamura-Nei (1993) genetic distances using the gamma value obtained in MODELTEST. Their significance was tested by multiple (1000) random permutations. When necessary, corrections for multiple tests were applied according to the sequential Bonferroni correction (Rice, 1989).

Times of divergence of population pairs (T) were estimated based on the mean nucleotide divergence between populations corrected for within-group variation (Nei, 1987) (i.e. net nucleotide divergence corrected for ancestral polymorphisms). Because there is no fossil record and no geological or climatic event that would be useful in calibrating a clock of mtDNA divergence specifically for mysids, we used a general molecular clock for crustacean COI mtDNA of 1.4% to 2.6% of nucleotide divergence per million years. These estimates of mutation rates were adopted from several calibrations for crustacean taxa thought to have been divided by the Isthmus of Panama (e.g. snapping shrimp, *Alpheus* sp., Knowlton & Weight, 1998) and crab species thought to have been subdivided since the trans-Arctic interchange (*Sesarama* sp., Schubart *et al*, 1998). A similar molecular clock calibration was used in molecular divergence studies of krill species (order Euphausiacea) (Patarnello, 1996; Zane *et al*, 2000), which are based on 28S rRNA sequences closely related to the Mysida order (Jarman *et al*, 2000).

Correlation of pairwise genetic distances over geographical distances for all pairs of samples were tested in order to determine if the pattern of genetic differentiation among sampling sites could be explained by geographical distance. This isolation by distance test was conducted using a regression of genetic distances between all sampling locations (Φ_{ST}) against minimum coastline distance between all pairs of sampling sites. The strength and statistical significance of associations between geographical distance and genetic differentiation was tested with reduced major axis regression and Mantel permutation tests using the program IBD v1.52 (Bohonak, 2002).

Nested Clade Analysis

A nested clade analysis (NCA, Templeton *et al*, 1995, 1998, 2004) was performed to test for associations between haplotypes and geography, and aims at separating patterns of population history and gene flow. The haplotype network obtained with TCS was nested into clades using the nesting rules given in Templeton *et al* (1987) and Crandall (1996). Ambiguities in the haplotype network were resolved following the criteria suggested by Crandall & Templeton (1993). Subsequently, an exact permutational contingency test was conducted for each clade and a chi-squared statistic was calculated from the contingency tables (clades vs. geographical locations) by treating sample locations as categorical variables. The statistical significance of the clade distance (Dc) and nested clade distance (Dn) was calculated by comparison with a null distribution (no geographical association of clades and clade dispersal distances are not significantly different from random) derived from 10 000 random permutations of clades against sampling locations using the program GEODIS 2.2 (Posada *et al*, 2000). The interpretation of the observed distance patterns was done using a revised version of the inference key of Templeton (1998), published by Templeton (2004).

Historical population dynamics

The distribution of pairwise differences ('mismatch distribution') was computed in ARLEQUIN. Mismatch distributions and Rogers' (1995) parameters of

mismatch distribution (τ, θ_0, θ_1) were assessed by Monte Carlo simulations of 1000 random samples using the ARLEQUIN 2.0 package. Additionally Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) neutrality test was used to infer the nature of sequence evolution (e.g. rapid selection or neutral) and probable historic population movements. Significant negative values of Tajima's D are expected to occur when there has been recent population expansion (Slatkin & Hudson, 1991; Fu, 1997; Knowles *et al*, 1999) or a selective sweep (Maruyama & Birky, 1991; Fu, 1997; Filatov *et al*, 2000) and significant negative Fu's Fs values are indicative for an excess of rare alleles, which might be caused by a recent population expansion (Fu, 1997). When a signature of a recent population expansion was detected, the corresponding time of expansion could be estimated using the formula $\tau = 2uT$ (Rogers & Harpending, 1992), where T is the number of generations since time of expansion, τ is the mode of the mismatch distribution and u is the mutation rate for the whole sequence ($u = 2\mu k$, with μ: mutation rate per nucleotide and k: the total number of nucleotides analysed).

RESULTS

SSCP and sequencing results

The SSCP technique distinguished 19 haplotypes within the COI-1 fragment and 20 haplotypes within the COI-2 fragment. The combined information led to the identification of 34 haplotypes among the 461 specimens analysed from 11 samples. All the differences observed using SSCP were confirmed by sequencing analysis. DNA sequencing detected a total of 35 polymorphic positions (7.8%) among the 34 different haplotypes. Most polymorphisms were due to single nucleotide changes, and constitute 29 transitions and 7 transversions; 20 characters were parsimony-informative (see Appendix I). Eight nucleotide changes resulted in an amino acid change. Pairwise DNA differences between haplotypes ranged from 0.22% (a single substitution) to 2.68% (12 base substitutions) nucleotide divergence.

Table 4.1: Geographic location, sample size, number of haplotypes (N_h), diversity measures (*h*: haplotype diversity, π: nucleotide diversity) and percentage of private haplotypes (%PH) for the 11 samples of *Neomysis integer*.

Sampling location	Code	Latitude	Longitude	Sample Size	N_h	*h* (SD)	π (SD)	%PH
Tvärminne	TV	59° 51' N	23° 12' E	41	3	0.0963 (0.0624)	0.00065 (0.00078)	33.3
Vistula	VI	54° 21' N	18° 56' E	41	1	0	0	0
Weser	WE	53° 25' N	08° 30' E	39	3	0.5263 (0.0688)	0.00534 (0.00330)	0
Ythan	YTH	57° 18' N	02° 00' W	39	4	0.5803 (0.0430)	0.00147 (0.00129)	75
Westerschelde	WS	51° 25' N	04° 00' E	60	6	0.4689 (0.0652)	0.00335 (0.00227)	50
East Looe	EL	50° 24' N	04° 26' W	36	5	0.3048 (0.0970)	0.00194 (0.00155)	80
Kilkeran Lake	KILK	51° 33' N	08° 57' W	43	5	0.2957 (0.0875)	0.00078 (0.00087)	100
Seine	SEI	48° 26' N	00° 10' E	48	4	0.4193 (0.0810)	0.00329 (0.00225)	25
Gironde	GI	45° 33' N	00° 55' E	44	3	0.4894 (0.0500)	0.00472 (0.00298)	100
Ria de Aveiro	RdA	40° 41' N	08° 45' W	30	5	0.6115 (0.0510)	0.00272 (0.00198)	100
Guadalquivir	GU	36° 55' N	06° 17' W	40	5	0.2359 (0.0880)	0.00128 (0.00118)	100

Seventeen unique haplotypes (50%) were observed (Table 4.1 & 4.2). The haplotypes strongly segregated with geographical origin. Only four haplotypes (12%) were shared between different sampling sites while the remaining 30 and thus the majority of the observed haplotypes (88%) were population-specific. Interestingly the shared haplotypes were only observed in sampling locations north of the English Channel with exception of the Irish population (KILK), which possessed only private haplotypes. The most common haplotype (Df) was observed in 5 different locations

and was present in 29.2% of the analysed individuals. All sampling sites except two (VI and WE) possessed private haplotypes and four locations (KILK, GI, RdA and GU) consisted solely of private haplotypes.

	TV	VI	WE	WS	YTH	SEI	EL	KILK	GI	RdA	GU	TOTAL
Df	39	41	1	42		3						135
Ee	1		25	1		36						63
Ga								36				36
Cc											35	35
Ha							30					30
Fh					20	7	3					29
Aa									28			28
Fg			4	13								17
Fa					16							16
Bb									15			15
Fd										14		14
Id										13		13
Ja								4				4
D'i				2								2
Fj						2						2
Kc											2	2
Fk					2							2
Fl										1		1
Fm										1		1
D'n				1								1
Eo				1								1
Lc											1	1
Mp											1	1
Cq											1	1
Ih					1							1
Gr								1				1
Je							1					1
Ds	1											1
Na								1				1
Ot									1			1
Pa								1				1
Qd										1		1
Ra							1					1
Rh							1					1

Table 4.2: Distribution of the COI haplotypes (columns) among populations (rows) of *Neomysis integer*. For sampling site abbreviations see Table 4.1.

Intraspecific evolution

Mean nucleotide diversity (π) among all locations was 0.002323, ranging from 0 to 0.00534 (Table 4.1). Lowest levels of nucleotide diversity were observed in the Baltic Sea (VI and TV) and in the Irish population (KILK). Highest levels were observed in the Weser estuary. Values of haplotype diversity (h) ranged from 0 to 0.6115 and had an average of 0.3662. Lowest h values were observed within the

Baltic Sea (TV & VI), while haplotype diversity in the Portuguese sample of Aveiro (RdA) and the North Sea samples from the Ythan and Weser were almost twice the average. No significant correlation was observed between haplotype diversity and latitude ($P = 0.49$).

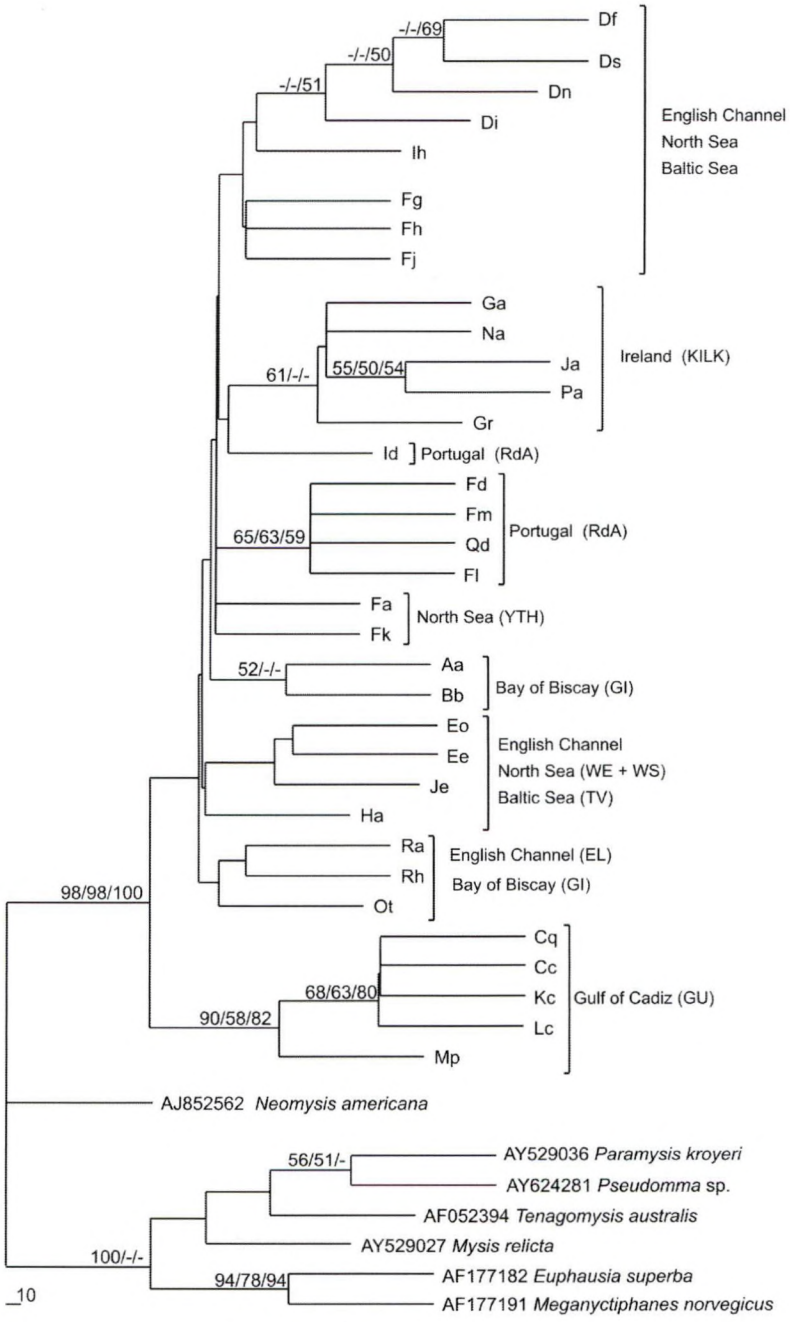

Fig. 4.2: Maximum parsimony consensus tree (519 steps) of the COI haplotypes of *Neomysis integer* obtained after a heuristic search of 100,000 random sequence addition replicates followed by tree-bisection-reconnection (TBR) branch swapping (CI = 0.6609; RI = 0.6349; RC = 0.4196). For each node the MP, ML and NJ bootstrap support is indicated, only bootstrap values > 50% are indicated.

The general time-reversible substitution model with a correction for significant invariable sites and rate heterogeneity (GTR + I + G) (Rodriguez *et al*, 1990) proved to be the best model fitting the COI data. The transition/transversion ratio (ti/tv), proportion of invariable sites (*i*) and gamma shape parameter (α) were estimated to be ti/tv = 2.45, i = 0.2366 and α = 0.5045 respectively. Base frequencies amounted to A = 0.3025, C = 0.1438, G = 0.1691, T = 0.3845; and the substitution matrix was [A-C] = 1.2112, [A-G] = 5.4729, [A-T] = 1.2653, [C-G] = 1.4337, [C-T] = 7.2169, [G-T] = 1.0000. Only minor topological differences were found between the NJ, MP and ML trees (Fig. 4.2). The low degree of sequence divergence between haplotypes resulted in a poorly resolved tree with low bootstrap support. Only one clade, containing all haplotypes observed in the Guadalquivir sample was supported by bootstrap values above 70%. The Portuguese (RdA) and the Irish (KILK) sample constituted separate clusters despite low bootstrap values.

The parsimony network is shown in Fig. 4.3. The center of the network consisted of haplotypes observed in the North Sea (YTH) and English Channel (EL & SEI). Most haplotypes were relatively closely related to each other, with the exception of all haplotypes observed in the Guadalquivir population (Cc, Kc, Lc, Mp, Cq), which formed a divergent subgroup, separated with at least six mutational steps from the central haplotypes (1.3% of uncorrected genetic divergence). Likewise the haplotypes from the Gironde population were more divergent from the central haplotypes (0.5 – 0.9% of uncorrected genetic divergence). Other subgroups within the haplotype network involved clustering of haplotypes from the same sample, as observed for the haplotypes of the Kilkeran Lake and the haplotypes (except haplotype Id) of the Portuguese Ria de Aveiro population.

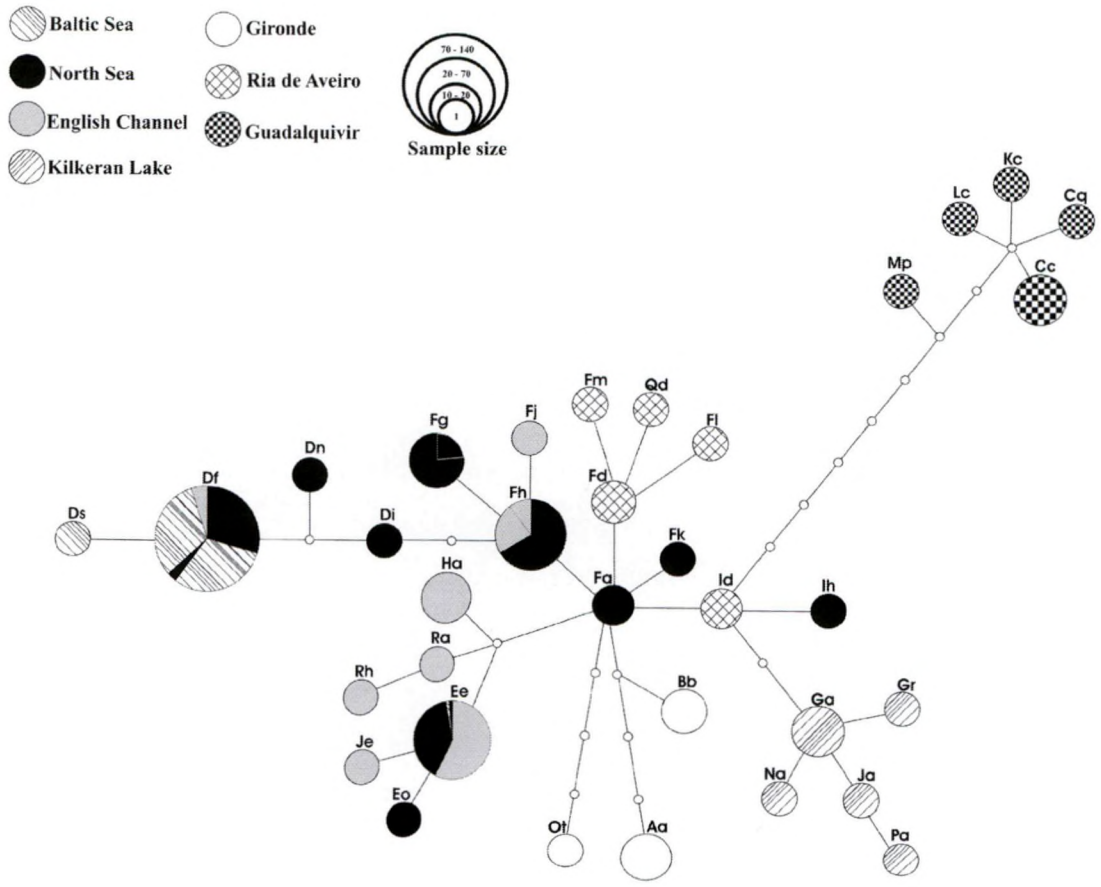

Fig 4.3: The 95% plausible parsimony network showing the mutational relationships among the COI haplotypes of *Neomysis integer*. Each line in the network represents a single mutational change and haplotypes are represented by a circle. The surface of each circle is proportional to its frequency of occurrence and the circles are shaded according to their geographic occurrence. Small empty circles indicate missing haplotypes.

Spatial genetic structure

Statistically significant differences were observed in haplotype frequencies among all samples (global test) and among all pairs of samples (P <0.0009 in all cases), except the two Baltic samples (P = 0.489). Genetic differentiation between sampling locations was assessed by an analysis of molecular variance (AMOVA) (Table 4.3). This analysis showed that a significant proportion of the genetic variation is partitioned among populations (78.67%). The global Φ_{ST} value across all samples amounted to 0.7867 (P < 0.001), indicating a significant differentiation between the samples. Additional significant geographic structuring was tested by grouping the different samples. The Guadalquivir was excluded to avoid distortion of the results

because of the large divergence of its haplotypes. When dividing the samples in two groups, one group with the samples north of the English Channel (TV, VI, WE, WS, YTH, SEI, EL) and one southern group (KILK, GI, RdA), a significant amount of between-group variation was observed ($\Phi_{CT} = 0.2488$, $P = 0.0078$). However, still the largest amount of variation was observed at the level among populations within groups. If a three group division was used, with one group comprising the Baltic samples (VI&TV) and the North Sea Westerschelde (WS) sample, a second group with the North Sea and English Channel samples (WE, YTH, SEI and EL) and a third group with the southern KILK, GI and RdA samples, a highly significant differentiation between groups was observed ($\Phi_{CT} = 0.4056$, $P = 0.001$) and now the among group variance component becomes slightly higher then the within group variation (see Table 4.3). Genetic distances between pairs of samples were calculated in order to identify those samples that might account for deviation from panmictic conditions, pairwise Φ_{ST} values are shown in Table 4.4. Virtually all pairwise comparisons were significant with the exception of the pairwise genetic distances between the WE and SEI samples and between both Baltic samples (VI and TV). Highest differentiation was found between the Guadalquivir (GU) and the Vistula (VI) population ($\Phi_{ST} = 0.976$). Genetic distances within the English Channel, North Sea and Baltic Sea ranged from low ($\Phi_{ST} = 0$) and moderate values ($\Phi_{ST} = 0.5$) (comparisons involving geographically closely located samples) to high values ($\Phi_{ST} > 0.8$) (comparisons involving the Baltic and the YTH and EL samples).

Table 4.3: Results of the hierarchical analysis of molecular variance (AMOVA). (For the sample codes see Table 4.1)

Analysis	Source of variation	% Total variance	Fixation indices	*P*
All samples	Among populations	78.67	$\Phi_{ST} = 0.7867$	< 0.001
	Within populations	21.33		
without GU sample	Among populations	71.39	$\Phi_{ST} = 0.7139$	< 0.001
	Within populations	28.61		
Two groups (TV, VI, WE, WS, YTH, SEI, EL) (KILK, GI, RdA)				
	Among groups	24.88	$\Phi_{CT} = 0.2488$	0.0078
	Among populations/ within groups	50.43	$\Phi_{SC} = 0.6713$	< 0.001
	Within populations	24.69	$\Phi_{ST} = 0.7531$	< 0.001
Three groups (TV, VI) (WE, WS, YTH, SEI, EL) (KILK, GI, RdA)				
	Among groups	28.55	$\Phi_{CT} = 0.2855$	0.019
	Among populations/ within groups	45.47	$\Phi_{SC} = 0.6363$	< 0.001
	Within populations	25.99	$\Phi_{ST} = 0.7401$	< 0.001
Three groups (TV, VI, WS) (WE, YTH, SEI, EL) (KILK, GI, RdA)				
	Among groups	40.56	$\Phi_{CT} = 0.4056$	0.001
	Among populations/ within groups	33.87	$\Phi_{SC} = 0.5697$	< 0.001
	Within populations	25.57	$\Phi_{ST} = 0.7442$	< 0.001

Table 4.4: Pair-wise Φ_{ST} values among the 11 samples of *Neomysis integer*. (ns, not significant: $P > 0.05$).

	TV	VI	WE	WS	YTH	SEI	EL	KILK	GI	Rda	GU
TV	-										
VI	0.000^{ns}	-									
WE	0.622	0.666	-								
WS	0.127	0.166	0.454	-							
YTH	0.816	0.874	0.381	0.521	-						
SEI	0.778	0.814	0.041^{ns}	0.624	0.517	-					
EL	0.882	0.916	0.440	0.726	0.692	0.505	-				
KILK	0.949	0.972	0.755	0.830	0.867	0.817	0.885	-			
GI	0.806	0.829	0.544	0.710	0.639	0.602	0.649	0.810	-		
Rda	0.833	0.874	0.452	0.641	0.457	0.551	0.663	0.790	0.612	-	
GU	0.963	0.976	0.842	0.901	0.932	0.879	0.916	0.961	0.871	0.911	-

The Mantel test showed a highly significant positive correlation between geographical distance and genetic distance (Φ_{ST}) among all samples ($P = 0.008$). This indicates an isolation by distance pattern, with geographical distance explaining 18.8% of the mitochondrial DNA variation found (r = 0.43346). At a smaller geographic scale isolation by distance was detected in the samples of the Baltic Sea, North Sea and English Channel (r = 0.4572, $P = 0.0158$), with 21% of the variation in genetic differentiation explained by geographic distance. A plot of the genetic distance vs. the geographical distance showing the pattern of isolation-by-distance is depicted in Fig. 4.4. Even when excluding the outliers from the analysis still a significant correlation could be detected ($P = 0.0014$).

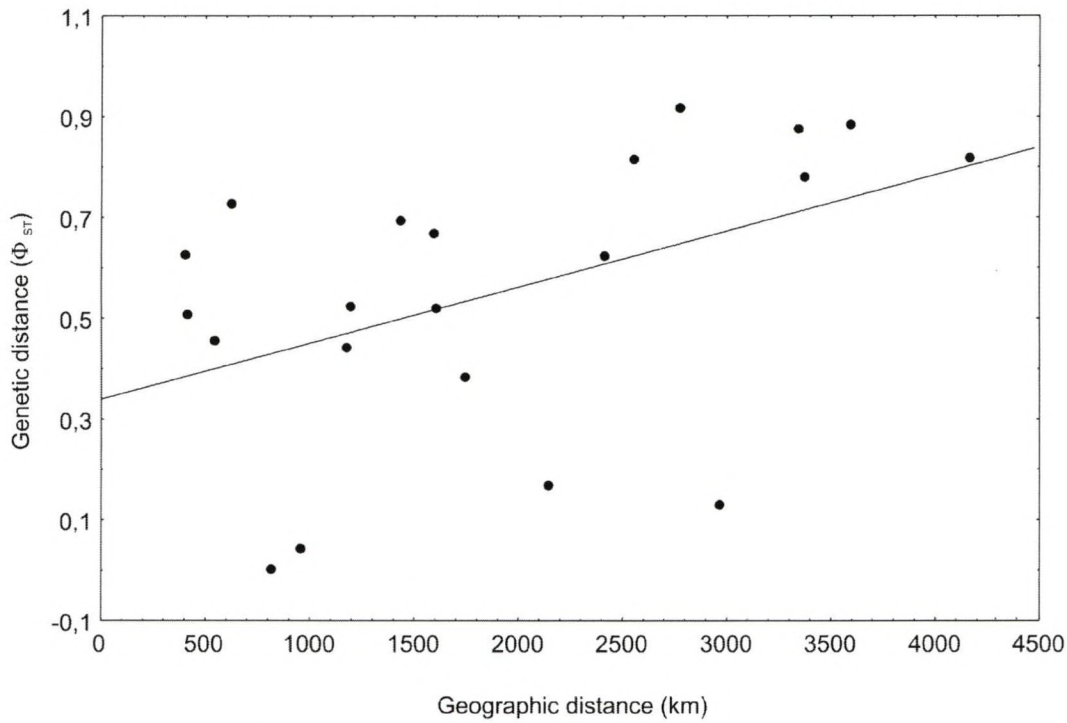

Fig 4.4: Pairwise genetic distances (Φ_{ST}) between the Baltic Sea, North Sea and English Channel samples plotted as function of the geographical distance (minimal coastline distance) between the samples. The slope had a value of 2.43×10^{-4} and R^2 of 0.209 with 95% confidence intervals for the slope (-8.33×10^{-4}, 1.19×10^{-3}) and R^2 (0.00295, 0.997).

Nested Clade Analysis

The 34 haplotypes fitted into 17 one-step clades, 8 two-step clades and 3 three-step clades (Fig. 4.5). The nested contingency analysis detected significant associations between haplotypes and geography within 8 nested haplotype clades (Table 4.5). NCA detected signals of contemporary processes such as restricted gene flow with isolation by distance (IBD) at lower level nesting groups (1-4 and 1-9). The distribution of these 2 one-step clades (1-4 and 1-9) seems to be restricted to the English Channel, North Sea and Baltic Sea samples (Fig. 4.6B). IBD was also detected at two higher nesting groups (2-7 and 3-2); however in these cases an effect of past fragmentation and/or range expansion could not be ruled out due to an inadequate sampling scheme (Table 4.5). At higher nesting groups (two-step and three-step clades) several historical demographic events, as past population fragmentation and range expansion could be inferred. A contiguous range expansion from the English Channel throughout the North Sea into the Baltic Sea is inferred for clade 2-4, which is restricted to the samples of the English Channel, North Sea and to a less extent the Baltic Sea (Fig. 4.6C). Similarly, the signals of past fragmentation and range expansion detected in nested clade 2-3 are caused by the restricted distribution of interior clade 1-5 (haplotypes Fa and Fk, found in the YTH sample) and the tip clade 1-6 (restricted to the RdA sample). However the polarity of the range expansion is rather dubious; the haplotype network suggests an expansion from the western North Sea (older interior clade 1-5) into the Portuguese coast (younger tip clade 1-6), which is opposed to the general believed expansion from southern to northern areas (Hewitt 2000). It should be noted that due to the lack of Iberian samples, the effect of long distance colonization cannot be ruled out. This process seems not plausible in the case of *N. integer* due to its presumably low dispersal capacities.

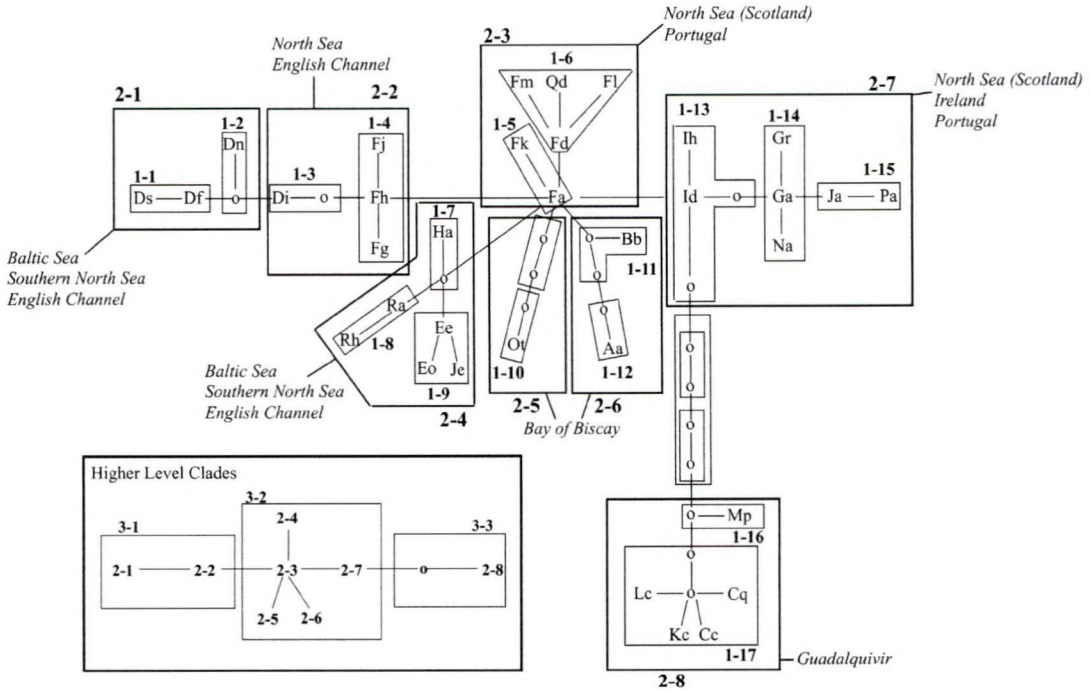

Fig. 4.5: Haplotype network among COI haplotypes of *Neomysis integer* with nesting design used in the Nested Clade Analysis. Geographical distribution of the 2-step clades is indicated.

Table 4.5: Nested contingency analysis of geographical associations (*: significant at the 5% level) with phylogeographic inferences from the Nested Clade Analysis (Templeton, 1998). For the nesting design see Fig. 4.5.

Clade	Chi-square statistics	Probability	Inference	
1-1	2.4178	0.4210		
1-4	57.3481	0.0000*	1-2-3-4-No	Restricted gene flow with isolation-by-distance
1-9	96.9841	0.0010*	1-2-11-17-4-No	Restricted gene flow with isolation-by-distance
1-13	14.0000	0.0760		
2-1	2.2021	1.0000		
2-2	6.1714	0.2650		
2-3	34.0000	0.0000*	1-2-11-12-13-14-Yes	Range expansion, Long distance colonization and/or Past fragmentation (sampling design inadequate)
2-4	92.6135	0.0000*	1-2-11-12-No	Contiguous Range Expansion
2-7	56.0000	0.0000*	1-2-3-5-15-16-18-No	Past fragmentation, Range expansion or Isolation by Distance (inadequate sampling scheme)
3-1	125.1341	0.0000*	1-2-11-12-No	Contiguous Range Expansion
3-2	609.6668	0.0000*	1-2-3-4-9-10-No	Past fragmentation or Isolation by Distance (inadequate sampling scheme)
Total	772.3222	0.0000*	1-2-11-13-13-14-Yes	Range expansion, Long distance colonization and/or Past fragmentation (sampling design inadequate)

At the level of the total cladogram the haplotype network shows a grouping into three clades (3-1, 3-2 and 3-3) that have a different geographical distribution (Fig. 4.6D). Clade 3-3 is restricted to the southern GU sample, clade 3-1 seems to have a northern distribution, dominating the Baltic and North Sea samples, whereas clade 3-2

dominates the more southern samples (RdA, GI, SEI, EL and KILK). Zones of geographical overlap of both clades are found in the English Channel (EL and SEI samples) and the North Sea (YTH and WE samples). NCA revealed that the pattern at the level of the total cladogram might be caused by a range expansion and past fragmentation. But again due to the lack of northern Iberian samples and intermediate samples between the Portuguese Ria de Aveiro sample and the southern Guadalquivir sample, a long distance colonization process might have caused a similar pattern.

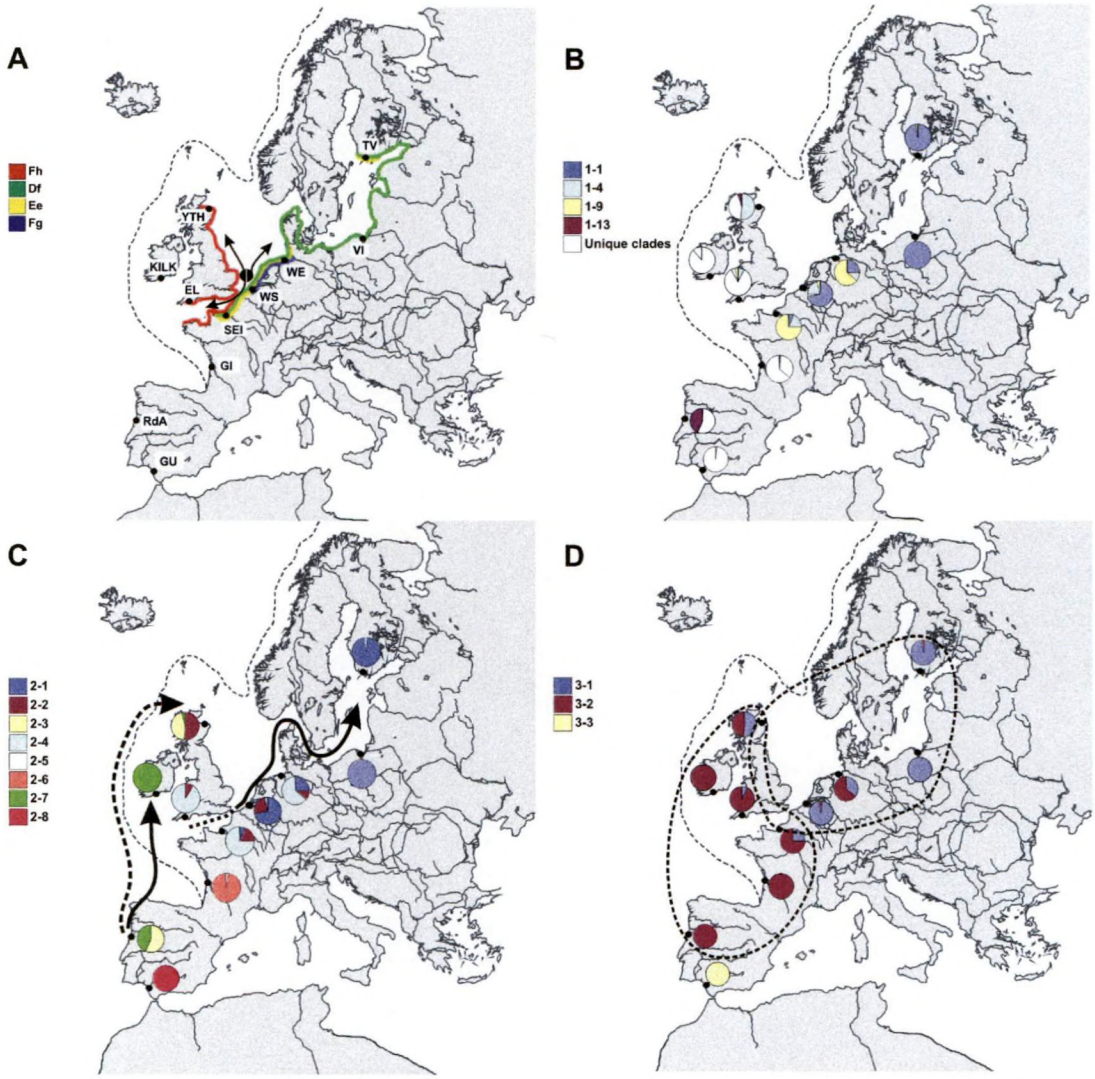

Fig. 4.6: Geographical distribution of 0-step, 1-step, 2-step and 3-step clades. Dashed line indicates the shoreline during the last glacial maximum (18 kya) (redrawn from Frenzel *et al*, 1992). A: distribution of the shared 0-step clades (i.e. haplotypes), the coloured shoreline indicates the hypothetical distribution range of a given haplotype, arrows indicate range expansion from glacial refugia B: distribution of the 1-step clades identified in the NCA (see the nested haplotype network in Fig. 4.5), C: distribution of the 2-step clades (arrows indicate putative colonisation routes), C: distribution of the 3-step clades.

Demographic history

The overall mismatch distribution was clearly multimodal (distribution not shown) and hence a fit to the sudden expansion model of Rogers (1995) was significantly rejected ($P(SSD_{obs}) = 0.04$). Likewise, signals of a postglacial population expansion could not be detected in the North Sea/English Channel region ($P(SSD_{obs}) = 0.02$). In addition both Tajima's *D* and Fu's *Fs* values were not significantly different from zero, which further supports the hypothesis of a stable population structure (Table 4.6). Only the mismatch distributions from the Irish (KILK) and the southern Guadalquivir sample seemed to fit the distribution underlying the sudden expansion model of Rogers (1995) ($P(SSD_{obs}) = 0.46$ and $P(SSD_{obs}) = 0.07$ respectively). However, the results of the Tajima's and Fu's neutrality tests were not in all cases congruent with the mismatch distributions, and hence did not always support the model of sudden population expansion. In case of the Guadalquivir sample, Fu's *Fs* value was negative, but nonsignificant, while for the Irish (KILK) sample Tajima's D value was marginally nonsignificant ($P = 0.051$). Estimations of the approximate time of expansion (T) for the samples fitting the model of sudden expansion could be calculated based on the mismatch distribution parameter τ and using a mutation rate of 1.4 - 2.6% per My for crustacean mitochondrial DNA COI (Knowlton & Weight, 1998; Schubart *et al*, 1998) and a generation time of approximately 4 months (Mees *et al*, 1994). Times of expansion for the Irish KILK and the Guadalquivir sample amounted to 14 - 22 kya (late Pleistocene) and 43 - 67.4 kya (late Pleistocene) respectively.

Table 4.6: Tests of neutrality within the pooled samples of the major geographical regions of *Neomysis integer*.

	samples	Tajima's D	*P*	Fu's Fs	*P*
All samples		-0.3415	0.3940	-5.8146	0.1120
Baltic Sea	VI & TV	-2.0403	0.0000	-1.7188	0.0950
North Sea	WE, WS & YTH	1.0602	0.8760	0.3368	0.6120
English Channel	EL & SEI	0.0436	0.6000	-0.0280	0.5310
Ireland	KILK	-1.4587	0.0510	-3.1766	0.0080
Bay of Biscay	GI	-0.4632	0.5790	4.8800	0.9750
Portugal	RdA	-0.0995	0.5130	-0.1123	0.4760
Gulf of Cadiz	GU	-1.8069	0.0080	-1.8330	0.0800

DISCUSSION

The present study of the mtDNA structuring along the distribution range of the brackish water mysid *Neomysis integer* revealed some striking patterns. Firstly, there is a clear geographic clustering of the haplotypes, showing a completely different picture in the sites north and south of the English Channel. Samples from the areas north of the English Channel share several common haplotypes, while the southern samples show a high amount of unique haplotypes per sample (see Fig. 4.6A and Tables 4.1 & 4.2). Secondly, the haplotypes observed in the southern Iberian Guadalquivir sample display a large divergence. Thirdly, the Baltic samples show an extremely low level of variability and consist solely of haplotypes that are predominant in the adjacent North Sea. Fourthly, no clear correlation between the molecular diversity and latitude is observed, except of a lower diversity at both the northern and southern edge of the distribution range.

Levels of genetic diversity

Values of haplotype and nucleotide diversity observed for *N. integer* along the NE Atlantic range from 0 to 0.6115 and from 0 to 0.005 respectively. These are comparable to values reported for other marine and brackish water invertebrates (Bucklin & Wiebe, 1998; Dahlgren *et al*, 2000; Small & Gosling, 2000; Wilke & Davis, 2000; Zane *et al*, 2000; Wares, 2001; Wares & Cunningham, 2001; Breton, 2003). The relatively low (< 0.5%) levels of sequence divergence between haplotypes within populations are thought to be typical for brackish water species (Bucklin *et al*, 1997). In these populations most of the variation is observed between populations rather than within populations (Cognetti & Maltagliati, 2000; Bilton *et al*, 2002; Maltagliati, 2002).

A complex phylogeographic pattern in *Neomysis integer*

The uniqueness of the Guadalquivir population

The Guadalquivir population is clearly distinguished from all the others through (1) its low variation compared to the other Iberian sample and (2) its

constituting solely of unique and highly divergent haplotypes. Taken together, these data point to a long-lasting isolation of this population as was already suggested earlier on for *N. integer* (see Chapter 3). Divergence times are estimated to be 500 – 700 kya or 320 - 450 kya using respectively a fast (2.6% per My) and slow molecular clock (1.66% per My), pointing to a middle-Pleistocene separation. Enhanced levels of genetic drift, due to the isolation of this population, could have lowered the levels of molecular diversity.

In addition, the low level of molecular diversity may also be due to the fact that this population is on the edge of the species' distribution. It is well-known that diversity declines at the edges of a species' distributional range, probably because of the enhanced selection under more extreme conditions (Lesica & Allendorf, 1995; Hewitt, 2000; Coyer *et al*, 2003; Hoffman & Blouin, 2004). The lower than expected densities of *N. integer* in the Guadalquivir estuary support the hypothesis that the habitat may not be optimal for the species (Drake *et al*, 2002). Alternatively, the reduced levels of molecular diversity may simply indicate the declining status of many southern populations caused by the post-glacial warming starting from about 11 500 years ago (Dahlgren *et al*, 2000; Consuegra *et al*, 2002; Coyer *et al*, 2003). Hence, the current diversity levels of the Guadalquivir populations may not be representative for those surviving in the glacial refugium during the last glacial maximum (LGM).

Evidence for multiple glacial refugia along the Atlantic coasts of western Europe and Great-Britain?

The Bay of Biscay

The divergence of the haplotypes in the Bay of Biscay population (GI) is estimated to have occurred around 170 – 350 or 100 – 220 kya (Holsteinian interstadial/ Saalian glaciation) using respectively a slow (1.66%) and fast (2.6%) molecular clock, and hence clearly predates the timing of the LGM (18kya). This points to a complementary refugium in that area. Although Pleistocene paleogeography of this region is lacking, the observed isolation is congruent with a previous study of *N. integer* (see Chapter 3) and with other marine species. Luttikhuizen *et al* (2003) found a significant differentiation of the Gironde population

of the bivalve *Macoma balthica*, which clearly predated the LGM and suggested a survival of this species in this area during the last glaciation. Similarly, a significant differentiation of the flatfish *Pleuronectes platessa* (plaice) in the Bay of Biscay was reported by Hoarau *et al* (2004).

The Iberian Peninsula

The high level of diversity in the Ria de Aveiro population apparently supports the hypothesis of a glacial refugium along the Iberian coast as suggested for many terrestrial and aquatic species (Hewitt, 1996, 1999; Taberlet, 1998). Higher levels of molecular diversity are typical for refugial areas (Hewitt, 2000) and is suggestive for an older age of this population since older populations are assumed to harbour more genetic diversity through persistent accumulation of alleles compared to younger ones (Crandall & Templeton, 1993). This hypothesis is also supported by the more central position of the common haplotype Id from the Ria de Aveiro sample in the haplotype network. However, not a single haplotype of the Ria de Aveiro sample was found in any other sampling location. This may indicate that, like the Guadalquivir and Gironde populations, the Ria de Aveiro estuary constitutes an isolated population. Alternatively, genetic drift and selection could have altered the genetic structure of the Iberian refugial population since the last ice age such that the modern populations in these areas are not representative of the population structure during the LGM (see Consuegra *et al*, 2002).

The North Sea or the English Channel

The presumably younger, northern populations are predicted to be less structured as a result of repeated founder-flush cycles during Pleistocene glaciations (Hewitt, 1996). However, our data shows a relatively high heterogeneity of the samples in that area (English Channel and North Sea). Moreover, the absence of any southern haplotypes, the high proportion of unique haplotypes, the levels of divergence between haplotypes of the northern samples, the rejection of a demographic expansion in the samples of the English Channel and North Sea, the absence of a star-like haplotype network and the detection of an isolation-by-distance pattern all reflect a temporally more stable demography and near mutation-drift

equilibrium conditions for these samples (Rogers & Harpending, 1992). This is suggestive of the presence of a northern refugium where *N. integer* survived the Pleistocene glaciations. Although it is generally accepted that the Pleistocene glaciations have dramatically altered the shoreline in northern Europe (see Fig. 4.6 for a reconstruction during the LGM) (Lambeck *et al*, 2002), paleogeographical data from several studies have provided evidence for the existence of a large ice lake, an extended network of rivers, estuarine-like environments and several small glacial lakes in the southern North Sea and English Channel region during the Elsterian (450-420 kya), Saalian (380-140 kya) and Weichselian (100-18 kya) glaciations (Gibbard, 1988; Cameron *et al*, 1992; Törnqvist *et al*, 2003; Ehler & Gibbard, 2004). Given that *N. integer* is a euryhaline and eurythermal species (Mauchline, 1971a), and provided it was able to withstand the lower temperature minima in these areas during glacial periods, populations may have survived compression of the distribution range in separate 'northern' refugia in these ice-free regions. Hence, different populations could diverge from each other during following glaciations and were able to retain their molecular identity. A similar scenario was suggested in a previous study of *N. integer* (see Chapter 3) and has been proposed as an explanation for the distribution of genetic variation in a number of other marine species along the European coasts including salmonids, gobies, polychaetes, gastropods, bivalves, and seaweed (Wilke & Davis, 2000; Consuegra *et al*, 2002; Breton *et al*, 2003; Coyer *et al*, 2003; Gysels, 2003; Luttikhuizen *et al*, 2003; Gysels *et al*, 2004; Jolly *et al*, 2005; Provan *et al*, 2005).

The British Isles

The high number of private alleles observed in the samples of the British and Irish coasts (75% in YTH, 80% in EL and 100% in KILK) is remarkable and surprising given that samples from the western European coasts at similar latitudes (SEI, WS and WE) possess much less private alleles. The divergent haplotypes uniquely observed in the Irish KILK sample suggest a refugium in the Celtic Sea or off the Atlantic shelf. The divergence time of the Irish haplotypes suggests that *N. integer* became isolated during the Saalian glacial (120 - 380 kya) and survived the subsequent glaciation (Weichselian). A similar isolation of the Irish populations was suggested for common goby (Gysels *et al*, 2004) and brown trout (Hynes *et al*, 1996).

Putative postglacial recolonisation routes along European Atlantic coasts

Throughout continental Europe, a continuous postglacial range expansion is assumed for many terrestrial species (Taberlet *et al*, 1998; Hewitt, 2000). A similar expansion has been confirmed for a variety of marine species along the NE Atlantic (Garcia-Marin *et al*, 1999; Consuegra, 2002; Gysels *et al*, 2004), NW Atlantic (Wares & Cunningham, 2001; Wares, 2002) and NE Pacific (Marko 1998, 2004; Dawson 2001; Hellberg *et al*, 2001; Hickerson & Ross, 2001; Johnson & Taylor, 2004). According to the present data it seems that the southern refugial populations of *N. integer* (e.g. from the Ria de Aveiro estuary) did not participate in the most recent range expansion to northern areas after the LGM. In contrast, NCA revealed only at higher clade levels evidence of a range expansion from the Iberian coast to northern European regions (see Fig. 4.6C, putative range expansion from the Iberian to the Irish and northern UK coasts). This could imply that during the Holsteinian or Eemian interglacials (400 – 370 and 120 - 100 kya respectively) mysids from a southern refugium, located on the Iberian coasts, colonized northern Europe. However, instead of being pushed back south during the following glaciations (Saalian and Weichselian); some of these populations might have survived the subsequent glaciations in northern refugia (see previous discussion). Alternatively, highly structured and diverse refugial populations may have inhabited a compressed southern distribution range (e.g. on the northern Iberian coasts) during glacial periods. Several subsequent postglacial colonization events of northern areas through different routes after the LGM may have caused the co-occurrence and/or the disparate distribution of different lineages. Although this hypothesis and the 'northern refugia' hypothesis are not exclusive, we cannot fully discriminate between them due to the lack of mysid fossil data and the absence of more northern Iberian and Bay of Biscay samples. Clearly, a good fossil record is important in determining the limits of refugial ranges (Hewitt, 2004).

The fact that the Scottish Ythan population does not share any haplotypes with the other North Sea samples (WE and WS) points to a colonisation of the North Sea from different refugia and in different phases. More than half of the analysed specimens of the Ythan estuary possessed the haplotype Fh, which is also common in English Channel, suggesting a colonisation of the eastern UK and the English Channel coasts from the same refugial population (see Fig. 4.6A). Remarkably, it seems that

this haplotype was not able to undertake a northward range expansion along the eastern North Sea coasts and within the Baltic Sea. On the other hand, the high proportion of unique haplotypes (75%) in an appreciable frequency (48.7%) within the Ythan estuary may be indicative of a colonisation from another refugium. Similarly a very high proportion of unique haplotypes (91%) was observed in the southern UK sample of East Looe. Hence, the northern, northeastern, and southern UK coasts may be zones of secondary contact between different mitochondrial lineages expanding from separate glacial refugia. In contrast, the coasts of the northern European continent and the Baltic Sea seem to be colonized by the same gene pool expanding from a glacial refugium probably located in the southern North Sea or English Channel (see Fig. 4.6A and C).

Latitudinal trends of genetic diversity

A gradient of declining genetic diversity from south to north of species in continental Europe and North America has been well established (Hewitt, 1996, 2000, 2004; Avise, 2000). In the present study the highest level of molecular diversity was observed in the southern (Portuguese) sample of the Ria de Aveiro (h=0.612), while the northernmost samples (Baltic Sea) showed much less diversity (h=0.096). However, no clear gradient in molecular diversity was observed for the samples in-between, considering that the average haplotype diversity of the North Sea samples was only slightly lower than in Ria de Aveiro (see Table 4.1). Whereas founder events may be the explanation for the low levels of genetic diversity in the Baltic Sea, the surprisingly high levels of diversity in the English Channel and the North Sea require another explanation. For example, high levels of genetic diversity at northern locations could be the result of a wholesale range shift caused by the extensive rapid dispersal, as has been suggested for the seagrass *Zostera marina* (Olsen *et al*, 2004). However, a similar mechanism seems unlikely for *N. integer* due to its discrete estuarine habitats and its lack of a dispersal stage, which might be necessary for an extensive rapid post-glacial colonization of northern areas. Alternatively, a very slow colonization process might be more plausible for *N. integer*. Computer simulations have shown that a slow post-glacial colonization ('phalanx' or diffusive expansions) tends to retain the levels of genetic diversity (Nichols & Hewitt, 1994; Ibrahim *et al*, 1996). This slow process involves a high proportion of individuals dispersing over

short distances in a continuous front, a pattern that would be expected in a strictly estuarine species as *N. integer*. Hence, the subsequent range expansion does not involve a subsampling of the genetic diversity of source populations through founder events and the effective population sizes remain much larger then in the case of a fast pioneer colonization process. As a consequence new populations will maintain the genetic diversity of the original population (Nichols & Hewitt, 1994). Additional evidence for this slow colonization process in *N. integer* are the rejection of a demographic expansion and the detection of an isolation-by-distance pattern which is suggestive for a temporally more stable population structure.

Species-specific attributes such as colonizing ability (related to dispersal capacities and/or physiological tolerance) may largely influence the general prediction of declining diversity with increasing latitude (Taberlet, 1998). Furthermore, migration behaviour of species during interglacial periods and the presence of more northern refugia may have blurred this pattern (Petit, 2003). Indeed, the admixture of divergent lineages colonizing northern areas from separate refugia may also be a large cause of the higher genetic diversity at intermediate latitudes. This has been observed for other marine taxa (Consuegra *et al*, 2002; Coyer *et al*, 2003) and in the case of *N. integer* the North Sea area may be a secondary contact zone between haplotypes from different refugia (see discussion above).

Contemporary gene flow and Isolation by Distance

The limited dispersal capacity of *N. integer* combined with the estuarine habitat, imply a reduced genetic neighbourhood and strong population differentiation. However, during occasional and stochastic events, such as exceptional rainfalls or floods, very low gene flow might occur between proximate estuaries by plumes of floodwater discharge that extend out to sea (see hydrodynamical model of Lacroix *et al* (2004) in the southern North Sea). This mechanism has been observed in several estuarine species (Maltagliati, 2002; Burridge *et al*, 2004). Low densities of *N. integer* (year average of 12 ind. 100 m^{-2}) have been reported in the surf zone hyperbenthos of Belgian sandy beaches (Beyst *et al*, 2001), especially during winter months, when floodwater discharge was higher and the salinity tolerance of *N. integer* increased with lower temperatures (Vlasblom & Elgershuizen, 1977; Kinne, 1955).

The present study corroborates these expectations. The high component of genetic variation attributed to among-population differences detected in the AMOVA (78.67%, see Table 4.3) may reflect these dispersal-limiting life-history traits. Also the pairwise Φ_{ST} values were in general relatively high indicating restricted gene flow. The majority of the comparisons revealed a pairwise Φ_{ST} value > 0.5 or even > 0.8. Pairwise Φ_{ST} values of the southern Iberian Guadalquivir population attained almost 1, which corresponds to a reproductive isolation of this population. Only in the case of both Baltic samples and the North Sea samples from the Seine and Weser estuaries a non-significant pairwise Φ_{ST} value was observed. Homogeneity of the Baltic samples could be caused be the recent colonization of the Baltic (last 8000 years) resulting in a migration-drift balance that has not yet attained equilibrium. Alternatively, high rates of gene flow within the Baltic Sea could be linked with the specific environmental characteristics of the Baltic Sea. The water in the Baltic Sea is brackish with an average salinity lower than 10 PSU, this could result in a higher connectivity between suitable habitats for *N. integer* leading to higher rates of gene flow within the Baltic.

In addition, the inference of restricted gene flow at different levels in the NCA is also consistent with high levels of population subdivision (Table 4.5). Surprisingly, two clades analysed in the NCA suggested long-distance dispersal among geographic regions as one of the possible inferences. It appears more likely to us that this inference is due to the inadequate sampling scheme, rather than a biological reality. Finally, the detection of an isolation-by-distance pattern, i.e. the decrease of genetic correlation with increasing geographic distance (Wright, 1943), provides further evidence for the restricted contemporary gene flow between adjacent estuaries, consistent with the one-dimensional stepping stone model of Kimura & Weiss (1964). This type of metapopulation structure has been observed in several other estuarine and brackish water species with disjunct distributions (e.g. Maltagliati, 1999; Burridge *et al*, 2004). It implies that the pattern of population divergence is maintained by very limited gene flow between adjacent populations and that genetic drift is primarily influencing the levels of genetic variability within populations (Maltagliati, 1999).

CONCLUSIONS

The present study of mitochondrial COI variation in populations of the mysid *Neomysis integer* revealed a significant differentiation throughout its distribution range with a complex phylogeographic structure. Despite the extensive population surveys across the whole distribution range, the historical dynamics of *N. integer* along the NE Atlantic coasts remains largely speculative. The levels of nucleotide divergence between the mitochondrial lineages is suggestive for a pre-LGM differentiation, even when taking into account a large error on the calibration of the molecular clock. Moreover, the heterogeneous distribution of the haplotypes in northern Europe points to a colonisation of these areas prior to the last glaciation and a survival in several northern refugia. This contradicts the general expectations derived from to current paleoclimatological and -oceanographic models. Although supported by a previous study of *N. integer* and some other marine species, additional analyses of samples from 'critical' areas such as the northern Iberian Peninsula, Bay of Biscay and coasts of Brittany may be useful the validate the current hypothesis. Likewise, additional analyses of unlinked nuclear loci might be needed since the genetic pattern observed at a single (mitochondrial) locus represents just one realization of an evolutionary process with a large stochastic component (Maddison, 1997; Nichols, 2001). Only then a distinction can be made between the genetic patterns generated by selective sweeps or the neutral variation shaped by random genetic drift and gene flow (Mishmar *et al*, 2003; Ballard & Whitlock, 2004).

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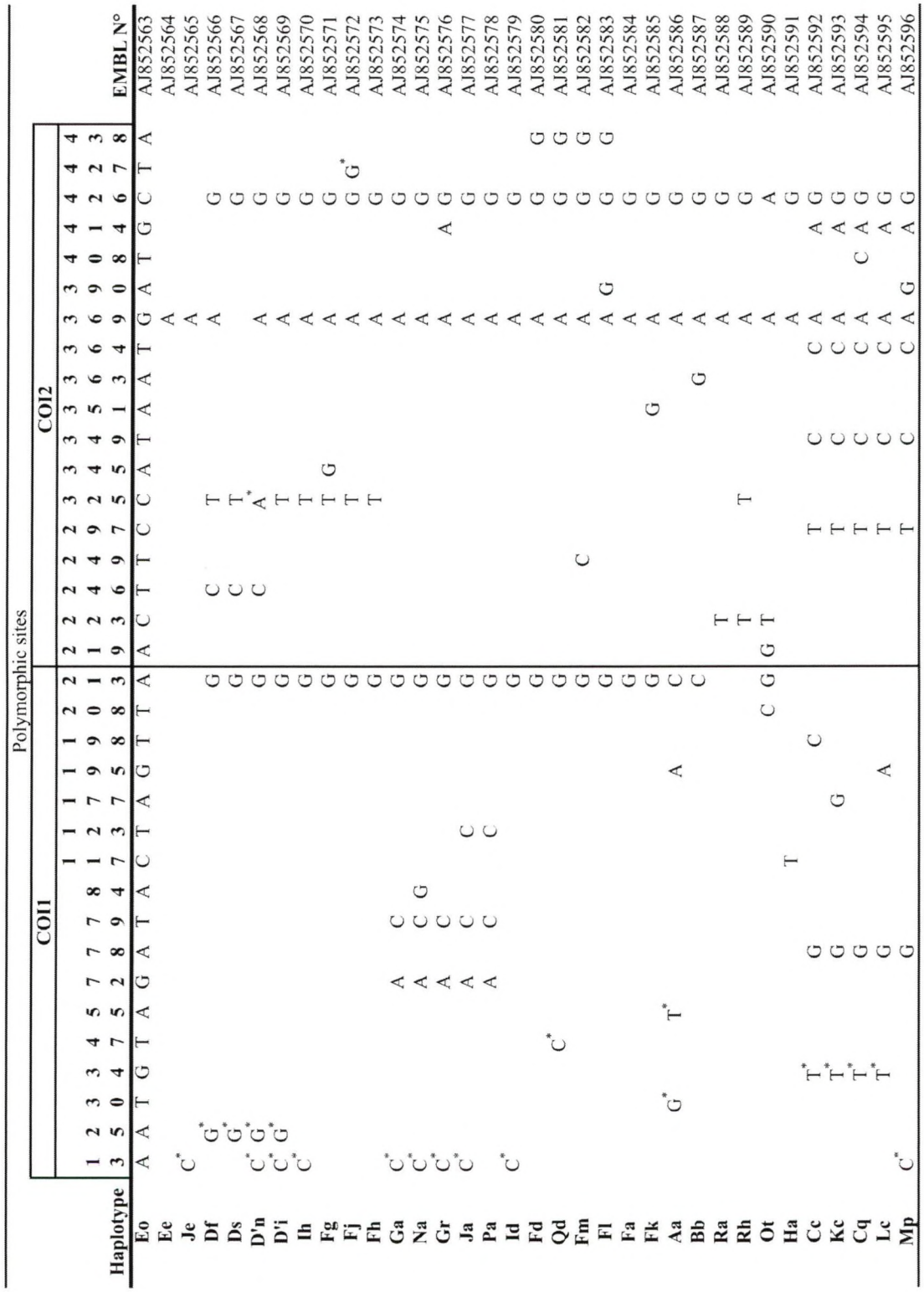
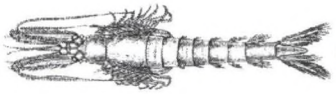
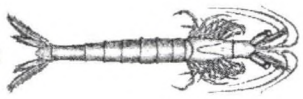
APPENDIX I: Variable nucleotide positions of the COI haplotypes observed in *Neomysis integer* with indication of the EMBL accession numbers of the haplotypes. The first letter of each compound haplotype indicates the COI-1 fragment, and the second corresponds to the COI-2 fragment. An asterisk at a nucleotide indicates a substitution resulting in an amino acid change.



CHAPTER V



Phylogeographic patterns within the mysid *Mesopodopsis slabberi* (Crustacea, Mysida): evidence for high molecular diversity and cryptic speciation



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ABSTRACT

The phylogeographic patterns among populations of *Mesopodopsis slabberi* (Crustacea, Mysida), an ecological important mysid species of marine and estuarine habitats, were analysed by means of DNA sequencing of a 458 bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and a 487 bp fragment of mitochondrial 16S ribosomal RNA gene. Samples of *M. slabberi* collected from five Atlantic and two Mediterranean populations were investigated. Very high levels of within-population molecular diversity were observed in all samples (mean $h = 0.807$ and $\pi = 0.0083$), with exception of the Mediterranean Ebro sample which contained just one haplotype. Differentiation among populations was high ($\Phi_{ST} = 0.9115$), and a clear phylogeographic break was observed between the Atlantic and Mediterranean populations. Moreover, a strong differentiation was detected between both locations in the Mediterranean basin (Alicante and Ebro delta), while two divergent lineages occurred in sympatry within the Atlantic Mondego sample. The high congruence between both the COI and 16S rRNA sequence data, the reciprocal monophyly of the different mitochondrial clades and the levels of nucleotide divergence between them suggest the presence of a complex of cryptic species. Estimations of divergence time between the different mitochondrial lineages indicate that a split occurred during the late Miocene/ early Pliocene, which could be concordant with sea-level changes within the Mediterranean region during that time. However within the Mediterranean, the potential of divergence through ecological diversification cannot be ruled out. The present phylogeographic patterns within the mysid *M. slabberi* are compared with other marine species with an Atlanto-Mediterranean distribution.

INTRODUCTION

Mesopodopsis slabberi van Beneden, 1861 is one of the most common mysid species (Crustacea, Mysida) along the European coasts. It tolerates a wide range of salinities (1.3 – 43 psu) and is therefore dominantly observed in the surf zone hyperbenthos of temperate beaches (Beyst *et al*, 2001), coastal zones (Dewicke *et al*, 2003) as well as estuaries (Gomoiu, 1978; Greenwood *et al*, 1989; Moffat & Jones, 1993; Mees *et al*, 1995). As prey for numerous species of fish (Greenwood *et al*, 1989; Hostens & Mees, 1999) *M. slabberi* is believed to be an important part of the food web in these ecosystems and is likely a key species regarding trophic interactions (Azeiteiro *et al*, 1999). Moreover, it has recently been proposed as a potential test organism for ecotoxicological research (Sardo *et al*, 2005). Seasonal variation in salinity preferences of *M. slabberi* has been described. During summer it prefers marine and metahaline conditions, while during the rainy season or winter it is more abundant in brackish conditions (Tattersall & Tattersall, 1951; Greenwood *et al*, 1989; Webb & Wooldridge, 1990; Wittmann, 1992; Azeiteiro *et al*, 1999). In addition, these seasonal migrations might also be triggered by changes in temperature, since a migration to deeper waters with decreasing temperature has been observed (Mauchline, 1980; Beyst *et al*, 2001; Dewicke, 2001). Diel migratory movements are also characteristic for *M. slabberi*. During daytime it is typically hyperbenthic, gathering in large and dense swarms or schools close to the substrate. At night or in turbid waters it becomes planktonic and disperses between bottom and surface waters (Wittman, 1992). However, little is known on long-range dispersal. *M. slabberi* might have restricted dispersal capacities since it possesses a brood pouch (marsupium) and hence lacks a planktonic dispersal stage.

M. slabberi has a wide geographical distribution. It was thought to be a monomorphic cosmopolitan species found in a wide area extending from the Baltic Sea, and the coasts of Europe, to the Mediterranean Sea, the Black Sea and south-eastern Africa (Tattersall & Tattersall, 1951; Pillai, 1968). However, the taxonomy of the genus *Mesopdopsis* Czerniavsky, and in particular of the species *M. slabberi*, has been a matter of controversy (see Bacescu, 1940; Tattersal & Tattersall, 1951; Pillai, 1968). After the most recent revision of the genus given by Wittmann (1992) based on morphogeographic variations, the formerly accepted cosmopolitan *M. slabberi* was split into the South African *M. wooldridgei*, the west African *M. tropicalis*, the

Mediterranean *M. aegyptia* and the nominal form from the NE Atlantic, Mediterranean and Black Sea. Variation within species of the genus *Mesopodopsis*, and in mysids in general, have not been profoundly studied. Wittmann (1992) reported small and statistically overlapping morphologic differences between Atlantic, Mediterranean and Black Sea populations of *M. slabberi*. The lack of morphological diversification or the confounding effects of high phenotypic plasticity in marine invertebrates has often hampered the assessment of biodiversity by using traditional morphological methods (e.g. Lee, 2000; Müller, 2000; Pfenninger *et al*, 2003; Witt *et al*, 2003). The advent of molecular and biochemical methods last decades has revealed a substantial amount of 'hidden' diversity within morphologically delimitated species. Broad geographical surveys of genetic variation within marine species, and in particular invertebrates, have led to the recognition of discrete evolutionary units, ranging from genetically divergent populations to cryptic species complexes (Knowlton, 1993, 2000). Within crustaceans genetic analyses of species boundaries have demonstrated surprisingly large genetic differences between cryptic species given their morphological similarity (e.g. Bucklin *et al*, 1995; Knowlton & Weight, 1998; Lee, 2000). The identification of cryptic species may have large consequences in the understanding of ecological patterns since cryptic species have independent population dynamics and may interact differently with other species and their environment (Knowlton, 1993, 2000).

In the present study the patterns of genetic differentiation of Atlantic and Mediterranean populations of the mysid *M. slabberi* were examined by means of DNA analyses of the mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rRNA (16S) genes. Owing to the relative large distribution range of *M. slabberi* and the fact that gene flow must be somewhat restricted, due to the lack of planktonic larvae, considerable genetic differentiation between populations and possibly the occurrence of cryptic species can be expected. In addition, the Atlantic-Mediterranean distribution of *M. slabberi* may be of special interest since this biogeographical transition is considered to have caused a strong genetic differentiation in a wide variety of marine taxa (e.g. Borsa *et al*, 1997b; Duran *et al*, 2004a, b; Peijnenburg *et al*, 2004). Lowered sea-level during the Quaternary glaciations, resulting in a significant restriction of gene flow between the Atlantic and Mediterranean basin, in combination with low levels of contemporary gene flow through the Straits of Gibraltar, is thought to have played a major role in the divergence between

populations of both basins. However, a recent study has shown that differences in the sensitivity to barriers or selective gradients, differences in effective population size and other ecological and/or demographical factors may have influenced the degree of Atlantic-Mediterranean divergence as well, even between closely related species with comparable dispersal capacities (Bargelloni *et al*, 2003). Since research has focused mainly on commercially important species, information on ecological important invertebrate species remains scarce. Hence, the present study may largely contribute to the knowledge of genetic differentiation between Atlantic and Mediterranean populations of invertebrate key species.

The aim of this study is to examine geographic patterns of genetic variation at different spatial scales, i.e. at a meso-geographic scale (50-400 km), at a macrogeographic scale within the Atlantic and Mediterranean basin (> 1000s km) and across across the Atlantic-Mediterranean biogeographic boundary. In addition, the time scales, isolation dynamics and historical demography involved in generating the intraspecific mitochondrial structure are assessed. Finally, the Atlantic-Mediterranean subdivision of *M. slabberi* populations is compared with those of other marine species.

MATERIALS & METHODS

Sampling

Specimens of *Mesopodopsis slabberi* were collected from 7 European locations (Fig. 5.1), comprising five northeast Atlantic estuaries, one Mediterranean estuary and one Mediterranean coastal site (Alicante), covering a significant range of the Atlantic and western Mediterranean distribution of the species' distribution. Specimens of *M. wooldridgei* were collected from the Gamtoos estuary (South Africa). Samples from each estuary were collected with a hyperbenthic sledge or a hand net (mesh size 1 mm). After collection, the samples were stored in ethanol (70 – 95%) or acetone at 4°C.

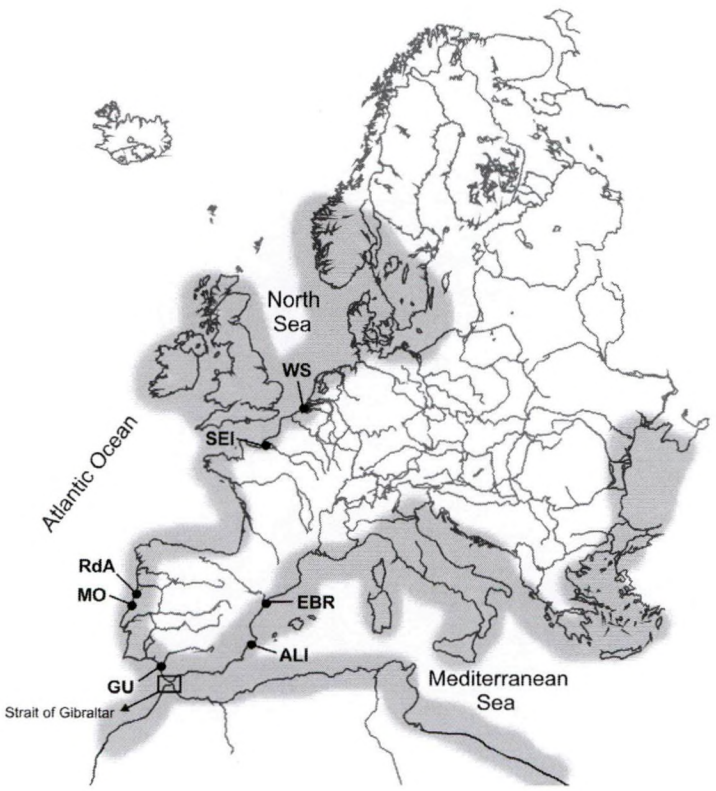

Fig. 5.1: Map of Europe showing the sample locations of *Mesopodopsis slabberi*. Shading represents the distribution range of *M. slabberi*. See Table 5.1 for sampling site codes.

DNA isolation, PCR amplification, and DNA sequencing

DNA was extracted using a modified CTAB protocol (Kocher *et al,* 1989). Mysid tissue was crushed using a beadbeater and afterwards incubated for minimum 3 hours at 60°C in 500 µl CTAB with 6 µl proteinase K (1 mg 100 μl^{-1}). After an overnight incubation at 37°C, the DNA was purified with a standard phenol/chloroform extraction protocol using phenol/chloroform/isoamylalcohol (25:24:1 PH 8) and chloroform:isoamylalcohol (24:1). Finally, DNA was isopropanol-precipitated and rehydrated in 25 µl bidi. A 651 bp fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) was amplified by polymerase chain reaction (PCR) using the universal primers LCO1490 and HCO2198 (Folmer *et al*, 1994). The conditions for the COI amplifications were as described in Chapter 4. A small aliquot (5 µl) of each amplification was loaded on a 1 % agarose gel, stained with ethidium bromide, and visualized under UV light. PCR products were purified with exonuclease I (10 U μl^{-1}; Amersham) and shrimp alkaline phosphatase (1 U μl^{-1}; Amersham). Purified products (forward and reverse) were cycle sequenced using BigDye Terminator Mix (PE Applied Biosystems) and following conditions: 25 cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Cycle sequencing products were electrophoresed on a Perkin-Elmer ABI Prism 377 DNA sequencer. Because of the poor amplification success of the universal COI primers LCO1490 and HCO2198 (Folmer *et al*, 1994), one set of species specific internal COI primers (COMSF 5'-GTA CTT TGC TTT TGG AGC CTG-3' and COMSR 5'-AGG TGC TGG TAT AGA ATA GGG-3') were designed. Conditions for the PCR were the same as for the universal primers, except for the annealing temperature which was 54°C.

After initial phylogenetic analysis (see below), three to four individuals for each clade inferred with COI sequences were chosen for additional analysis with partial mitochondrial 16S ribosomal RNA sequences. The 16S fragment was amplified using the primers 16Sar5' (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16Sbr3' (5'-CCG GTY TGA ACT CAG ATC AYG T-3') (Palumbi *et al*, 1991) and under the following thermocycle profile: initial denaturation at 94°C for 2 min, followed by 40 cycles (94°C for 30 s, 48°C for 90 s and 72°C for 2 min) and final extension of 5 min at 72°C. Amplified 16S fragments were sequenced as described above. Identities of all sequences were confirmed with BLAST searches in GENBANK and were thereafter deposited in GENBANK.

Data analysis

Sequences were aligned using Clustal X (Version 1.74, Thompson *et al*, 1997) followed by manual adjustment. Gaps resulting from the alignment (indels) were treated as missing data. Phylogenetic relationships were estimated separately for the two datasets (COI and 16S rRNA) with PAUP* 4.0b10 (Swofford, 1998) using the neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) method of phylogenetic inference. Sequences of the species *Neomysis integer* and *M. wooldridgei* were used as outgroup. The appropriate best-fit substitution model of DNA evolution was determined by a likelihood ratio test implemented in MODELTEST 3.06 (Posada & Crandall, 1998). Parsimony analysis was performed by heuristic searches under TBR branch swapping and 10 000 random taxon addition replicates. Maximum likelihood analyses were also run in PAUP, using the model and parameters selected by MODELTEST through neighbour-joining or heuristic searches. Robustness of the resulting phylogenetic trees was tested by bootstrapping (Felsenstein, 1985), with 1000 replications for the NJ and MP analyses and 100 replications for the ML analysis. Each gene was analysed independently. In addition, a network between the COI haplotypes was constructed using the minimum spanning tree algorithm (MST) (Excoffier *et al*, 1992) implemented in ARLEQUIN 2.0 (Schneider *et al*, 2000).

Population genetic statistics were estimated for the COI dataset using ARLEQUIN 2.0 (Schneider *et al*, 2000). Standard diversity values as haplotype diversity (h; Nei, 1987) and nucleotide diversity (π; Nei, 1987) were calculated for each sample. A hierarchical analysis of molecular variance (AMOVA, Excoffier *et al*, 1992) was performed in order to quantify the geographical differentiation of haplotypes. In addition, pairwise genetic divergences between populations were estimated using the fixation index ϕ_{ST} (Excoffier *et al*, 1992). Significance of variance components and pairwise population comparisons was tested by 10 000 permutations. Mismatch distributions, which represent the frequency distribution of pairwise difference among haplotypes in a sample, were analysed to further explore the historical demography of the populations and the species (Slatkin & Hudson, 1991; Rogers & Harpending, 1992; Schneider & Excoffier, 1999). A fit of the observed mismatch distribution to the model of a sudden population expansion was calculated by quantifying the sum of

squared deviations (SSD) between the observed and simulated distributions on one hand and the expected distribution on the other. This distribution is usually unimodal for lineages that experienced a recent bottleneck or population expansion, and multimodal for a lineage whose population is in demographic equilibrium or is subdivided into several units. Rogers' (1995) parameters of mismatch distribution (τ, θ_0, θ_1) were assessed by Monte Carlo simulations of 1000 random samples. Additionally Tajima's D statistic (Tajima, 1989) and Fu's F_s test (Fu, 1997) for selective neutrality were calculated. For neutral markers significant negative values can be expected in case of a population expansion (Knowles *et al*, 1999). All analyses were performed using the ARLEQUIN 2.0 package. Isolation-by-distance was evaluated by plotting pairwise genetic distances over geographical distances for all pairs of samples. The mean sequence divergence between samples corrected for within-sample divergence (d_A) was used as genetic distance measure. d_A was calculated as $d_A = P_{xy} - (P_x + P_y)/2$, where P_{xy} is the mean sequence divergence between populations, and P_x and P_y are the mean sequence divergence within population x and y (Nei & Li, 1979). Geographical distance was calculated as the shoreline distance between sites. The strength and statistical significance of associations between geographical distance (calculated as minimal shore-line distance) and genetic differentiation was tested with reduced major axis regression and Mantel permutation tests using the program IBD v1.52 (Bohonak, 2002).

RESULTS

Sequence variation

A fragment of 458 bp from the mitochondrial COI gene was obtained for 101 individuals of the mysid *Mesopodopsis slabberi* from five Atlantic and two Mediterranean locations (Table 5.1 and Fig. 5.1). A total of 148 variable sites (32%) were detected, of which 124 were parsimony informative (see Appendix 1). No indels were observed. Most substitutions involved transitions, with a transition/transversion ratio amounting to 5.25. Changes at the third codon position were more then six times more common then first codon changes (86% and 14% respectively), while substitutions at the second codon position were non-existent. Only eight substitutions caused a replacement mutation resulting in an amino acid substitution (see Appendix 1). The mutation rate among sites along the COI fragment of *M. slabberi* was heterogeneous yielding a low value for the gamma shape parameter, alpha (α = 0.898). The 101 individual sequences yielded a total of 79 different haplotypes of which the majority was only represented once, resulting in a very high haplotype diversity (h= 0.9835 ± 0.0061). Pairwise differences between haplotypes ranged from 0.21% (a single substitution) to 19.43% nucleotide divergence (89 substitutions).

Table 5.1: Geographical location and sampling date of the different sampling locations. The number of individuals analyzed per sampling location for each molecular marker are specified.

Sampling site	Code	Latitude	Longitude	Sampling date	COI	16S
Westerschelde (Atl)	WS	51° 25' N	4° 0' E	Aug 2001	25	3
Seine (Atl)	SEI	48° 26' N	0° 10' E	May 2001	19	
Mondego (Atl)	MO	40° 09' N	8° 49' W	Jul 2000	10	6
Ria de Aveiro (Atl)	RdA	40° 41' N	8° 45' W	Jun 2002	16	
Guadalquivir (Atl)	GU	36° 55' N	6° 17' W	May 2001	18	3
Alicante (Med)	ALI	38° 18' N	0° 27' W	Dec 2003	8	3
Ebro (Med)	EB	40° 43' N	0° 54' E	Apr 2002	7	3

The analysis of the 16S rRNA fragment yielded a fragment of 487 bp. With inclusion of the congeneric species *M. wooldridgei*, a total of 64 variable sites (13%) were detected, of which 42 were parsimony informative, defining 13 distinct

haplotypes (see Appendix 2). The transition/transversion amounted to 3.63. Three indels were observed, of which two were specific to *M. wooldridgei* and the third deletion was specific to the haplotypes of the Mediterranean Alicante (ALI) population. Pairwise differences between haplotypes ranged from 0.21% (a single substitution) to 6.16% nucleotide divergence (30 substitutions).

Phylogenetic relationships

The hierarchical likelihood ratio test and the Akaike Information Criterion (AIC) test performed with MODELTEST 3.06 (Posada & Crandall, 1998) indicated the the transversion model (TVM) with correction for invariable sites (*I*) and rate heterogeneity (*G*) and TVM + *G* as the appropriate nucleotide substitution model for the COI and 16S dataset respectively. For the COI dataset the proportion of invariable sites (*i*) and the gamma shape parameter (α) were 0.3724and 0.6187 respectively. The base frequencies were estimated to be A = 0.3000, C = 0.1442, G = 0.1673, T = 0.3886. In case of the 16S dataset the model parameters were: $\alpha = 0.7757$ and the base frequencies A = 0.3424, C = 0.1513, G = 0.1604 and T = 0.3459.

mitochondrial COI gene

Phylogenetic analysis of the mtCOI sequences under the parsimony criterion yielded 36 most parsimonious trees of 895 steps (Consistency index (CI) = 0.4737, Homoplasy index (HI) = 0.5263, Retention index (RI) = 0.7354). The bootstrap 50% majority-rule consensus tree is shown in Fig. 5.2. The maximum likelihood and distance (neighbour-joining) heuristic search resulted in a tree nearly identical to the MP topology. The (single) most likely tree had a likelihood of -ln *L* = 4369.34396. Bootstrap support for the NJ and ML tree are indicated on the consensus MP tree (see Fig. 5.2). The phylogenetic tree was characterised by four major clades which all had moderate to relatively high bootstrap support. The different clades showed a clear geographic structuring. A first, large, clade contained the majority of the Atlantic haplotypes (hereafter called the 'Atlantic clade'). Some degree of substructuring was apparent, like the existence of a small subclade containing Portuguese haplotypes (Mondego & Ria de Aveiro), however most nodes lacked a relevant bootstrap support.

A second highly supported clade included four haplotypes of the Portuguese Mondego sample. The two divergent sympatrical clades in the Mondego (MO) sample is remarkable, and therefore the codes MO-A and MO-B will be used in the future analyses to refer to the Mondego haplotypes belonging to the Atlantic clade and belonging to this second divergent clade respectively. A third clade contained the single haplotype found in the Mediterranean Ebro sample ('MEDIT 1 clade'). And finally, a fourth clade included all haplotypes of the Mediterranean Alicante sample ('MEDIT 2 clade').

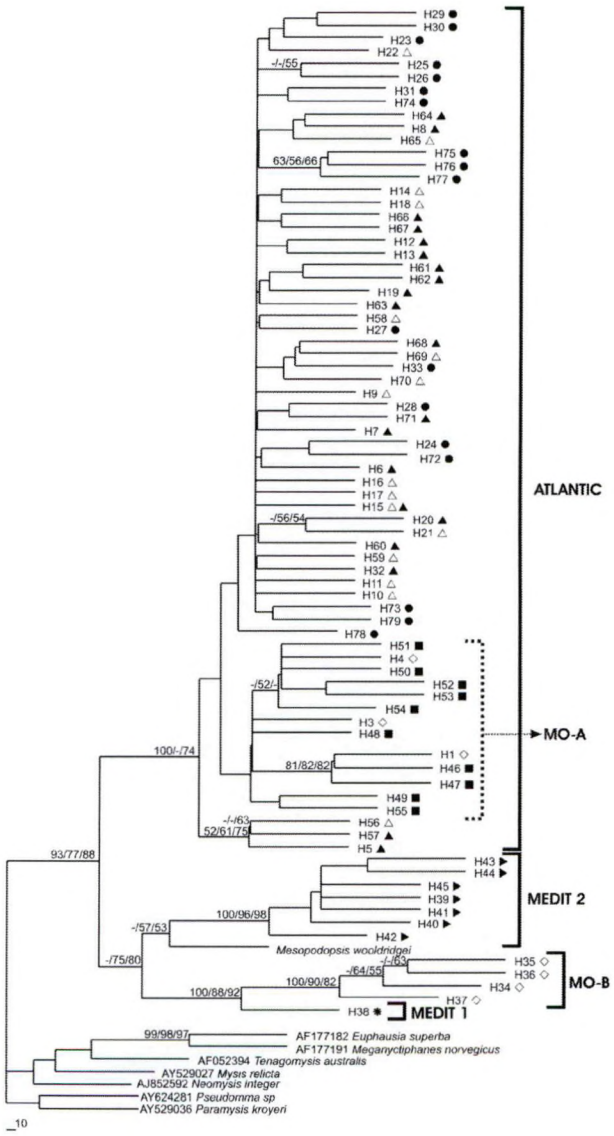

Fig. 5.2: Maximum parsimony consensus tree (895 steps) of the mitochondrial COI haplotypes of *Mesopodopsis slabberi* obtained after a heuristic search of 1,000 random sequence addition replicates. For each node the MP, ML and NJ bootstrap support is indicated, for clearness only bootstrap values > 50% are indicated. ▲ = Westerschelde, △ = Seine, ◇ = Mondego, ■ = Ria de Aveiro, ● = Guadalquivir, ▶ = Alicante, ✱ = Ebro.

This phylogeographic structure of *M. slabberi* was also highly supported by the minimum spanning haplotype network (Fig. 5.3). The two haplotypes that were common to the Westerschelde and Seine samples (H9 and H15) had a central position in the network. All other haplotypes of the Westerschelde and Seine samples were more or less related to these central haplotypes (uncorrected sequence divergence ranging from 0.22 – 1.09%) generating a star-like phylogeny. The majority of the haplotypes belonging to the Iberian Guadalquivir sample were also related to the central haplotype H9, albeit more divergently (1.09 – 2.4%). The haplotypes of the Portuguese Ria de Aveiro and Mondego samples formed a subgroup with a minimal uncorrected sequence divergence of 2.18% between the central haplotype H9 and this subgroup. The three other highly divergent subgroups within the network correspond to the 'MO-B clade', 'MEDIT 1' and 'MEDIT 2' clades. Average sequence divergence within and between the major clades are listed in Table 5.2. The deepest split was observed between both Mediterranean clades (MEDIT 1 and MEDIT 2) and the Atlantic clade, with an average net divergence (i.e. sequence divergence corrected for ancestral polymorphism according to Nei & Li [1979]) of 16.31%. However, net divergences between both Mediterranean clades (14.93%), between the MEDIT 2 and MO-B clades (15.53%) and between the Atlantic and MO-B clades (14.63%) fell in a similar range. The split between the MO-B and MEDIT 1 clades seemed to be younger (net divergence of 6.53%). Using a mutation rate of 1.4 - 2.6% per My for crustacean mitochondrial DNA (Knowlton & Weight, 1998; Schubart *et al*, 1998; Patarnello, 1996; Zane *et al*, 2000) the split between the Atlantic and Mediterranean lineage was estimated at 6.3 – 9.8 million years ago.

Table 5.2: Average sequence divergence (%) between major mitochondrial COI clades. *Diagonal*: average uncorrected sequence divergence within clades. *Above diagonal*: average uncorrected sequence divergence between clades. *Below diagonal:* average sequence divergence between clades corrected for ancestral polymorphism.

	Atlantic	MO-B	MEDIT 1	MEDIT 2
Atlantic	**1.80**	15.68	17.01	17.65
MO-B	14.63	**0.31**	6.68	16.12
MEDIT 1	16.11	6.53	**0.00**	15.37
MEDIT 2	16.31	15.53	14.93	**0.88**

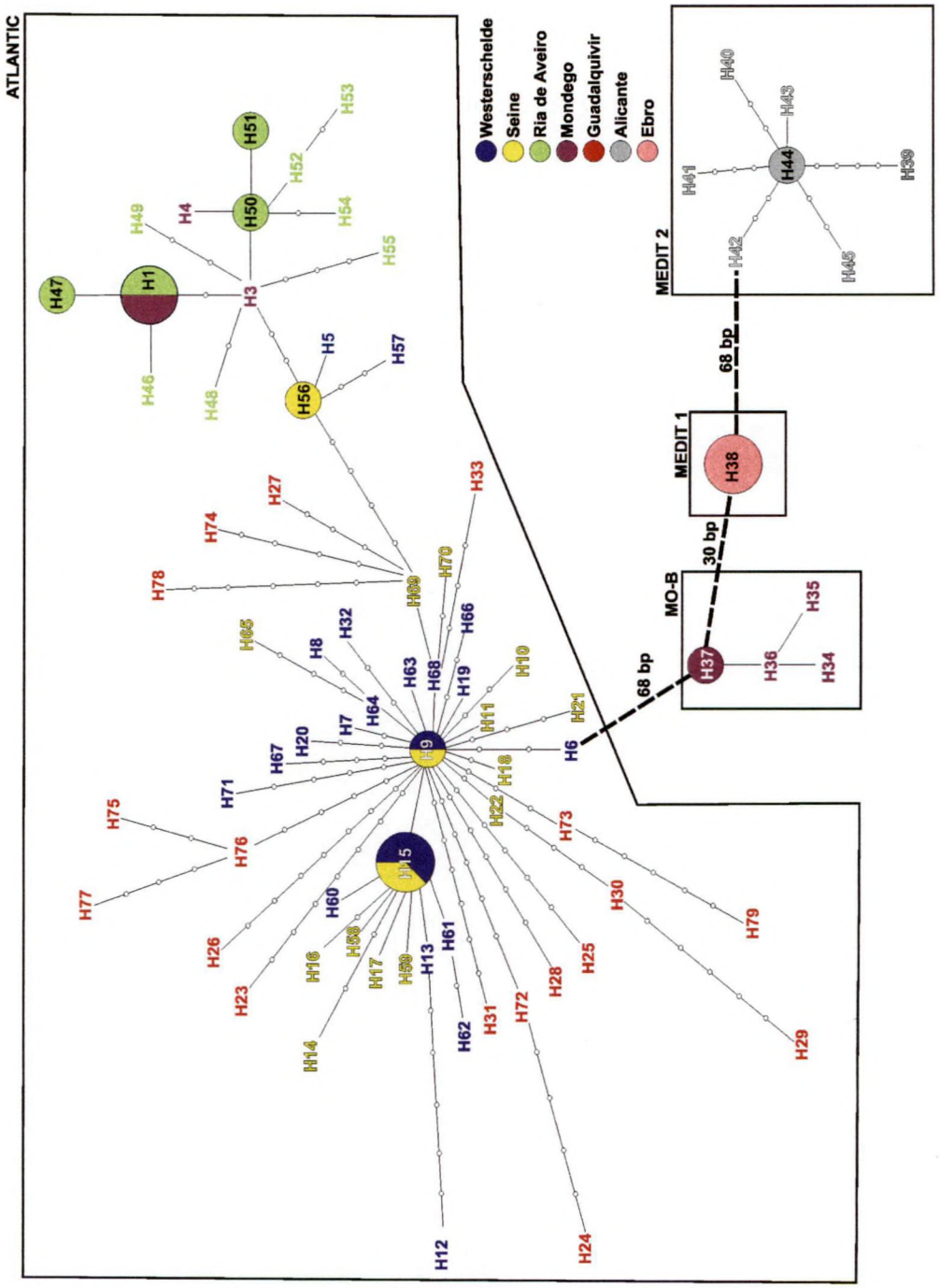

Fig. 5.3: Minimum spanning network showing the mutational relationships among the mitochondrial COI haplotypes of *Mesopodopsis slabberi*. Each line in the network represents a single mutational change and haplotypes are represented by a circle if the haplotype frequency > 1. The surface size of each circle is proportional to its frequency of occurrence and the circles are shaded according to their geographic occurrence. Small empty circles indicate missing haplotypes.

16S rRNA

MP analysis on the 16S rRNA sequences resulted in a single most parsimonious tree of 682 steps (CI = 0.8211, HI = 0.1789, RI = 0.7399). One most likely tree with a likelihood of -Ln *L* = 3151.17927 was obtained by the ML analysis. The tree topology of the 16S phylogeny (Fig. 5.4) is highly congruent with the COI phylogeny (Fig. 5.3). Four clades were supported by high bootstrap values, and correspond to the 'Atlantic', 'MO-B', 'MEDIT 1' and 'MEDIT 2' mtCOI clades. Levels of nucleotide divergence between those clades showed the same patterns as for the COI dataset, however they were much lower; divergences between the Atlantic and Mediterranean clades, the Atlantic and MO-B clades and both Mediterranean clades ranged from 4.7 to 6.4%, and now the split between the MO-B and MEDIT 1 clades (1.23%) seemed to fall in the range of nucleotide divergence within the Atlantic clades (0.2 – 1.32%).

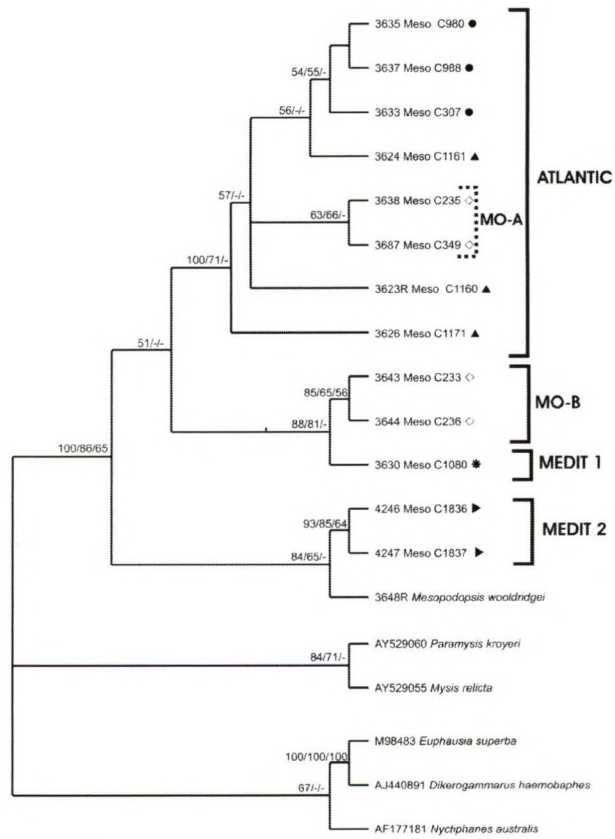

Fig. 5.4: Maximum parsimony consensus tree (682 steps) of the mitochondrial 16S rRNA haplotypes of *Mesopodopsis slabberi* obtained after a heuristic search of 1,000 random sequence addition replicates. For each node the MP, ML and NJ bootstrap support is indicated, only bootstrap values > 50% are indicated. ▲ = Westerschelde, ◇ = Mondego, ● = Guadalquivir, ▶ = Alicante, ✱ = Ebro.

Population diversity and structure

Mitochondrial COI haplotype diversity within the samples was very high, since almost every individual analysed possessed a unique haplotype (Table 5.3), with exception of the Mediterranean Ebro sample where the five analysed specimens bear the same haplotype. In addition, only three out of the 79 haplotypes were found in more then one sample (see Appendix 3). Nucleotide diversity ranged, when excluding the invariable Ebro sample, from 0.008461 (RdA) to 0.089956 (MO). The very high levels of nucleotide diversity in the Mondego sample are caused by the existence of two divergent mitochondrial lineages (MO-A and MO-B). Separately, haplotype and nucleotide diversity of these mtDNA lineages amounted respectively to 0.7000 (± 0.2184) and 0.004367 (± 0.003429) for MO-A and 0.9000 (± 0.1610) and 0.003057 (± 0.002601) for MO-B.

Table 5.3: Standard diversity values per sampling location. N_h= number of haplotypes, h= haplotype diversity, π= nucleotide diversity. Standard deviations of haplotype and nucleotide diversity values are indicated between brackets.

Sampling location	Sampling location	Sample size	N_h	h	π
Westerschelde	WS	25	21	0.9667 (0.0292)	0.010888 (0.006104)
Seine	SEI	19	16	0.9766 (0.0267)	0.010483 (0.005985)
Mondego	MO	10	7	0.9111 (0.0773)	0.089956 (0.048351)
Ria de Aveiro	RdA	16	11	0.9500 (0.0364)	0.008461 (0.005022)
Guadalquivir	GU	18	18	1.0000 (0.0185)	0.019993 (0.010789)
Alicante	ALI	8	7	0.9643 (0.0772)	0.008812 (0.005599)
Ebro	EBR	5	1	0	0

An AMOVA using the Tamura & Nei (1993) distance performed on the mtDNA COI sequence data set of the Atlantic clade (comprising the WS, SEI, RdA, MO-A and GU samples) revealed significant heterogeneity among the Atlantic populations. Although the variance component within populations (59.92%) was higher, a highly significant amount of variation was observed between populations ($\Phi_{ST} = 0.4001$, $P < 0.001$).

Pairwise Φ_{ST} values between the Atlantic samples are listed in Table 5.4. Of all the possible Φ_{ST} comparisons only two were not significant. These comparisons involved the geographical proximate samples WS & SEI and RdA & MO-A. A Mantel test detected a marginally significant correlation of pairwise genetic distance and geographical distance ($r = 0.7040$, $P < 0.05$) pointing to an isolation-by-distance pattern. The RMA regression showed that almost 50% of the observed variance was explained by this correlation.

Table 5.4: *Below diagonal*: pairwise Φ_{ST} values between sampling sites based on pairwise Tamura & Nei (1993) distances. Significant values at 99% level (***) were calculated from 10,000 permutations and are indicated. *Above diagonal*: Average pairwise differences between sampling sites corrected for within-sampling site ancestral polymorphism (D_A) calculated based on the Tamura & Nei (1993) model.

	WS	SEI	RdA	MO-A
WS	-	0	6.75	7.12
SEI	0^{ns}	-	6.83	7.21
RdA	0.5904^{***}	0.6035^{***}	-	0
MO-A	0.5931^{***}	0.6119^{***}	0^{ns}	-

Patterns of historical demography

The mismatch distribution for the pooled sample was clearly not unimodal, and hence deviated significantly from the expected distribution under the sudden expansion model (Fig. 5.5). The first peak (around 10 bp of pairwise differences) represents differences within the major mitochondrial clades, while the smaller peaks around 30, 70 and 80 bp of pairwise differences represent differences between different mtDNA clades. However, within the different mitochondrial clades and at a more regional scale evidence of population expansion could be detected as shown by the mismatch distributions and the Tajima's *D* and Fu's Fs tests of neutrality (Fig. 5.5 and Table 5.5). Because of the lack of differentiation between the Westerschelde and Seine samples (WS+SEI) and the Ria de Aveiro and Mondego samples (RdA + MO-A), these samples were pooled and considered as panmictic metapopulations for the demographic analyses. A fit to the sudden expansion model of Rogers (1995) could not be significantly rejected for all regional samples, however only the mismatch distributions of the WS+SEI and GU samples were clearly unimodal. These samples had also significantly negative Tajima's *D* and Fu's *Fs* values, further supporting a

population expansion. The estimates of the pre- and post-expansion effective population sizes (θ_0 and θ_1) showed a very large increase in size for the GU sample, suggesting rapid population expansion (Table 5.5). In contrast, the much lower increase of the population size of the WS+SEI sample might be indicative for a more recent expansion. The Mediterranean ALI sample (belonging to the MEDIT 2 clade) had a clearly more ragged mismatch distribution, which together with the marginally non-significant Fu's F_S value ($P = 0.051$) could be due to the smaller sample size (n = 8). Mismatch distributions of the MEDIT 1 and MO-B clades were not calculated due to the low sample size and the presence of only one haplotype in the Ebro population (MEDIT 1 clade).

An approximate time of expansion was estimated for the WS+SEI and GU samples using the equation $T= \tau/2\mu$ (Rogers & Harpending, 1992). Based on a mutation rate (μ) of 1.66 - 2.6% per My for crustacean mitochondrial DNA (Knowlton & Weight, 1998; Schubart *et al*, 1998; Patarnello, 1996; Zane *et al*, 2000) and a mean generation time of approximately 4 months (Delgado *et al*, 1997; Uppabullung, 1999), the expansion was estimated to have occurred 44-69 kya in the WS+SEI sample and 133-200 kya in the GU sample.

Table 5.5: Mismatch distribution parameters for the regional samples of *Mesopodopsis slabberi*. (θ_0 and θ_1 = pre- and post-expansion effective population sizes; τ = time in number of generations, elapsed since the sudden expansion episode; SDD, sum of squared deviations. *P*-values for the rejection of the sudden expansion model are indicated. For the neutrality tests, the statistical significance are shown (***: $P < 0.01$, ^{ns}: not significant, $P > 0.05$).

	WS+SEI	RdA+MO-A	GU	ALI
Mismatch mean	4.873	3.414	9.157	4.036
θ_1	2.245	0.000	0.000	0.000
θ_2	37.017	14.990	66655.000	4682.500
τ	3.153	4.532	9.485	4.547
Test of goodness-of-fit				
SSD	0.0009	0.0144	0.0169	0.0293
P	0.900	0.290	0.060	0.430
Neutrality tests				
Tajima's *D*	-2.1701***	-1.1809^{ns}	-1,8516***	-1,5473***
Fu's Fs	-25.5756***	-5.5425***	-10,9733***	-2,4358^{ns}

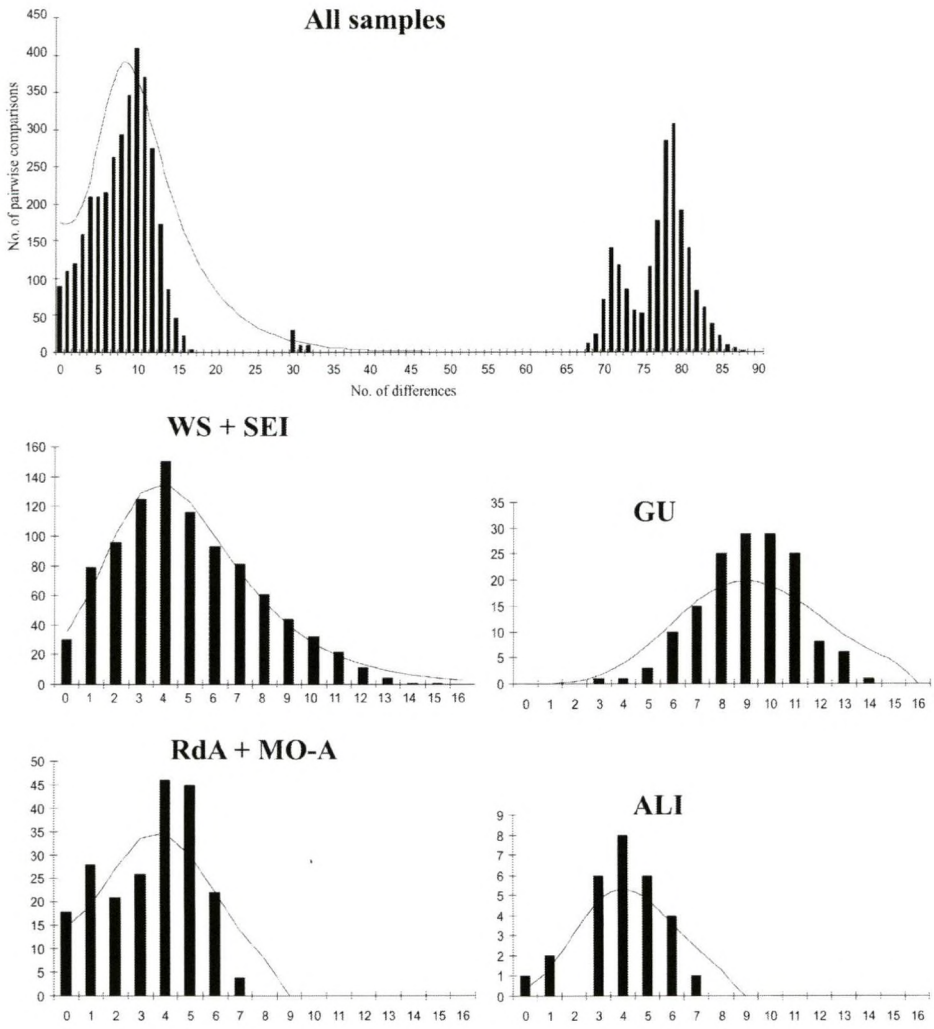

Fig. 5.5: Mismatch distribution of the samples; for sampling site abbreviations see Table 5.1. In each case the bar represents the observed frequency of the pairwise differences among haplotypes, while the solid line shows the distribution expected under a model of sudden demographic expansion (Rogers, 1995).

DISCUSSION

Patterns of molecular variation

The analysed fragment of the mtDNA COI gene of *Mesopodopsis slabberi* was extremely variable. The DNA sequence analysis revealed that more than 32 % of the nucleotide positions were variable, whereas in the mitochondrial COI gene of the related mysid *Neomysis integer* only 8.5% of the nucleotide positions were polymorphic (see Chapter 4). Extremely high values of haplotype diversity were observed in the majority of the samples, with a very high proportion of unique haplotypes present in low frequencies. This high variability is not uncommon in marine species (see Bucklin & Wiebe, 1998; Duran *et al*, 2004b; Peijnenburg *et al*, 2004; Stamatis *et al*, 2004; Zardoya *et al*, 2004) and could be attributed to the enormous population sizes, resulting in the retention of many unique haplotypes during population growth or expansion (Watterson, 1984; Bucklin & Wiebe, 1998). The large difference in levels of molecular variation compared with *N. integer* could be partly explained by the existence of highly divergent mitochondrial COI lineages within *M. slabberi*, and the larger spatial sampling of the present study. However, levels of molecular diversity within the different mtCOI clades of *M. slabberi* (mean h = 0.807 and π = 0.0083) are still considerably larger than in *N. integer* (mean h = 0.366 and π = 0.0023). A possible explanation for these differences could be the constraints on gene flow between populations of the strictly brackish *N. integer* in combination with the biological adaptations to the unpredictable instability of chemical-physiological parameters in brackish-water environments (Battaglia *et al*, 1978; Röhner, 1997). The genetic impoverishment of the brackish water fauna has been widely reviewed (Cognetti, 1994; Maltagliati, 1999; Cognetti & Maltagliati, 2000; Bilton *et al*, 2002). On the other hand, the higher environmental heterogeneity of the habitats where *M. slabberi* is found (estuaries, coastal zones, surf zones, salt marches), and hence the increase in available niches and microhabitats, might result in a higher level of genetic variation, as reported for marine gobies (Wallis & Beardmore, 1984).

Apart from ecological processes (e.g. differences life history, population dynamics, environmental interactions), also evolutionary dynamics (mutation, genetic drift, natural selection) which act on a species over different temporal and spatial scales may have caused different patterns of population genetic diversity in species (Bucklin & Wiebe, 1998). For instance, the differences in the number of haplotypes and the levels of nucleotide diversity within *M. slabberi* and *N. integer* might be related to the different age of both species. Based on coalescence theory it is generally assumed that older lineages harbour more genetic diversity through persistent accumulation of alleles compared to younger ones (Crandall & Templeton, 1993). Differences in the relative age of both species may also be reflected in the level of species diversity of the genus *Mesopodopsis* and *Neomysis* within the Atlantic Ocean and the different biogeographical distribution of both genera. The genus *Mesopodopsis* harbours the highest level of species diversity within the Atlantic and Indian Ocean, while the diversity 'hot-spot' of the genus *Neomysis* lies within the Pacific Ocean (see NeMys database, http://intramar.ugent.be/nemys, Deprez *et al*, 2004). Hence, this could imply a 'more recent' colonization of the northern Atlantic by *Neomysis* from the Pacific following the opening of the Bering Strait in the late Pliocene (Vermeij, 1991), with a subsequent radiation into the NW Atlantic *N. americana* and the NE Atlantic *N. integer*. In addition, historical changes in effective population size, variance in reproductive success, differential response to historical range compression and changes in the selective regime are also thought to affect species differently, resulting in interspecific variations of the levels of molecular diversity (Avise *et al*, 1988; Hedgecock, 1994a; Bucklin & Wiebe, 1998).

The Mediterranean Ebro population of *M. slabberi* seems to be an exception to the general trend of high genetic diversity within *M. slabberi* populations. Only one haplotype (H 38) was shared by the five specimens analysed. Since this reduced level of molecular diversity could be caused by the lower sample size (n = 7), a more extended sample of 25 specimens was screened with Single-Strand Conformation Polymorphism (SSCP) analyses and the preliminary results confirm the pattern of reduced diversity. Low levels of mitochondrial DNA diversity can be attributed to several events such as severe population fluctuations, inbreeding, strong natural selection, population extinctions and recolonizations due to environmental modification of natural and/or anthropogenic origin, or alternatively caused by a

recent founding event (Cognetti & Maltagliati, 2004; Stamatis, 2000). The latter event, a recent colonization and founding event, seems unlikely since *M. slabberi* is widely distributed throughout the whole Mediterranean Sea. On the contrary, strong demographic bottlenecks resulting in the removal of the genetic diversity seems more plausible. Temporal fluctuations in population size, sometimes leading to a complete removal of *M. slabberi* from the Ebro Delta, has been observed (Ribera, pers. com.) probably as a result of the treatment of rice fields within the delta with toxic chemicals (organophosphorous pesticides such as fenitrothion) (Solé *et al*, 2000). Consequently, these temporal population crashes could lead to reduced levels of genetic diversity.

Intra- or interspecific variation?

Analysis of the mitochondrial COI and 16S rRNA genes revealed extremely high levels of genetic divergence between morphologically indistinguishable populations of *M. slabberi*. Four clades were apparent in the COI and 16S phylogenies and in the COI haplotype network. The levels of nucleotide divergence for the COI gene between these clades as listed in table 5.2 clearly shows that the different clades are largely differentiated from each other. Moreover, most values seem to fall within the range of nucleotide divergence between the morphological distinguishable species *M. wooldridgei* and *M. slabberi* (14.9 – 17.9% of uncorrected nucleotide divergence). Furthermore, these values are much higher than the intraspecific divergences reported for the brackish water mysid *N. integer* (0.22 – 2.68%) or even between the congeneric *N. integer* and *N. americana* (10%) (see Chapter 4). On the other hand, divergences within the different *M. slabberi* clades are similar to the intraspecific divergences observed within *N. integer*. When compared to other marine crustaceans, these values seem to be equivalent to those of closely related species or between cryptic species (see Lee, 2000; Rocha-Olivares *et al*, 2001; Holland, 2004). Thus, the reciprocal monophyly of the different clades in the mitochondrial COI phylogeny as well as in the more conserved 16S rRNA gene tree, the levels of nucleotide divergence and the presence of a high number of fixed differences between the different *M. slabberi* clades (see Table 5.2 and Appendices I and II) indicate that this nominal species is most probably a complex of cryptic species. Clearly, analysis of additional molecular markers (e.g. nuclear genes) and a

more extensive sampling would be needed to validate the exceptional degree of divergence among *M. slabberi* clades and to draw further conclusions on the taxonomic status of this species.

Phylogeographic patterns along the northeast Atlantic and Mediterranean coasts

M. slabberi populations of the Atlantic and Mediterranean Sea are clearly differentiated as shown by the AMOVA. The pairwise Φ_{ST} values indicate a long-term interruption of gene flow and suggest that current lineages undergo no genetic exchange. The degree of genetic divergence found between the Mediterranean and Atlantic lineages suggests that the different clades have evolved independently in vicariance. Last decade the Atlanto-Mediterranean transition has been studied for a variety of marine taxa, showing a clear break between both basins for several species, whereas for other no differentiation at all was detected (see overview in Table 5.6). The observed nucleotide divergence between the Atlantic and Mediterranean *M. slabberi* clades are, together with those for *Carcinus maenas* (Roman & Palumbi, 2004), amongst the highest reported thus far for marine invertebrates (see Table 5.6). Historically, the connection between the Atlantic and Mediterranean through the narrow Strait of Gibraltar has been blocked on several occasions. Firstly, during the Messinian salinity crisis (6 – 5.5 Mya) when the Mediterranean basin desiccated and transformed into a series of hypersaline lakes with thick evaporate deposition (Krijgsman *et al*, 1999). Secondly, during the Quaternary glaciations (1.8 Mya – 18 kya) when glacio-eustatic sea level drops (115-120 m below the present-day level) resulted in a fragmentation of the Atlantic and Mediterranean (Nilsson, 1982; Maldonado, 1985). On a more contemporary time scale a restriction of exchange exists between both basins caused by an oceanographical density front located in the Alboran Sea (the Oran-Almeria Front; Tintore *et al*, 1998; Millot, 1999), as demonstrated for a number of species (Quesada *et al*, 1995; Sanjuan *et al*, 1996; Zane *et al*, 2000). The estimations of divergence time between the Atlantic and Mediterranean mitochondrial lineages of *M. slabberi* suggest that the split occurred

Taxon	Mol. Marker	Atl/Medit divergence (%)	Timing	Author
Marine invertebrates				
Mesopodopsis slabberi - mysid	mt COI gene	16%	6.3 - 9.8 Mya	this study
Carcinus maenas - crab	mt COI gene	11%	5 - 8 Mya	Roman & Palumbi 2004
Cerastoderma glaucum - cockle	mt COI gene	1.27 - 6.2%	100 - 360 kya	Nikula & Väinölä 2003
Sagitta setosa - chaetognath	mt COII gene	6.1 %	1.7 Mya	Peijnenburg et al. 2004
Nephrops norvegicus - lobster	mtDNA RFLP	no differentiation	NA	Stamatis et al. 2004
Homarus gammarus - lobster	mtDNA RFLP	NA, strong differentiation	NA	Triantafyllidis et al. 2004
Meganyctiphanes norvegica - euphausiid	mt NADH gene	NA, distinct gene pools	NA	Zane et al. 2000
Monocelis lineata - flatworm	allozymes	NA, sharp separation	6.3 Mya	Casu & Curini-Galletti 2004
Sepia officinalis - cuttlefish	allozymes	NA, strong differentiation	NA	Perez-losada et al. 1999
Chthamalus montagui - barnacle	allozymes	NA, strong differentiation	NA	Pannacciulli et al. 1997
C. stellatus - barnacle	allozymes	NA, strong differentiation	NA	Pannacciulli et al. 1997
Paracentrotus lividis - sea urchin	mt COI gene	NA, significant differentiation	NA	Duran et al. 2004a
Crambe crambe - sponge	mt COI gene	no differentiation	NA	Duran et al. 2004b
Marine fish				
Pomatoschistus microps - goby	mt cyt *b* gene	0.3 - 2.4%	Pleistocene isolation	Gysels et al. 2004
Aphanius iberus - killifish	mt cyt *b* gene	4.7 - 6.4%	5.5 Mya	Perdices et al. 2001
Lithognathus marmyrus - seabream	mt D-loop	17%	1.2 - 1.8 Mya	Bargelloni et al. 2003
Spondyliosoma cantharus - seabream	mt D-loop	16.4%	1.2 - 1.8 Mya	Bargelloni et al. 2003
Dentex dentex - seabream	mt D-loop	13%	1.2 - 1.8 Mya	Bargelloni et al. 2003
Pagurus bogaraveo - seabream	mt D-loop	no differentiation	NA	Bargelloni et al. 2003
Pagurus pagurus - seabream	mt D-loop	no differentiation	NA	Bargelloni et al. 2003
Xiphias gladius - swordfish	mt D-loop	3.8%	Pleistocene isolation	Bremer et al. 1995
Scomber japonicus - chub mackerel	mt D-loop	no differentiation	NA	Zardoya et al. 2004
Scomber scombrus - mackerel	mt D-loop	no differentiation	NA	Zardoya et al. 2004

Table 5.6: Overview of phylogeographic studies of marine taxa with an Atlanto-Mediterranean distribution. For each study the used molecular marker is indicated and, if available, the degree of nucleotide divergence and timing of the split between the Atlantic and Mediterranean populations. NA = not available.

about 9.8 – 6.3 Mya. Hence it is clear that the Atlanto-Mediterranean divergence predates the onset of the Pleistocene and date back to the late Miocene, even when taking into account the large stochastic errors associated with the estimates of divergence time.

Within the Mediterranean samples two divergent lineages could be identified; one was restricted to the Ebro sample (MEDIT 1) and the other lineage was observed in the Alicante sample (MEDIT 2). It remains unclear how these different Mediterranean clades evolved. One possible hypothesis could be that both clades originated in allopatry in separated basins which were formed within the Mediterranean Sea when sea-level dropped during the Messinian salinity crisis (late Miocene, 5.5 – 6 Mya) (Hsü *et al*, 1977; Por, 1989; Krijgsman *et al*, 1999; Duggen *et al*, 2003). After sea-level rise the different lineages colonized separate regions within the Mediterranean Sea where they remained isolated from each other due to restricted gene flow. Estimates of divergence time between both clades corroborate such an old split (9 - 5.7 Mya, using a molecular clock of 1.66 – 2.6% per My). However, thus far no molecular research has been done on other mysid populations throughout the Mediterranean Sea. The only evidence of differentiation between mysid populations within the Mediterranean comes from a detailed morphological and ecological study of *Diamysis mesohalobia* populations, which are thought to be reproductive isolated and evolved in allopatry during the Messinian sea-level drops (Ariani & Wittmann, 2000). On the contrary, recent simulations has shown that the creation of large divergences doesn't necessary imply an evolution in allopatry. Deep phylogeographic breaks can be formed within a continuously distributed species even when there are no barriers to gene flow, but given that the individual dispersal distance and population size are low enough (Irwin, 2002). Likewise, at some loci extreme divergences can occur by stochastic events (Rosenberg, 2003).

Alternatively, the two clades could have originated from a parapatric speciation (see Gavrilets *et al*, 2000) and/or ecological speciation (see Schluter, 2001; Doebeli & Dieckmann, 2003) between populations in fully marine conditions (e.g. MEDIT 2 clade in the Alicante population), and those in more sheltered, brackish water environments (e.g. MEDIT 1 clade in the Ebro population). This speciation event could have been driven by divergent selection for characteristics that allow a better adaptation to this particular kind of environment, resulting in a 'quick' genetic

divergence between marine and brackish populations of *M. slabberi*. A similar hypothesis of ecological radiation has been used for explaining the differentiation between cryptic species of the interstitial flatworm *Monocelis lineata* separately occurring in marine and brackish habitats (Casu & Curini-Galletti, 2004), and the ascidian *Clavelina lepadiformis* from inside harbours and from the rocky littoral (Tarjuelo *et al*, 2001).

Finally, the co-distribution of two divergent mtDNA lineages within the Mondego estuary (MO-A and MO-B) is remarkable. A more detailed morphological examination of the remaining specimens from this sample revealed no morphological differences between them suggesting that this divergent lineage is morphologically cryptic with *M. slabberi*. The large divergence between both lineages suggests that they are reproductively isolated. Although sympatric speciation has become more accepted in the past decade (see Vai, 2001), for several reasons it seems an unlikely cause for the present pattern. Firstly, both lineages seemed to co-occur under the same environmental conditions, however further analysis are necessary to validate this. Secondly, if the observed pattern is caused by sympatric speciation it remains unclear why this pattern is not more widespread in *M. slabberi*. Thirdly, the phylogenetic and network analysis suggests that the MO-B lineage has not an Atlantic origin but seems to be more related to the Mediterranean haplotypes, and more specifically to those of the MEDIT 1 clade. The nucleotide divergence between the MO-B and MEDIT 1 clades was more than half the divergence between the MO-B and the Atlantic clades for the COI dataset (6.53 and 14.63% respectively), and for the 16S gene the MO-B/MEDIT 1 divergence (1.23%) even seemed to fall within the Atlantic intra-clade divergence range (0.2 – 1.32% respectively). The result are suggestive for an invasion of the MO-B lineage, with Mediterranean origin, by natural means or caused by ballast water of ships into the Mondego estuary (Carlton, 1985; Carlton & Geller, 1993; Lavoie *et al*, 1999; Wonham *et al*, 2000). Ship ballast water transport might be an efficient mechanism for the transfer and dispersal of most taxonomic groups (Carlton and Geller, 1993), and could have a homogenization effect on the genetic pattern or lead to the existence of highly divergent haplotypes within a local population (see Roy & Sponer, 2002; Nobrega *et al*, 2004; Caudill & Bucklin, 2004; Shefer *et al*, 2004). Within mysids the anthropogenic transport by means of ship ballast water has been invoked to explain some recent invasions, e.g. the invasion of

the Mediterranean endemic mysid *Diamysis bahirensis* in the Atlantic Ria de Aveiro estuary (Cunha *et al*, 2000). Clearly, the limited number of specimens analysed doesn't allow us to make firm conclusions and hence detailed molecular and morphological studies are needed to resolve the identity and evolutionary origin of this divergent lineage.

Population structure within the Atlantic clade of Mesopodopsis slabberi

Within the Atlantic clade the null hypothesis of panmixia could be significantly rejected (Φ_{ST} = 0.40, $P < 0.001$). The genetic heterogeneity along the Atlantic coasts is also supported by the highly significant pairwise Φ_{ST} values. The very high proportion of population-specific haplotypes and the existence of few shared haplotypes, only distributed in geographically closely located samples, suggest a restriction of gene flow on a large geographic scale (i.e. > 500 km). However, on a meso-geographical scale (i.e. between the northern Westerschelde and Seine, and the Iberian Ria de Aveiro and Mondego populations) no significant differentiation was observed, indicating high levels of gene flow. The detection of isolation-by-distance, i.e. the decrease of genetic correlation with increasing geographic distance (Wright, 1943), further corroborates this pattern. Hence, the large tolerance range and seemingly continuous habitat of *M. slabberi* enables an exchange of mysids between adjacent populations, while the absence of a dispersal stage (such as pelagic larvae) tends to restrict gene flow on a larger geographic scale. The observed genetic differentiation along the Atlantic coasts could be the result of this isolation by distance pattern whereby no obvious barriers to gene flow are necessary to explain the genetic heterogeneity. On the other hand, the effect of latitudinal differences in selective forces or the existence of historically separated populations (e.g. in multiple glacial refugia), as observed for the mysid *N. integer* (see Chapter 4), cannot be ruled out as a potential cause for the pattern of genetic differentiation.

Demographic analysis of the Atlantic mtCOI clade point to a population expansion in the northern samples (WS and SEI), as shown by the unimodal mismatch distribution and the highly significant negative Tajima's D and Fu's F_S values. In addition, the haplotype network showed a star-shaped genealogy for the haplotypes of these samples, which is also thought to be a signature of a recent demographic

expansion (Slatkin & Hudson, 1991). On the contrary, a temporal stable population structure was suggested for the Iberian samples of the Ria de Aveiro and Mondego estuary. The demographic expansion of the northern populations could be dated back to the Pleistocene epoch, which is concordant to the currently accepted paleoclimatological model of the NE Atlantic during the Quaternary. During the last glacial maximum (about 18 kya) the polar front is hypothesized to have been located near the present-day northern coast of the Iberian peninsula (Frenzel *et al*, 1992), sea level lowered 100-120 m (Lambeck *et al*, 2002) and as a result the North Sea and English Channel were mainly dry land (Andersen & Borns, 1994). These drastic climatological changes forced most temperate species to the south where they survived in glacial refugia (e.g. off the Iberian Peninsula). After the last glacial maximum when the conditions in northern Europe ameliorated and sea level rose, new habitats became available and were rapidly colonised by mysids from the southerly located refugia, followed by a demographic population expansion in these areas (Hewitt, 1999, 2004). These results contrast with the patterns observed in the mysid *N. integer*, where no evidence of a sudden population expansion was found in the North Sea and English Channel populations, probably caused by the existence of multiple northern refugia (see Chapter 4). This could be indicative for the higher susceptibility to climate oscillations, and in particular lowered temperatures, of *M. slabberi* compared to *N. integer*. However, these results and conclusions require future validation by means of more extended geographic sampling (e.g. along the UK coasts, North Sea and western Baltic Sea).

CONCLUSIONS

In conclusion, the present phylogeographic study revealed very high levels of genetic divergence with a strong geographic pattern among morphological identical populations of the mysid *Mesopodopsis slabberi*. The levels of divergences observed in the mitochondrial COI gene and in the more conserved 16S rRNA gene suggest that populations of *M. slabberi* have evolved into possible cryptic species during the late Miocene/ early Pliocene. Hence, the current species status within the genus *Mesopodopsis*, may still be an underestimate of the actual species diversity of this genus. Since mitochondrial DNA evidence alone should not justify taxonomic decisions (Hudson & Coyne, 2002), evidence from unlinked molecular markers (e.g. nuclear genes) might be appropriate. The discovery of cryptic species is not uncommon in the marine realm and the existence of cryptic species, especially in invertebrates seems to be a far more widespread phenomenon as previously thought (see Knowlton, 1993, 2000). However, the present study is to our knowledge the first in reporting evidence of cryptic speciation within a mysid species. Continued molecular studies of *M. slabberi* with a more complete geographic sampling of habitats of *M. slabberi* within the Atlantic and Mediterranean Sea, will undoubtedly yield more insights into the phylogeographic patterns and cryptic speciation of this ecological important key species. Moreover, analyses of nuclear markers could be useful to detect reticulate patterns, such as resulting from hybridization and introgression among the different lineages detected in the present study.

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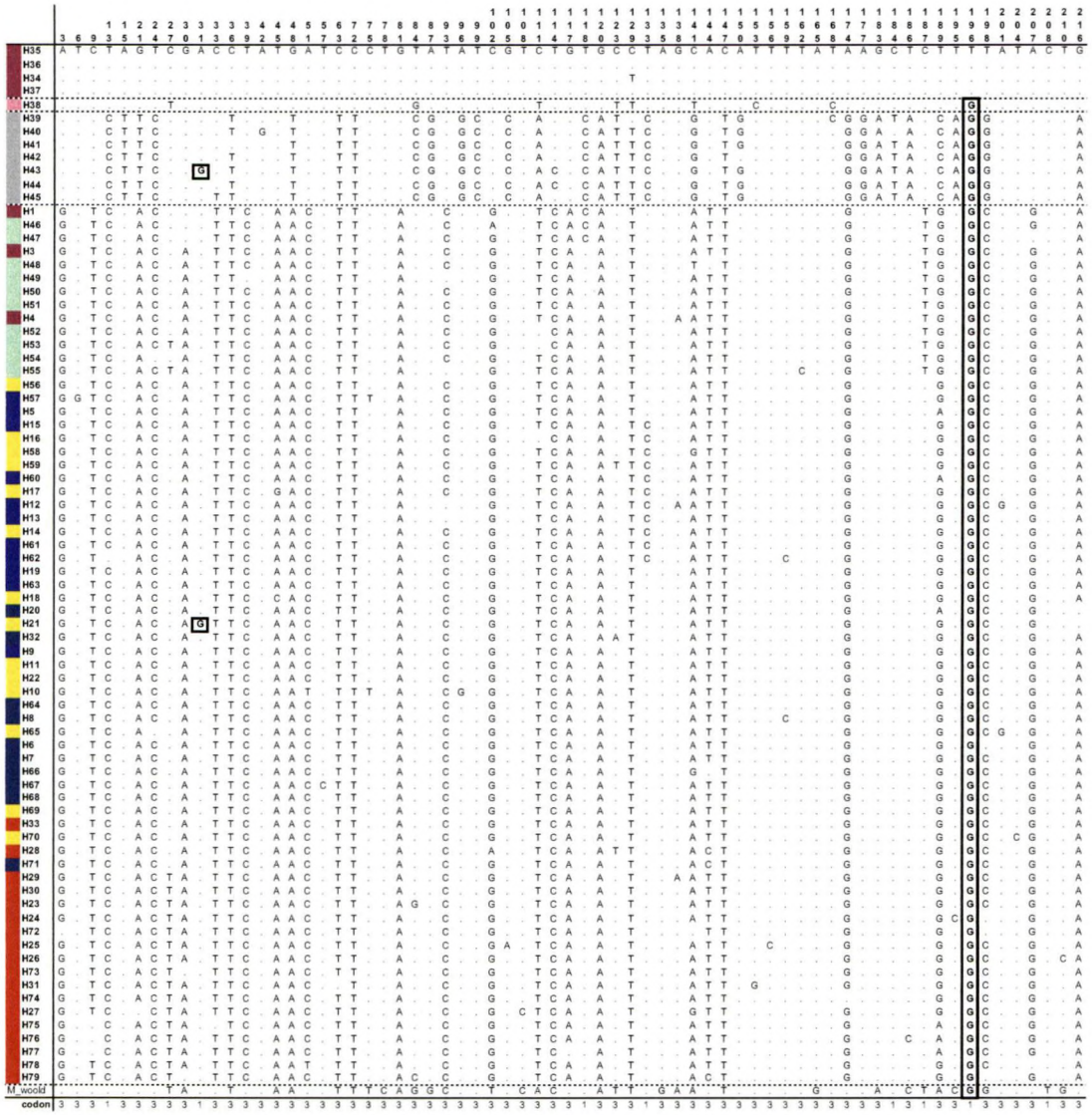
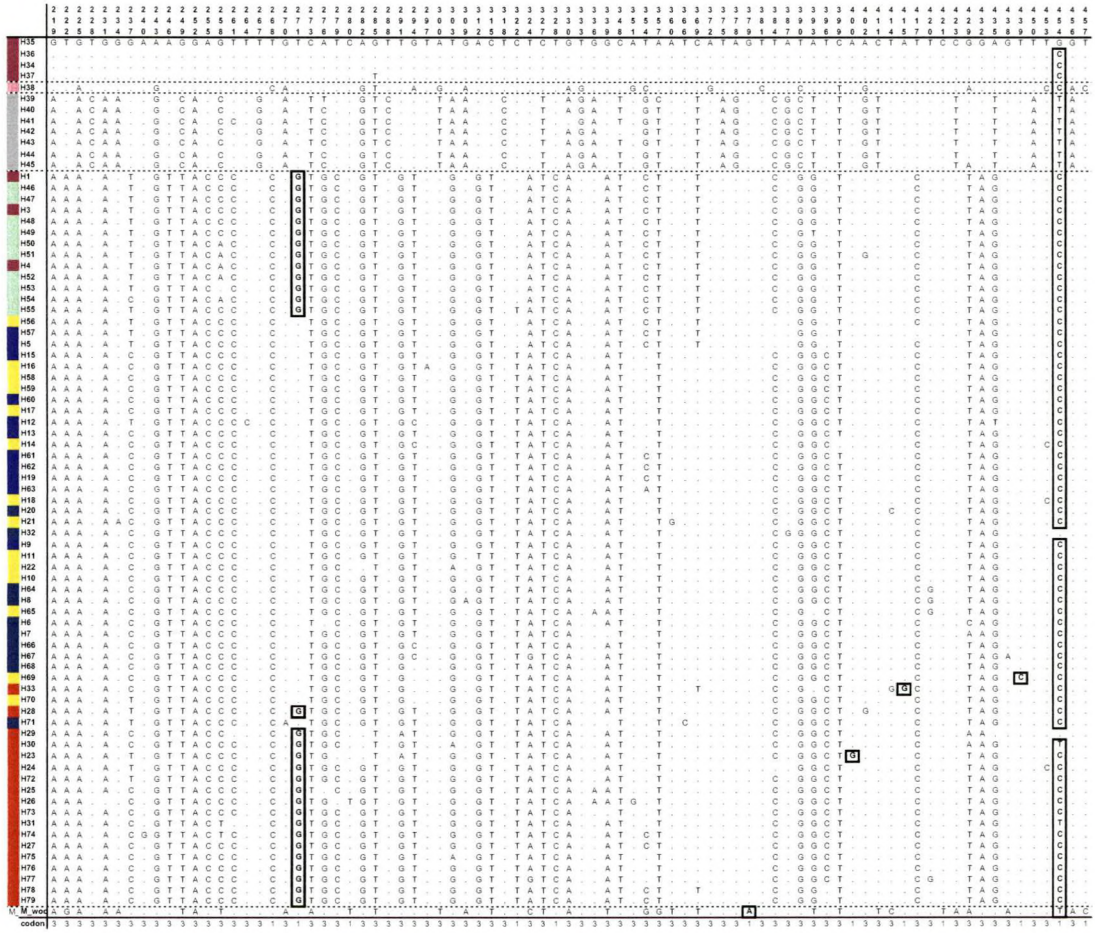
APPENDIX 1: Variable nucleotide positions observed in the 458 bp fragment of the mitochondrial COI gene of the *Mesopodopsis slabberi* and *M. wooldridgei* (M_woold) haplotypes. Colors in the first column indicate geographical origin of the haplotypes (same colour scheme as in Fig. 5.3), dots indicate an identical nucleotide base, dotted horizontal lines delimitate the different mtDNA clades as observed in the MP tree (see Fig. 5.2). Squares represent nucleotide substitutions resulting in an amino acid change. For each nucleotide substitution the codon position at which a change occurred is indicated in the last row.



APPENDIX 1 (continued)



APPENDIX 2: Variable nucleotide positions observed in the 487 bp fragment of the 16 rRNA gene of the *Mesopodopsis slabberi* and *M. wooldridgei* (M_woold) haplotypes. The first column indicates the geographical origin of the haplotypes (for sampling site abbreviations see Table 5.1), dots indicate an identical nucleotide base, dotted horizontal lines delimitate the different mtDNA clades as observed in the MP tree (see Fig. 5.4). - : gap, ? : unknown nucleotide.

		18	19	20	23	31	32	46	48	109	139	145	149	151	152	154	156	157	159	166	172	173	188	232	234	247	248	249	250	251	252	253
MO	3643MesoC233	A	A	A	T	G	G	T	G	G	G	C	A	T	A	A	A	C	T	A	C	T	A	T	T	T	A	T	G	T	T	T
MO	3644MesoC236	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EB	3620MesoC1080	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
GU	3635MesoC980	T	G	G	.	.	.	.	.	.	.	.	C	G	.	.	T	C	.	T	.	.	.	.	G	G	G	C	.	.	C	.
GU	3637MesoC988	T	G	G	.	.	.	.	.	.	.	.	C	G	.	.	T	C	.	T	.	.	.	.	G	G	G	C	.	.	.	.
GU	3633MesoC307	T	G	G	.	.	.	.	.	.	.	.	C	G	.	.	T	C	.	T	.	.	.	.	G	G	G	C	.	.	.	.
WS	3624MesoC1161	T	G	G	.	.	.	.	.	.	.	.	C	G	.	.	T	C	.	T	.	.	.	.	G	G	.	C	.	.	.	.
WS	3623MesoC1160	T	G	G	.	.	.	.	.	A	.	.	C	G	.	.	T	C	.	T	.	T	.	.	G	G	.	C	.	.	.	.
MO	3638MesoC235	T	G	G	.	.	.	.	.	.	.	.	C	.	.	.	T	C	.	T	.	.	.	.	G	G	.	C	.	.	.	.
MO	3687MesoC349	T	G	G	C	.	.	.	.	.	.	.	C	G	.	.	T	C	.	T	.	.	.	.	G	G	.	C	.	.	.	.
WS	3626MesoC1171	T	G	G	.	.	.	.	.	.	.	.	C	G	.	.	T	C	.	T	.	.	.	.	G	G	.	C	.	.	.	.
ALI	4246MesoC1836	.	.	G	A	.	.	A	A	.	.	T	G	.	G	.	G	T	C	T	.	C	.	C	C	.	.	A	.	.	A
ALI	4247MesoC1837	.	.	G	A	.	.	A	C	?	.	T	G	.	G	?	G	.	C	T	.	?	.	C	C	.	.	C	A	C	.	.
	M. wooldridgei	.	.	G	A	A	A	.	T	A	A	T	.	.	G	G	G	.	.	.	.	.	.	C	.	.	.	A	A	A	C	.

		262	277	276	278	291	294	296	299	308	312	313	314	315	316	317	333	338	361	377	379	380	388	402	406	419	423	424	427	429	446	461	475
MO	3643MesoC233	G	C	G	A	A	G	C	A	C	A	C	T	A	T	C	T	G	G	T	T	A	C	A	C	A	A	C	A	-	A	A	A	T	T
MO	3644MesoC236	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	-	.	.	.	.	.	.
EB	3620MesoC1080	.	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	-	.	.	.	.	.	.
GU	3635MesoC980	.	.	A	G	.	C	.	.	.	.	A	.	G	.	T	.	A	.	C	C	G	.	G	T	G	.	.	G	-	.	.	.	A	C
GU	3637MesoC988	.	.	A	G	.	C	.	.	.	.	A	.	G	.	T	.	A	.	C	C	G	.	G	.	G	.	.	G	-	.	.	.	A	C
GU	3633MesoC307	.	.	A	.	.	C	.	.	.	.	A	.	G	.	T	.	A	.	C	C	G	.	G	.	G	G	.	G	-	.	.	.	A	C
WS	3624MesoC1161	.	.	A	G	.	C	.	.	.	.	A	.	G	.	T	.	A	.	C	C	G	.	G	.	G	.	.	G	-	.	.	.	A	C
WS	3623MesoC1160	.	.	A	G	.	C	.	.	.	.	A	.	G	.	T	.	A	A	C	C	G	.	.	.	G	.	.	G	-	.	.	.	A	C
MO	3638MesoC235	.	.	A	G	.	C	.	.	.	.	A	.	G	.	T	.	A	.	C	C	G	.	.	.	G	.	.	G	-	.	.	G	A	C
MO	3687MesoC349	.	.	A	G	.	C	.	.	.	.	A	.	G	.	T	.	A	.	C	C	G	.	.	.	G	.	.	G	-	.	.	G	A	C
WS	3626MesoC1171	.	.	A	G	.	C	.	.	.	.	A	.	G	.	T	.	A	.	.	C	G	.	.	.	G	.	.	G	-	.	.	.	A	C
ALI	4246MesoC1836	.	.	A	.	.	-	.	.	.	G	G	A	.	.	T	A	A	.	.	C	.	.	.	.	G	.	.	G	-	C	.	.	.	.
ALI	4247MesoC1837	.	.	A	.	.	-	.	.	.	G	A	A	.	.	T	A	A	.	.	C	?	.	.	.	G	.	.	.	-	C	.	.	.	.
	M. wooldridgei	A	T	A	.	T	C	T	T	T	G	G	A	.	.	T	A	A	.	.	C	.	.	.	.	.	.	T	-	C	.	T	G	.	.

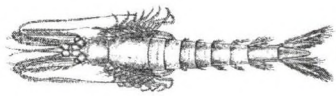
APPENDIX 3: *Mesopodopsis slabberi*: distribution of haplotypes per sampling site (for abbreviations see Table 5.1)

	WS	SEI	MO	RdA	GU	ALI	EBR
H1			3	3			
H3			1				
H4			1				
H5	1						
H6	1						
H7	1						
H8	1						
H9	1	1					
H10		1					
H11		1					
H12	1						
H13	1						
H14		1					
H15	5	3					
H16		1					
H17		1					
H18		1					
H19	1						
H20	1						
H21		1					
H22		1					
H23					1		
H24					1		
H25					1		
H26					1		
H27					1		
H28					1		
H29					1		
H30					1		
H31					1		
H32	1						
H33					1		
H34			1				
H35			1				
H36			1				
H37			2				
H38							7
H39						1	
H40						1	
H41						1	
H42						1	
H43						1	
H44						2	
H45						1	
H46				1			
H47				2			

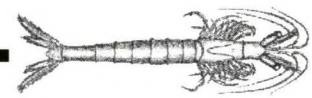
APPENDIX 3 (continued)

	WS	SEI	MO	RdA	GU	ALI	EBR
H48				1			
H49				1			
H50				2			
H51				2			
H52				1			
H53				1			
H54				1			
H55				1			
H56		2					
H57	1						
H58		1					
H59		1					
H60	1						
H61	1						
H62	1						
H63	1						
H64	1						
H65		1					
H66	1						
H67	1						
H68	1						
H69		1					
H70		1					
H71	1						
H72					1		
H73					1		
H74					1		
H75					1		
H76					1		
H77					1		
H78					1		
H79					1		

CHAPTER VI



Patterns of genetic diversity of the brackish water mysid *Neomysis integer* (Crustacea, Mysida) within the Westerschelde estuary: panmictic population or local differentiation in a highly variable environment?



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ABSTRACT

In the present study, the spatiotemporal variability in the genetic population structure of the brackish water mysid *Neomysis integer* within the Westerschelde estuary was assessed by means of Single Stranded Conformation Polymorphism (SSCP) analyses on a fragment of the mitochondrial cytochrome *c* oxidase subunit 1 gene (COI). Intra-estuarine patterns of genetic variation were examined by analysing different samples along an environmental gradient (salinity, pollution) and from different habitats (subtidal, brackish lake, harbour site). The temporal genetic variation within the Westerschelde estuary was examined on samples collected over 3 consecutive years (9 generations). Samplings were carried out in 2001, 2002, 2003 and a total of 480 mysids were analysed. Within two of the three years surveyed (2001 and 2002) a small, but significant genetic differentiation was observed within the Westerschelde estuary. However, there was no evidence for temporal stability of this genetic structure, and it remains unclear if this is a result of stochastic events, sampling error, or unpredictable environmental changes within an estuary. Furthermore, the effective female population size of *N. integer* within the Westerschelde estuary was estimated to be 2 to 3 orders of magnitude below the estimates of the census female population size, resulting in very low N_{ef}/N_f ratios. Hence, this could serve as a warning that large population sizes, as in *N. integer*, do not necessarily confer a high level of genetic diversity.

INTRODUCTION

Estuaries are harsh ecosystems subject to highly variable environmental conditions such as large fluctuations in submersion, salinity, temperature, etc., which generally result in a low species diversity and a high adaptability of the estuarine fauna. Located at the interface between sea and land, estuaries have as ecosystems several vital functions (e.g. nursery areas for juvenile fish and shrimp, habitats for estuarine residents) (Day *et al*, 1989). Most European estuaries are subjected to high anthropogenic stress, and the Westerschelde estuary seems to be no exception to this. Moreover, due to the high degree of industrialisation and urbanisation, this estuary has been transformed into a major drain for industrial and domestic wastes. Consequently, the Westerschelde is believed to be one of the most heavily metal polluted estuaries of the world (Bayens, 1998). Despite the numerous ecological surveys of the hyperbenthic communities of several major European estuaries, including the Westerschelde (e.g. Mees & Hamerlynck, 1992; Mees *et al*, 1993a,b; Mees *et al*, 1995; Cunha *et al*, 1999; Mouny *et al*, 2000; Drake *et al*, 2002), the levels of molecular diversity and degree of genetic population structuring of typical estuarine species, and in particular of hyperbenthic invertebrates such as mysids, are poorly studied. Yet a detailed knowledge of the distribution of genetic variation within populations of a species is of great importance since the ability of a species to respond to variable environmental conditions may depend to a large extent on the genetic variability (diversity) that exists within populations of that species. An adequate knowledge of marine biodiversity is also a basic requirement in planning conservation efforts on intraspecific levels of biological diversity (Cognetti & Maltagliati, 2004).

The brackish water mysid, *Neomysis integer*, is one of the most common mysid species in Europe, where it typically dominates the hyperbenthic communities of estuaries (Mees *et al*, 1995). Its ecological relevance has been studied profoundly (Fockedey & Mees, 1999; Hostens & Mees, 1999). As phytoplankton, zooplankton and detritus consumers and as important prey item for fish, bird and larger crustacean species, *N. integer* is believed to be a key species in estuarine food webs where it is an important link in the energy transfer to higher trophic levels (Mees *et al*, 1994). *N. integer* is also a relevant organism for ecotoxicological research and it has recently been proposed as a potential test organism for the evaluation of environmental

endocrine disruption (Roast *et al*, 1998; Verslycke *et al*, 2004). Recent studies on the distribution of molecular diversity throughout the whole distribution range of *N. integer* revealed a large genetic heterogeneity of populations where most variability is observed among-populations rather than within-populations (see Chapters 3 & 4). This pattern of natural fragmentation of a single species into genetically differentiated populations, adapted to different environmental conditions, is increasingly observed in the marine environment, and above all in brackish water populations (see Cognetti & Maltagliati, 2000; Bilton *et al*, 2002). In addition, the dispersal limiting life history traits of *N. integer*, and of mysids in general (absence of pelagic larvae), might limit the introduction of lost and/or new genetic variation into these naturally fragmented populations. Populations of *N. integer* may therefore be especially prone to rapid loss of genetic diversity under changing environmental conditions. An adequate knowledge of the levels of molecular diversity, as well as of the temporal variation of the genetic structure of a species are of major scientific importance when safeguarding these estuarine habitats and in gaining knowledge on the dynamics of genetic change in natural populations.

The scale at which genetic differentiation occurs in the marine environment seems difficult to predict from dispersal capacities of a species alone. The general assumption that the high dispersal potential of most marine species and the lack of obvious barriers to gene flow in the marine realm results in low population structure and high molecular homogeneity does not hold true for many species (see Palumbi, 1997; Lessios *et al*, 1999; Luttikhuizen *et al*, 2003). Moreover, several surveys have shown that, even in high gene flow species, sometimes a clear fine-scale structure can be observed (e.g. Stepien, 1999; Lemaire *et al*, 2000; Beheregaray & Sunnucks, 2001; McPherson *et al*, 2003; Pampoulie *et al*, 2004). In estuarine species, several studies have even shown differentiation within a single estuary system (see Bilton *et al*, 2002 and references therein). This genetic heterogeneity on a microgeographic scale could be the result of a combination of factors acting on the genetic population structure of a species, such as genetic drift, temporal variation in reproductive success, differential selection on several environmental gradients (e.g. salinity, pollution) or local adaptation (Beheregaray & Sunnucks, 2001; Planes & Lenfant, 2002).

The objectives of this study were: (1) to assess the levels of genetic variability of the mysid *N. integer* within the Westerschelde estuary, and (2) to examine the temporal variation of the genetic structure of *N. integer* within the Westerschelde

estuary by analysing mitochondrial DNA variation in samples collected over 3 consecutive years (9 generations). In order to test for intra-estuarine differentiation, different samples along an environmental gradient (salinity, pollution) and from different habitats (subtidal, brackish lake, harbour site) were analysed. The temporal analysis may be important in assessing the role of microevolutionary processes in producing genetic divergence among populations, as well as in giving insight in the degree of population stability and the effect of habitat heterogeneity in maintaining genetic variability (Lessios *et al*, 1994).

MATERIALS AND METHODS

Sampling

Mysids were sampled from the Westerschelde estuary over a 3-year period (February /March 2001, March 2002 and July 2003). In total nine different stations were sampled, from which seven are subtidal (HA, WA, ZUI, SAE, BA, DO and AP) covering the major distribution range of *Neomysis integer*. In addition samples were taken twice (October 2002 and July 2003) from a site within the harbour of Antwerp at the dock of BASF (DOCK) and once (July 2003) from Galgenweel (GAL), a brackish water pond near the river Sheldt, Antwerp (Fig. 6.2, Table 6.1).

Table 6.1: List of all sampling sites within the Westerschelde estuary, with indication of the abbreviation code and the total number of individuals analysed in each year.

		No. of individuals analysed		
Locality	code	2001	2002	2003
Hansweert	HA	0	28	29
Schaar van Waarde	WA	31	0	0
Zuidergat	ZUI	30	30	0
Saeftinghe	SAE	25	0	30
Bath	BA	62	30	30
Doel	DO	0	0	30
Antwerp	AP	0	0	29
Antwerp harbour dock (BASF)	DOCK	0	39	29
Galgenweel	GAL	0	0	28
Total		148	127	205

Most subtidal samples were collected actively by trawling a hyperbenthic sledge (mesh size 1x1 mm) over the bottom in front of the tidal current, except for the most upstream stations of Doel (DO) and Antwerp (AP) where the sampling was done passively by putting the hyperbenthic sledge on the bottom with the opening orientated against the current flow. Neighbouring sampling stations in the upstream part of the estuary were collected in the same tidal phase of subsequent days. This was done to avoid sampling of the same water mass moving longitudinally with the tide over the sampling traject. All samples were taken during daytime when hyperbenthic

animals are known to be concentrated near the bottom. Adult *N. integer* specimens were sorted out on board and the collected mysids were kept at –80°C or stored in aceton until molecular analysis. Mysids from the BASF dock and Galgenweel were collected by hand net (mesh size 1x1 mm). Salinity, dissolved oxygen concentrations and temperature were measured at all sampling sites (see Table 6.2).

Table 6.2: Temperature, salinity and dissolved oxygen concentrations for all sampling sites (ns = not sampled, NA = not available).

	HA	WA	ZUI	SAE	BA	DO	AP	DOCK	GAL
2001									
Temperature (°C)	ns	7.9	8.4	8.3	8.3	ns	ns	ns	ns
Salinity (PSU)	ns	10	8	7	5	ns	ns	ns	ns
Dissolved oxygen (mg/l)	ns	8.84	8.39	8.3	7.11	ns	ns	ns	ns
2002									
Temperature (°C)	7.2	ns	7.7	ns	7.9	ns	ns	19.3	ns
Salinity (PSU)	15.3	ns	7.5	ns	5.5	ns	ns	6.3	ns
Dissolved oxygen (mg/l)	8.46	ns	9.0	ns	5.8	ns	ns	7.6	ns
2003									
Temperature (°C)	20.2	ns	ns	20.9	21.2	21.4	21.8	21.8	NA
Salinity (PSU)	20.1	ns	ns	17.5	12.9	10.8	5.8	6.8	NA
Dissolved oxygen (mg/l)	7.9	ns	ns	7.3	6.7	5.9	2.4	3.9	NA

DNA extraction, PCR and single-stranded conformation analysis.

DNA was extracted using a modified CTAB protocol (Kocher *et al*, 1989). Mysid tissue was crushed using a beadbeater and immediately incubated for minimum 3 hours at 60°C in 500 µl CTAB buffer (2% (w/v) CTAB, 1.4M NaCl, 0.2% (v/v) mercaptoethanol, 20 mM EDTA, 100 mM Tris/HCl pH 8) with 6 µl proteinase K (1 mg 100 μl^{-1}). After an overnight incubation at 37°C, the DNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1 PH 8) and chloroform:isoamylalcohol (24:1). Finally, DNA was isopropanol-precipitated and rehydrated in 25µl water. A 235 bp fragment of the COI gene was amplified using the COIF2 (see Chapter 4) and HCO2198 (Folmer *et al*, 1994). The conditions for the COI amplifications were as previously described in Chapter 4. The amplified fragments were analysed with the single-strand conformation polymorphism (SSCP) technique (Orita *et al*, 1989). SSCP analyses were performed using 0.5 mm thick nondenaturing polyacrylamide gels (250 x 110 mm) (T=12.5%, C=2%). Electrophoresis was performed at a constant power of 8 W at 5°C for 3.5h. Bands were visualized with a DNA silver staining kit

(Amersham Biosciences) and scored by their relative mobility. Samples showing mobility differences were sequenced on a Perkin-Elmer ABI Prism 377 DNA sequencer under the conditions described in Chapter 4. At least two replicates of each haplotype were sequenced, with the exception of haplotypes found only in one individual.

Statistical analysis

Alignment of the haplotype sequence data was produced with the Clustal X program (Version 1.74, Thompson *et al*, 1997). When needed, the alignment was manually corrected with the program GeneDoc (Version 2.6, Nicholas & Nicholas, 1997). A minimum spanning network (Excoffier & Smouse, 1994) was constructed using the ARLEQUIN 2.0 software (Schneider *et al*, 2000) in order to visualize the phylogenetic relationships among the different COI haplotypes. Levels of mtDNA diversity were assessed by calculating mitochondrial haplotype diversity (h), nucleotide diversity (π) and Tajima's D using the ARLEQUIN software. Temporal and spatial genetic variation among samples was evaluated by an Analysis of Molecular Variance (AMOVA, Excoffier *et al*, 1992) using Φ-statistics based on haplotype frequencies and Tamura-Nei (1993) genetic distances, and F-statistics (based on haplotype frequencies only). Genetic variation among samples was quantified by estimating an analogue of Wright's F_{ST}, Φ_{ST} (Excoffier *et al*, 1992). Pair-wise Φ_{ST} values were calculated following Tamura-Nei (1993). The statistical significance of Φ_{ST} estimates was determined by using a permutation test (1000 permutations) in ARLEQUIN 2.0 (Schneider *et al*, 2000). To visualize the genetic relationships among samples, we performed a multidimensional scaling analysis (MDSA) on the pair-wise Tamura-Nei (1993) genetic distances in STATISTICA 6.0 (STATSOFT 2001).

Effective population size (N_e), which in case of mtDNA represents the effective female population size (N_{ef}) since it is maternally inherited, was calculated using two approaches. First, N_{ef} was calculated using the formula $\theta = 2\ N_{ef}\ v$ (Tajima, 1993). Where theta, θ, was estimated from the mean number of pairwise differences (Tajima, 1983) under the infinite-sites model, as implemented in ARLEQUIN 2.0 (Schneider *et al*, 2000). The parameter v could be calculated as $m\mu$, where m is the

sequence length and μ the mutation rate per generation. When assuming a general molecular clock for crustacean mitochondrial DNA ranging from 1.4 to 2.6% per My (Knowlton and Weight, 1998; Schubart *et al*, 1998; Patarnello, 1996; Zane *et al*, 2000) and a mean generation time of 4 months (Mees *et al*, 1994), the mutation rate per generation (μ) of *M. slabberi* ranged from 5.53×10^{-9} to 8.66×10^{-9} bp^{-1} $generation^{-1}$. A second approach, based on coalescent theory (Kingman, 1982), estimates N_{ef} from the genealogical structure of the mitochondrial haplotypes. For this analysis, we used the program MIGRATE (Beerli & Felsenstein, 1999, 2001), where a maximum-likelihood approach that considers the history of mutations and the uncertainty of the mtDNA genealogy is used to estimate θ by means of Markov chain Monte Carlo (MCMC) sampling of gene trees (Beerli & Felsenstein, 1999). The female effective population size can be determined using the formula $\theta = 2 N_{ef} \mu$ with μ being the mutation rate per generation. F_{ST} estimates of effective population sizes were used as initial values. Ten short chains with 100,000 sampled genealogies each and three long chains with 1,000,000 sampled genealogies each were run. One of every 20 reconstructed genealogies was sampled. A heating scheme with four temperatures (1.0, 1.5, 3.0 and 6.0) was used. The contemporary-method and coalescent-based estimates of N_{ef} can differ in some cases, since the coalescent method estimates the historical N_e which is a long-term estimate integrated over time to common ancestry of all alleles in the population (Avise, 2000). On the other hand, historical and contemporaneous estimates of N_e may agree if population effective size has remained stable over a long period (see Turner *et al*, 2002).

The census population size of *N. integer* in the Westerschelde estuary was calculated as follows: first, the densities (ind./ 1000 m^2) in 20 stations located along the salinity gradient were averaged over a period of one year (April 1990 – April 1991) using data collected by Mees (1994) and consulted through the Integrated Marine Environmental Readings and Samples (IMERS) database (VLIZ, 2004) (see Fig. 6.1). Then, the 20 stations were grouped according to the 13 compartments of the Westerschelde as presented by Soetaert & Herman (1995) (Fig. 6.1) and the average density per compartment was calculated. By multiplying the average densities (ind./1000 m^2) by the compartment surface, as reported in Soetaert & Herman (1995), a total number of mysids could be estimated in each compartment (see Table 6.3).

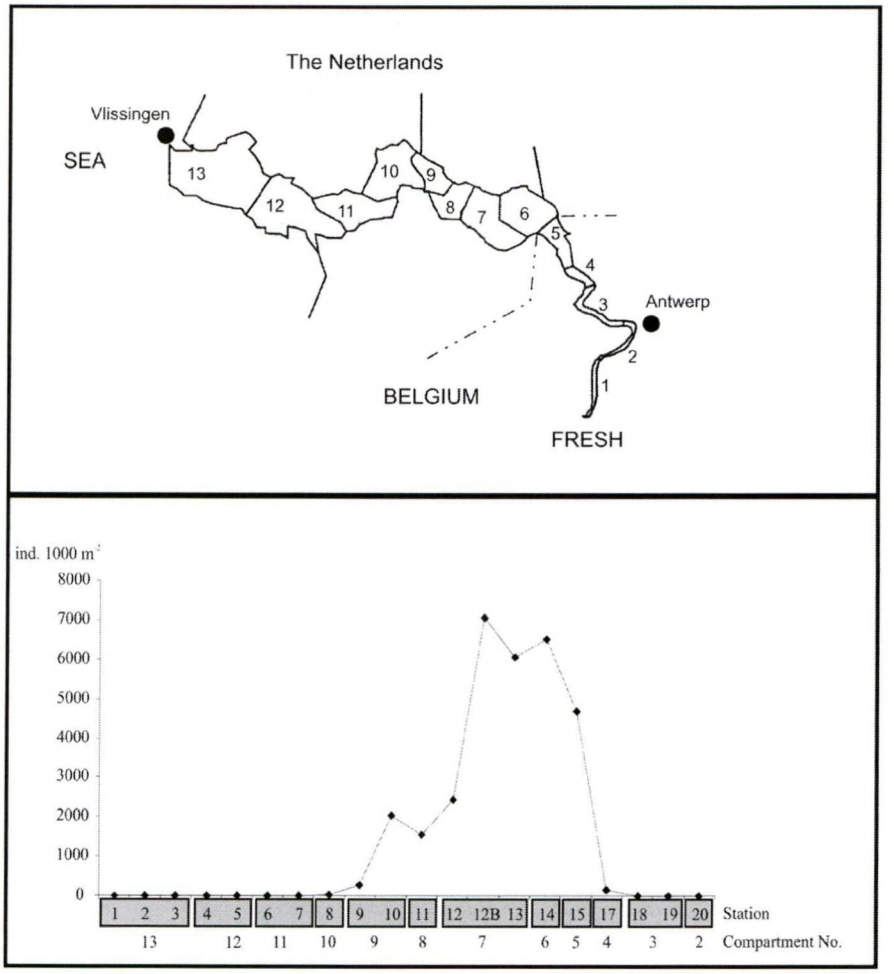

Fig. 6.1: Top: Map of the Westerschelde estuary with indication of the 13 compartments (redrawn after Soetaert & Herman, 1995). Below: average densities in each sampling station of the Westerschelde over a period of one year (April 1990 – April 1991), with indication of the grouping of the different samples in each of the 13 compartments.

Table 6.3: Surface size and density of *Neomysis integer* in the 13 compartments of the Westerschelde estuary

Comp. No.	Surface (10^3 m^2)	Density ind. 10^3 m^{-2}
1	2973	ns
2	3075	0
3	6387	1
4	2854	138
5	7772	0
6	16420	6507
7	14380	5187
8	14380	1563
9	13360	1556
10	34600	42
11	30300	4
12	49360	0
13	63620	0

RESULTS

The SSCP analyses of a 235 bp fragment of the mitochondrial COI gene of 480 *Neomysis integer* specimens detected six haplotypes (Table 6.4). Two haplotypes (n & v) were singletons, while 65% and 29% of all specimens possessed the haplotypes f and g. The distribution of the haplotypes within the samples in each year is indicated in Fig. 6.2. The overall haplotype and nucleotide diversity values were relatively low (h = 0.4891 and π = 0.0046). Interannual variation in levels of molecular diversity was low, except for a slightly higher haplotype diversity in the 2002 samples (h = 0.5713). In contrast, nucleotide diversity was similar between the different years when taking into account the large standard deviations (Table 6.4). Polymorphism within each year was consistent with neutral expectations, as evidenced by the non-significant Tajima's D values ($P > 0.05$; Table 6.4).

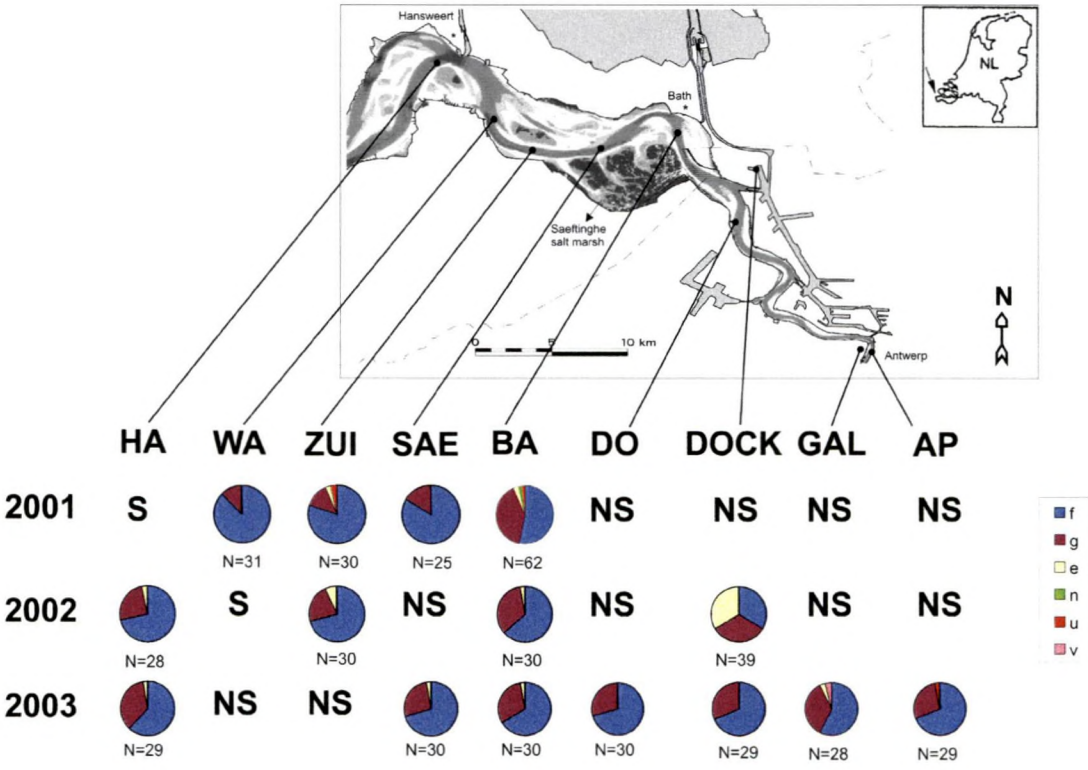

Fig. 6.2: Map of the sampling locations within the brackish to freshwater part of the Westerschelde estuary, with indication of the haplotype frequencies per sampling site (NS = not sampled, S = sampled, not analysed). Sampling years are indicated in the left column, N= total number of mysids analysed, for sampling location abbreviations see Table 6.1.

Table 6.4: Genetic diversity values with indication of the number of specimens analysed (N), number of haplotypes (N_h), haplotype and nucleotide diversity (*h* and π) and Tajima's *D* value. Standard deviations for *h* and π are given in parentheses.

	N	Nh	*h*	π	Tajima's *D*
2001	148	5	0.4365 (0.0376)	0.0039 (0.0030)	0.0504 (P = 0.591)
2002	127	3	0.5713 (0.0308)	0.0059 (0.0040)	1.6463 (P = 0.946)
2003	205	5	0.4673 (0.0262)	0.0041 (0.0031)	0.4900 (P = 0.533)
Overall	480	6	0.4891 (0.0186)	0.0046 (0.0031)	0.3848 (P = 0.719)

Among the six different haplotypes, a total of 6 polymorphic sites were observed (2.55%) (Table 6.5). All haplotypes were closely related as observed in the haplotype network (Fig. 6.3), with a maximum divergence of 6 substitutions (2.55% of uncorrected nucleotide divergence). Most haplotypes were connected by one mutation, except for the haplotypes g and e which were connected to the others by 2 and 3 mutations respectively.

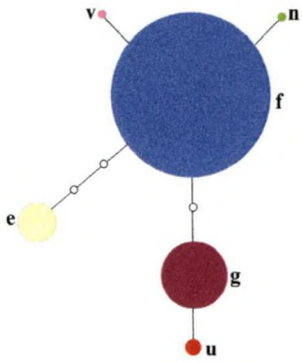

Fig. 6.3: Minimum spanning network among COI haplotypes of *Neomysis integer* within the Westerschelde estuary.. Branches connecting circles are mutation steps and the small open circles indicate missing haplotypes. The area of each circle is representative of the frequency with which the haplotypes occurred in the total sample.

Table 6.5: Polymorphic positions observed in the 235 bp fragment of the COI gene screened for *Neomysis integer*. Dots indicate that the same nucleotide is present as in haplotype g.

	Polymorphic sites					
			1	1	1	1
	1	9	0	1	9	9
Haplotype	8	7	7	7	5	8
g	T	T	C	G	T	G
u	.	.	.	.	C	.
f	C	.	.	A	.	.
v	C	.	T	A	.	.
n	C	A	.	A	.	.
e	.	C	.	A	.	C

The AMOVA analysis indicated that, when pooling all data per year, a low but significant amount of genetic variance occurred among temporary samples (1.75%, Φ_{ST} = 0.0175; P = 0.0106) (Table 6.6). The same pattern was observed when performing an AMOVA solely based on haplotype frequencies (F-statistic), but now the between-years variance component was reduced to 1.19% (F_{ST} = 0.0119; P = 0.0469). Within years, a significant rejection of panmixia was detected, both using the Φ and F-statistics, of the 2001 and 2002 samples (Table 6.6). Although most variance was detected within the samples, a small fraction of the total variance was attributed to the among-samples component within these years. Finally, when performing a hierarchical AMOVA incorporating the temporal (among years) and spatial component (among samples, within years), the spatial variance was greater than the temporal component (Φ_{SC} = 0.0537, $P < 0.001$ and Φ_{CT} = 0.0030, P = 0.3566).

The multidimensional scaling analysis (MDSA) based on the pairwise Φ_{ST} distances clearly revealed this heterogeneity between the samples (Fig. 6.4). The low stress value (0.0827, i.e. < 0.10 see Clarke, 1993) indicated a good and useful 2D-representation of the structuring of the samples. The 2003 samples formed a homogenous group (as evidenced by the very low, non-significant pairwise Φ_{ST} distances; see Appendix I) comprising as well the 2002 samples, with exception of the 2002 DOCK sample. The divergence of this sample is mainly due to the higher frequency of the haplotype e (see Fig. 6.2). When excluding this sample in the AMOVA analysis, the 2002 samples appeared to be homogenous (Φ_{ST} = -0.024; P = 0.800). Similarly, the heterogeneity of the 2001 samples is only caused by the divergence of the Bath sample ('BA 2001'), which is mainly the result of a higher frequency of the haplotype g within this sample.

Table 6.6: Results of the analysis of molecular variance (AMOVA) for spatial and temporal samples of *Neomysis integer* within the Westerschelde estuary, displaying the Φ-statistics (based on haplotype frequencies and molecular divergence based on Tamura-Nei (1993) genetic distances) and F-statistics (based on haplotype frequencies only).

Source of variation	% total	Φ-statistics	*P*	% total	*F*-statistics	*P*
Pooled per year						
Among years	1.75	$\Phi_{ST} = 0.0175$	0.0106	1.19	$F_{ST} = 0.0119$	0.04692
Within years	98.25			98.81		
2001 samples						
Among samples	9.11	$\Phi_{ST} = 0.0917$	0.0017	10.16	$F_{ST} = 0.1016$	0.0006
Within samples	90.89			89.84		
2002 samples						
Among samples	9.4	$\Phi_{ST} = 0.0940$	< 0.001	7.82	$F_{ST} = 0.0782$	0.0039
Within samples	90.6			92.18		
2003 samples						
Among samples	-2.83	$\Phi_{ST} = -0{,}0282$	0.9955	-2.62	$F_{ST} = -0.0262$	0.9831
Within samples	102.82			102.62		
Total dataset, grouped per year						
Among groups	0.3	$\Phi_{CT} = 0.0030$	0.3566	0.35	$F_{CT} = 0.0035$	0.3831
Among populations/ within groups	5.36	$\Phi_{SC} = 0.0537$	< 0.001	3.8	$F_{SC} = 0.0382$	< 0.001
Within populations	94.34	$\Phi_{ST} = 0.0566$	< 0.001	95.84	$F_{ST} = 0.0416$	< 0.001

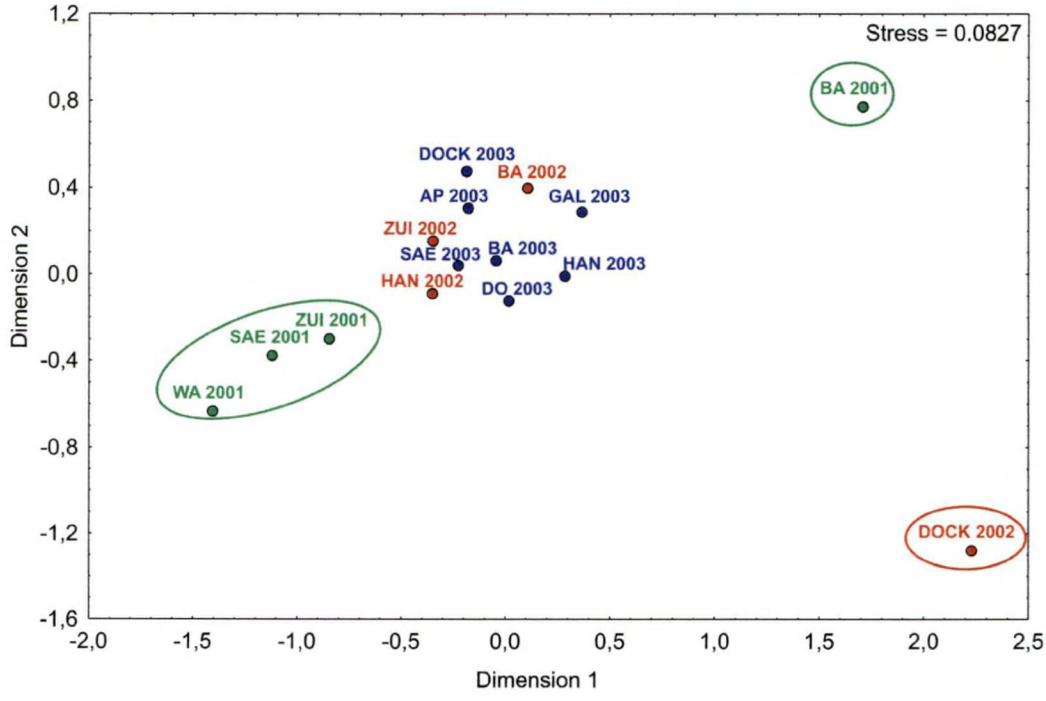

Fig. 6.4: Multidimensional scaling analysis on the samples of *Neomysis integer* collected within the Westerschelde and based on pairwise Tamura-Nei genetic distances. Colour scheme: green = 2001, red = 2002 and blue = 2003 samples.

Table 6.5: Polymorphic positions observed in the 235 bp fragment of the COI gene screened for *Neomysis integer*. Dots indicate that the same nucleotide is present as in haplotype g.

	Polymorphic sites					
			1	1	1	1
	1	9	0	1	9	9
Haplotype	8	7	7	7	5	8
g	T	T	C	G	T	G
u	.	.	.	.	C	.
f	C	.	.	A	.	.
v	C	.	T	A	.	.
n	C	A	.	A	.	.
e	.	C	.	A	.	C

The AMOVA analysis indicated that, when pooling all data per year, a low but significant amount of genetic variance occurred among temporary samples (1.75%, Φ_{ST} = 0.0175; P = 0.0106) (Table 6.6). The same pattern was observed when performing an AMOVA solely based on haplotype frequencies (F-statistic), but now the between-years variance component was reduced to 1.19% (F_{ST} = 0.0119; P = 0.0469). Within years, a significant rejection of panmixia was detected, both using the Φ and F-statistics, of the 2001 and 2002 samples (Table 6.6). Although most variance was detected within the samples, a small fraction of the total variance was attributed to the among-samples component within these years. Finally, when performing a hierarchical AMOVA incorporating the temporal (among years) and spatial component (among samples, within years), the spatial variance was greater than the temporal component (Φ_{SC} = 0.0537, $P < 0.001$ and Φ_{CT} = 0.0030, P = 0.3566).

The multidimensional scaling analysis (MDSA) based on the pairwise Φ_{ST} distances clearly revealed this heterogeneity between the samples (Fig. 6.4). The low stress value (0.0827, i.e. < 0.10 see Clarke, 1993) indicated a good and useful 2D-representation of the structuring of the samples. The 2003 samples formed a homogenous group (as evidenced by the very low, non-significant pairwise Φ_{ST} distances; see Appendix I) comprising as well the 2002 samples, with exception of the 2002 DOCK sample. The divergence of this sample is mainly due to the higher frequency of the haplotype e (see Fig. 6.2). When excluding this sample in the AMOVA analysis, the 2002 samples appeared to be homogenous (Φ_{ST} = -0.024; P = 0.800). Similarly, the heterogeneity of the 2001 samples is only caused by the divergence of the Bath sample ('BA 2001'), which is mainly the result of a higher frequency of the haplotype g within this sample.

Table 6.6: Results of the analysis of molecular variance (AMOVA) for spatial and temporal samples of *Neomysis integer* within the Westerschelde estuary, displaying the Φ-statistics (based on haplotype frequencies and molecular divergence based on Tamura-Nei (1993) genetic distances) and F-statistics (based on haplotype frequencies only).

Source of variation	% total	Φ-statistics	*P*	% total	*F*-statistics	*P*
Pooled per year						
Among years	1.75	$\Phi_{ST} = 0.0175$	0.0106	1.19	$F_{ST} = 0.0119$	0.04692
Within years	98.25			98.81		
2001 samples						
Among samples	9.11	$\Phi_{ST} = 0.0917$	0.0017	10.16	$F_{ST} = 0.1016$	0.0006
Within samples	90.89			89.84		
2002 samples						
Among samples	9.4	$\Phi_{ST} = 0.0940$	< 0.001	7.82	$F_{ST} = 0.0782$	0.0039
Within samples	90.6			92.18		
2003 samples						
Among samples	-2.83	$\Phi_{ST} = -0,0282$	0.9955	-2.62	$F_{ST} = -0.0262$	0.9831
Within samples	102.82			102.62		
Total dataset, grouped per year						
Among groups	0.3	$\Phi_{CT} = 0.0030$	0.3566	0.35	$F_{CT} = 0.0035$	0.3831
Among populations/ within groups	5.36	$\Phi_{SC} = 0.0537$	< 0.001	3.8	$F_{SC} = 0.0382$	< 0.001
Within populations	94.34	$\Phi_{ST} = 0.0566$	< 0.001	95.84	$F_{ST} = 0.0416$	< 0.001

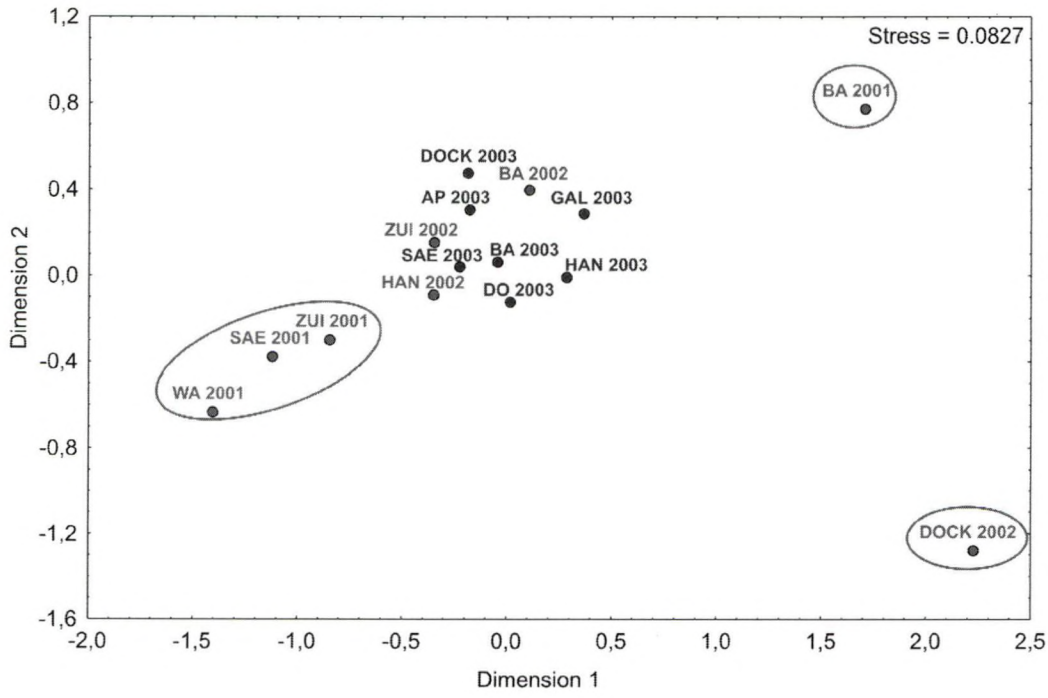

Fig. 6.4: Multidimensional scaling analysis on the samples of *Neomysis integer* collected within the Westerschelde and based on pairwise Tamura-Nei genetic distances. Colour scheme: green = 2001, red = 2002 and blue = 2003 samples.

The estimation of the census population size resulted in a total population size of 226.7 × 10^6 mysids (see Table 6.3). An estimation of the sex ratio using data from the same database (number of females/ number of males) provided, despite minimal temporal fluctuations, an average value close to 1. Hence, the total female population size (N_f) was estimated to be 113.4 × 10^6 females. The estimations of θ based on the mean number of pairwise differences ranged from 0.920 ± 0.708 to 1.376 ± 0.945, with an overall θ amounting to 1.076 ± 0.786. The overall effective female population size (N_{ef}) was calculated to be 264157 to 413740 (Table 6.7). The maximum-likelihood estimates of θ using the coalescence based approach are listed in Table 6.8, the corresponding estimates of the female effective population size ranged from 476,885 to 1,419,579. The N_{ef}/N_f ratios based on the summary statistics and on the maximum-likelihood coalescence approach ranged between 0.0016 – 0.0035 and 0.0041 – 0.0125 respectively (Tables 6.7 and 6.8).

Table 6.7: Estimates of θ based on the mean number of pairwise differences, with indication of the standard deviation in parentheses, effective female population size (N_{ef}) and N_{ef}/N_f ratio within the samples pooled per year.

	2001	2002	2003	Overall
θ	0.920 (0.708)	1.376 (0.945)	0.974 (0.735)	1.076 (0.786)
N_{ef}	220335 - 345103	181179 - 283773	249673 - 391054	217513 - 340682
N_{ef}/N_f	0.0019 - 0.0030	0.0016 - 0.0025	0.0022 - 0.0035	0.0019 - 0.0030

Table 6.8: Estimates of θ obtained by the coalescence method, with indication of the 95% confidence intervals, effective female population size (N_{ef}) and N_{ef}/N_f ratio within the samples pooled per year.

	2001	2002	2003
θ	0.00981	0.01571	0.00811
95% CI	0.00873 - 0.0104	0.01068 - 0.02442	0.00716 - 0.00922
N_{ef}	565962 - 886446	906347 - 1419579	467885 - 732832
N_{ef}/N_f	0.0050 - 0.0078	0.0080 - 0.0125	0.0041 - 0.0065

DISCUSSION

Spatiotemporal variation in the genetic population structure of Neomysis integer?

The analysis of molecular variance (AMOVA) detected a significant amount of variation among the different years (see Table 6.6). The largest temporal variation was observed between the years 2001 – 2002 and 2002 – 2003, while the years 2001 – 2003 did not show significant variation. However, when considering the whole Westerschelde estuary not as a single panmictic population unit for *N. integer*, and taking into account possible spatial variations between the Westerschelde samples of *N. integer* within sampling years, only a very small, albeit not significant, amount of variation (0.3%) was observed between years (see results of the AMOVA using three hierarchical levels in Table 6.6). Hence, the spatial pattern of the samples within the Westerschelde estuary seems to override the (limited) temporal fraction. As evidenced by the AMOVA and the MDS plot of the pairwise genetic distances between samples, a clear pattern of genetic differentiation was apparent within the years 2001 and 2002. In contrast, all the samples collected in 2003 were genetically homogeneous notwithstanding a high sampling intensity along the whole salinity range over which *N. integer* is distributed and the extensive molecular analysis (# analysed specimens per sampling station $\geq$ 28). Interestingly, the semi-closed brackish water pond Galgenweel (GAL 2003) does not seem to be isolated from the subtidal *N. integer* population. This implies that the occasional (seasonal) opening of this pond enables sufficient gene flow between the populations in the Westerschelde estuary and Galgenweel, counteracting genetic differentiation within this pond. Within 2001 the significant genetic structure was caused by the shift in frequencies of the haplotypes f and g in the Bath sample (BA 2001). The differentiation of this sample is remarkable and surprising since the distance to the Saefthinge sample is less then 10 km. The genetic structure within 2002 was caused by the divergent genetic composition of the sample collected within the harbour site of BASF (DOCK 2002). However, finding possible causes for these genetic heterogenities will be puzzling since there was no evidence for temporal stability of the spatial genetic patterns within the Westerschelde estuary (see Fig. 6.2).

Genetic differentiation within a single estuary system has been demonstrated in a wide range of taxa (see review in Bilton *et al*, 2002). Although some of these

assumed intraspecific studies may in fact represent the differential distribution of several reproductively isolated cryptic species or different ecotypes (Schizas *et al*, 2002; Takahashi *et al*, 2003; Caudill & Bucklin, 2004; Derycke *et al*, submitted), others attribute this microgeographic genetic differentiation to inbreeding or stochastic events (drift – gene flow) (see references in Bilton *et al*, 2002). Alternatively, clinal variation in the frequency of alleles has also been linked to differential selection along several environmental gradients within an estuary (e.g. salinity, pollution) (see references in Bilton *et al*, 2002; De Wolf *et al*, 2004). It is clear, however, that the different haplotypes observed within *N. integer* of the Westerschelde samples represent closely related variants which are not reproductively isolated. Neither seems environmentally induced selection to have an influence on the observed spatial variation in *N. integer*. First of all, the mitochondrial COI gene screened is assumed to be selectively neutral (but see Ballard & Kreitman, 1995; Blier *et al*, 2001; Ballard *et al*, 2004), which was confirmed by the overall test of neutrality showing no differences from neutral expectations (see non-significant Tajima's *D* values in Table 6.4). Moreover, the discordance in the genetic pattern of the subtidal samples between years (see Fig 6.2, structure in 2001 vs. homogeneity in 2002 & 2003) does not add to the hypothesis of selection playing a role in maintaining a genetic differentiation along an environmental gradient. However, additional (seasonal) sampling would be appropriate to further unravel any environment – genotype interactions and potential seasonal fluctuations in the selective pressure.

Could the specific life history traits (brooding behaviour, absence of pelagic larvae) and aggregation (swarming) behaviour of *N. integer* promote intra-estuarine differentiation? Field observations have shown that swarming behaviour of *N. integer* may be extensive, resulting in a patchy distribution of *N. integer* within an estuary (Mauchline, 1971a; Lawrie *et al*, 1999; Roast *et al*, 2004). Swarming behaviour in marine invertebrates, such as in krill species, seems to promote genetic differentiation even on very small geographic scales (< 20 km) (Zane & Patarnello, 2000; Jarman *et al*, 2002). Studies on within-swarm variability in Antarctic krill has shown that these swarms represent associations of krill that are more related to each other than to individuals from other swarms (Jarman *et al*, 2002). Although our results of the 2001 samples are in line with such a hypothesis, the results of the 2002 and especially the 2003 samples do not corroborate this. Hence, it seems that such a 'differentiation between swarms' pattern doesn't hold true for a shallow water mysid. Firstly, the

swarming behaviour observed in *N. integer* is probably more a random aggregation of individuals swimming in the same direction, triggered by physical environmental parameters (e.g. tidal flow; Roast *et al*, 1998; Lawrie *et al*, 1999), rather than a breeding aggregation as observed in deep water mysids and krill species (Mauchline & Fisher, 1969; Mauchline, 1971a). Secondly, if different breeding entities could be formed within a population of *N. integer*, it seems unlikely that these aggregations are able to maintain a long-term cohesion of related individuals, especially in a highly turbulent environment as the Westerschelde estuary.

Although selection in relation to different environmental parameters does not seem to be a plausible explanation the spatial and temporal variation in the genetic structure of the harbour samples of BASF (DOCK 2002 & DOCK 2003), temporal variations in dissolved oxygen (DO) concentrations could provide more insight in the dynamics of the population structure of *N. integer* at this harbor site. Dissolved oxygen concentrations below 40% of the saturation value (~ 4.2 mg/l at 15°C) are a critical threshold for hyperbenthic life (Mees *et al*, 1995). Detailed recordings of the seasonal variation in the DO concentrations at the BASF dock site have shown regular drops in DO concentrations, sometimes well below 40% of the saturation value (Verween, pers. comm.). Hence, the situation at the harbour site of BASF could resemble a dynamic metapopulation structure, with frequent extinctions of the *N. integer* population and followed by a recolonisation after environmental amelioration. As a consequence, large temporal shifts in the genetic composition at the BASF site may not be unexpected. In addition, the restricted connectivity of the harbour docks with the Westerschelde estuary through the presence of ship locks may be responsible for restricted gene flow between the subtidal population of *N. integer* and the population present within the harbor docks. Furthermore, the watermass composition within the harbour docks of BASF is also influenced by the inflow of the nearby Scheldt – Rhine Canal (Verween, pers. comm.). Consequently, episodic immigration from sources with different allele frequencies compared to the subtidal Westerschelde population (e.g. a small *N. integer* population within the Scheldt – Rhine canal) may cause rapid, although sometimes transient, shifts in allele frequencies (cfr. BAS 2002 pattern). However, without further spatial and temporal sampling these hypotheses remain speculative.

Effective female population size estimates

Although the effective population size is rarely measured for natural populations of marine invertebrates, it is one of the most important parameters in evolutionary biology and population genetics. It is defined as the size of an ideal Wright-Fisher model population subject to the same rate of random genetic change as the studied population (Wright, 1931; Hartl & Clark, 1989) and hence determines the genetic properties of a population (e.g. population fitness). Generally, the effective population size is much smaller than the census population size, since not all individuals contribute progeny to the next generation with equal probability (Frankham, 1995).

The estimates of the effective female population size (N_{ef}) of the Westerschelde population of *N. integer* obtained in the present study ranged from 1,81 $\times 10^5$ to 1.42 $\times 10^6$ females. The estimates of N_{ef} obtained by the two methods were different, with the N_{ef} obtained by the coalescent method being slightly higher. However, when taking into account the large variances associated with the calculation of θ, both estimates seem to fall within the same range (see Tables 6.7 & 6.8). Interannual variation in N_{ef} was low. The estimates of the current census female population sizes (N_f) were made based on average densities of *N. integer* within the Westerschelde estuary over a period of one year. However this value (226.7 $\times 10^6$ mysids) should be treated with caution since it probably is an underestimate of the actual census population size. Firstly, our calculations were based only on density data of subtidal samples, hereby extrapolating these densities for probable denser shallow areas (Mees & Hamerlynck, 1992). Secondly, the vertical distribution of *N. integer* in the water column was not inferred since only densities in a zone of 1 m above the bottom were used. Moreover, densities per compartment were calculated using compartment surface (m^2) instead of compartment volumes (m^3). Especially in the maximum turbidity zone, the *N. integer* population is evenly distributed over the complete water column (Mees & Hamerlynck, 1992; Fockedey & Mees, 1995). Finally the net efficiency of the hyperbenthic sledge may not be 100%, since mysids could be capable of avoiding nets (Mauchline, 1980; Mees & Hamerlynck, 1992). Nevertheless, since no one has yet attempted to provide a good estimate of the *N.*

integer census population size, our values may be useful for comparative purposes with the molecular estimates, bearing in mind the possible underestimations.

The effective female population size estimations obtained for *N. integer* are about 2 to 3 orders of magnitude below the estimates of census female population size, with a N_{ef}/N_f ratio ranging from 0.0016 to 0.0125 (Table 6.7 & 6.8). These values are very low when compared to N_e/N ratios obtained from theoretical studies ranging between 0.25 and 0.75 for most organisms (Nunney & Elam, 1994). In a review of empirical studies that estimated N_e, Frankham (1995) found that the average N_e/N ratio across 102 species was 0.11, thus one or two orders of magnitude larger than those found for *N. integer*. However, low N_e/N ratios are not that uncommon in marine species, even in populations with a census size of several millions of individuals (Carvalho & Hauser, 1994). Estimates of the N_e/N ratio as low as 0.001 and 0.25×10^{-5} have been reported for the red drum (*Sciaenops ocellatus*) and the New Zealand snapper (*Pagrus auratus*) (Hauser *et al*, 2002; Turner *et al*, 2002). Temporal genetic analysis of North Sea cod (*Gadus morhua*) gave a N_e/N ratio of 3.9×10^{-5} (Hutchinson *et al*, 2003). The few studies that estimated N_e for marine invertebrates such as for krill and copepods, reported much lower N_e/N ratios: $5.28 - 8.30 \times 10^{-10}$ for Antarctic krill (*Euphausia superba*; Zane *et al*, 1998), $8.30 - 13 \times 10^{-4}$ for European krill (*Meganyctiphanes norvegica*; Zane *et al*, 2000) and $3.62 - 5.38 \times 10^{-11}$ for pelagic copepods (*Nannocalanus minor* and *Calanus finmarchicus*; Bucklin & Wiebe, 1998). A case study of the Pacific oyster (*Crassostrea gigas*) showed that the effective population size was about 10,000 times less than the number of oysters harvested per year (Hedgecock 1994a). These low N_e/N ratios suggest that only a small portion of the actual population contributes successfully to the next generation, as could be the case in species with very high fecundity and high mortality of early life stages (Hedgecock, 1994a; Li & Hedgecock, 1998). Several ecological and evolutionary factors could be responsible for low N_e/N ratios: large variance in female reproductive success, fluctuations in population size through time or unequal sex ratio (Avise *et al*, 1988; Hedgecock, 1994a,b; Nunney, 1996; Vucetich *et al*, 1997). All these factors have a complex combined effect, which makes it difficult to assign the low N_e/N ratio of *N. integer* to one specific cause only. *N. integer* has a moderate fecundity, with females producing ~20 – 80 larvae/brood (Mauchline, 1973; Mees *et al*, 1994). Although *N. integer* has some mechanisms to improve survival of the offspring, e.g. simultaneous release of the juveniles from the female brood pouch at

an advanced development stage, social aggregations in a shoal and behavioral adaptations in order to prevent displacement from the estuary (Mauchline, 1971a; Roast *et al*, 1998), variance in reproductive success could be high since the success or failure of each entire brood may depend on the fate of the mother. Moreover, the ecology of *N. integer* which seems to be a compromise between an *r*-strategy (i.e. a relative unspecialized opportunist, poor competitor, large salinity tolerance, small in size and intense reproduction during most favorable period of the year) and a *K*-strategy (i.e. brooder with small number of offspring) might be fallible for species inhabiting unstable brackish habitats, and hence *N. integer* could experience large fluctuations in population density (Parker & West, 1979). In addition, since the N_{ef} estimation using DNA sequence genealogies reflects the end results of processes that may have occurred over a very long time period. Pleistocene changes in the population size of *N. integer* may also have played a role in reducing N_{ef} (Avise *et al*, 1988; Neigel, 1996).

Conclusions and recommendations

In conclusion, within two of the three years surveyed (2001 & 2002) a significant rejection of panmixia was observed within the Westerschelde estuary. However, there was no evidence for temporal stability of this genetic structure. Whether the temporal instability of the population structure results from stochastic events, sampling error or unpredictable environmental changes, which are not uncommon in estuarine habitats, remains largely unanswered and demands further research. In addition, research of small scale, intra-estuarine genetic variation within other mysid or invertebrate species could be very valuable in order to quantify the signal:noise ratio of the molecular marker with more precision, leading to a more accurate estimation of the spatial population structure.

The estimations of the effective female population size of *N. integer* we obtained in the present study were about 2 to 3 orders of magnitude below the estimates of the census female population size, resulting in very low N_{ef}/N_f ratios. Further estimates of the effective female population size of other brackish water invertebrates would be useful to find out if such low N_e/N ratios are commonplace in these species. But the present results could already serve as a warning for conservation biologists that large population sizes, as in *N. integer*, do not necessarily

confer a high level of genetic diversity. Such populations could be prone to genetic erosion.

Furthermore, future estimates of the spatial genetic structuring, as well as of the effective population sizes of *N. integer* should preferably make use of a multilocus approach. Not only does it reveal genetic differences that remained undetected in the present study, it would also increase the accuracy of the effective population size estimations (Nunney & Elam, 1994; Neigel, 1996; Roman & Palumbi, 2003). Screening a larger amount of genetic information in both coding and noncoding regions, and tracking several unlinked loci may also provide information on whether any regions of the genome are under selective pressure (see Nevo, 2001).

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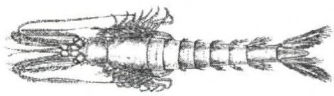
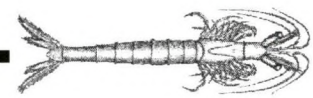
APPENDIX I: Pairwise Φ_{ST} distances between samples, calculated using Tamura-Nei (1993) genetic distances. Significant values at the 95% level are indicated in bold. For sampling site abbreviations see Tabel 6.1.

	WA 2001	ZUI 2001	BA 2001	DOCK 2002	SAE 2001	HA 2002	ZUI 2002	BA 2002	AP 2003	DO 2003	BA 2003	SAE 2003	HA 2003	DOCK 2003	GAL 2003
WA 2001	0														
ZUI 2001	0	0													
BA 2001	**0.1507**	**0.0863**	0												
DOCK 2002	**0.2979**	**0.219**	**0.1233**	0											
SAE 2001	0	0	**0.1117**	**0.2607**	0										
HA 2002	0.0253	0	0.0242	**0.1633**	0										
ZUI 2002	0.031	0	0.0266	**0.1402**	0.004	0									
BA 2002	**0.0899**	0.0257	0	**0.1314**	0.0499	0	0								
AP 2003	0.0587	0.0053	0.0061	**0.1762**	0.0225	0	0	0	0						
DO 2003	0.0524	0.0033	0.0124	**0.1852**	0.0168	0	0	0	0	0					
BA 2003	0.0621	0.0059	0.0005	**0.1456**	0.0264	0	0	0	0	0	0				
SAE 2003	0.0366	0	0.0168	**0.1612**	0.0058	0	0	0	0	0	0	0			
HA 2003	**0.1009**	0.0325	0	**0.1263**	0.0592	0	0	0	0	0	0	0	0		
DOCK 2003	0.061	0.0078	0.0067	**0.1791**	0.0236	0	0	0	0	0	0	0	0	0	
GAL 2003	**0.108**	0.0391	0	**0.1189**	0.0658	0	0	0	0	0	0	0	0	0	0

CHAPTER VII



Morphological differentiation between geographically separated populations of *Neomysis integer* and *Mesopodopsis slabberi* (Crustacea, Mysida)



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ABSTRACT

Morphological variation was examined in *Neomysis integer* and *Mesopodopsis slabberi*, two abundant, low dispersal mysid species of the European coasts. Both species dominate the hyperbenthic communities in the northeast Atlantic, and *M. slabberi* is also widely distributed in the Mediterranean and Black Sea. Three populations of each species were sampled throughout their distribution range. Samples of *N. integer* were collected in the northeast Atlantic Eems-Dollard, Gironde and Guadalquivir estuaries. In the case of *M. slabberi*, mysids were sampled in two northeast Atlantic estuaries (Eems-Dollard and Guadalquivir) and one Mediterranean site (Ebro Delta). A total of 12 morphometric and two meristic characters were measured from 30 – 64 mysids per sample. Multivariate analysis showed clear morphometric differences between populations of both species. The morphological differentiation within *M. slabberi* was highly concordant with the available genetic data from mitochondrial loci, pointing to a large divergence between the Atlantic and Mediterranean populations. However, due to overlap between populations, the morphometric analysis does not suffice to assign the populations to a separate species status. In the case of *N. integer*, the morphometric patterns showed a divergence of the Gironde population. Potential interactions of the mysid morphology and environmental conditions are discussed.

INTRODUCTION

Multivariate analysis of a set of morphometric and meristic characters has been widely used in stock identification of freshwater and marine fish species (Mamuris *et al*, 1998; Cadrin, 2000; Murta, 2000; Pakkasmaa & Piironen, 2001; Cabral *et al*, 2003), and to a lesser extent in marine invertebrates (e.g. Henderson *et al*, 1990; Kassahn *et al*, 2003). The method is regarded more appropriate than the use of single morphological characters for investigating taxonomic problems in determining relationships between populations or closely related (cryptic) species (e.g. Scapini *et al*, 1999; De Grave & Diaz, 2001; Clark *et al*, 2001; Debuse *et al*, 2001; Doadrio *et al*, 2002; Lee & Frost, 2002). Moreover, morphometric analyses can be a tool in assessing habitat-specific differentiation of populations, such as differentiation related to predation pressures, salinity, temperature, food availability, etc. (e.g. Gee, 1988; Scapini *et al*, 1999; Maltagliati *et al*, 2003). Differences in morphometric and meristic characters among populations of a species are thought to be the result of genetic differences or environmental factors, or their interactions (Lindsey, 1988; Scheiner, 1993; Hoffman & Merilä, 1999). Strong genetic differentiation of populations, accompanied with reproductive isolation, may lead to local adaptation. On the other hand, changing environmental conditions may produce phenotypic plasticity in genetically similar populations (Thompson, 1991). Hence, the comparison of the degree of variation in molecular markers with morphological characters may be important in assessing the degree of phenotypic plasticity shown by a species (O'Reilly & Horn, 2004).

Neomysis integer and *Mesopodopsis slabberi* are two of the most common mysid species in European coastal (*M. slabberi*) and brackish (*M. slabberi* and *N. integer*) habitats, where they are believed to play a key role (Mees *et al*, 1995; Azeiteiro *et al*, 1999; Hostens & Mees, 1999). Both species are euryhaline and eurythermic, and have a wide distribution: *N. integer* occurs along the NE Atlantic from the Baltic Sea to the North African coasts of Morocco (Tattersall & Tattersall, 1951) and *M. slabberi* is distributed from the western Baltic, the NE Atlantic, up to the entire Mediterranean, Marmara, Black and Azov Seas (30 - 59°N, 10°W – 41°E) (Wittmann, 1992). This wide distribution of both species spanning different biogeographical regions (Subarctic, Celtic, Lusitanian and Mediterranean region, *cfr* Adey & Steneck, 2001) with varying environmental conditions, combined with the

limited dispersal capacities of these mysids (brooding behavior and lack of free-living larvae), may be expected to produce differences in both molecular and morphological traits among populations (Planes, 1998; O'Reilly & Horn, 2004).

The taxonomy of the genus *Mesopodopsis*, and in particular of the species *M. slabberi* has been a matter of controversy, mainly due to the limited phylogenetic resolution of the morphological characters used to describe and diagnose different species within this genus. Based on a study by Wittmann (1992) on the morphogeographic variations within the genus *Mesopodopsis*, the cosmopolitan *M. slabberi* was split into four species: *M. slabberi* (NE Atlantic, Mediterranean, Black Sea), *M. aegyptia* (Mediterranean), *M. tropicalis* (equatorial W-Africa) and *M. wooldridgei* (South Africa). Morphological differences between Atlantic, Mediterranean and Black Sea populations of *M. slabberi* were reported by Wittmann (1992). However, the observed variation was small and statistically overlapping, without any consistent pattern related to environment or geography. It must be noted that this study did not use a multivariate statistical analysis of morphometric characters to elucidate variation between populations. On the other hand, morphological variation within *N. integer* is considered to be small (Tattersall & Tattersall, 1951; Parker & West, 1979), but has not been studied in detail. A number of 'forms' or varieties within the species *N. integer* were introduced by Czerniavsky (1882), but since these varietal divisions were based on trivial differences, they have been largely ignored in subsequent descriptions (Tattersall & Tattersall, 1951). However, given the slight taxonomic differences observed between populations of the North American congeneric *N. americana* (Williams *et al*, 1974), morphometric variation between populations of *N. integer* may be expected.

Previous studies on genetic variation between populations of *N. integer* and *M. slabberi*, based on several mitochondrial loci, have shown significant heterogeneity within both species (see Chapters 3, 4 & 5). Analysis of Atlantic and Mediterranean populations of *M. slabberi* showed a clear differentiation between both basins, with very high genetic distances, probably pointing to the existence of different cryptic species (see Chapter 5). Phylogeographic analyses of *N. integer* identified a large genetic break at the southern distribution range (= divergent Guadalquivir population) and showed a genetic isolation of each population south of the English Channel, including the Irish population (see Chapters 3 & 4). In this respect, a morphometric

analysis within both species could lead to a better understanding of the intraspecific evolutionary and systematic diversity and its biological significance.

The aims of this study were to (i) examine the pattern and the extent of morphometric variation in populations of the mysids *N. integer* and *M. slabberi*, and (ii) compare these results with the available genetic data. For this purpose, three population samples of each species, covering, at least for *N. integer*, most of its geographical distribution range, were examined morphologically and analysed using multivariate methods.

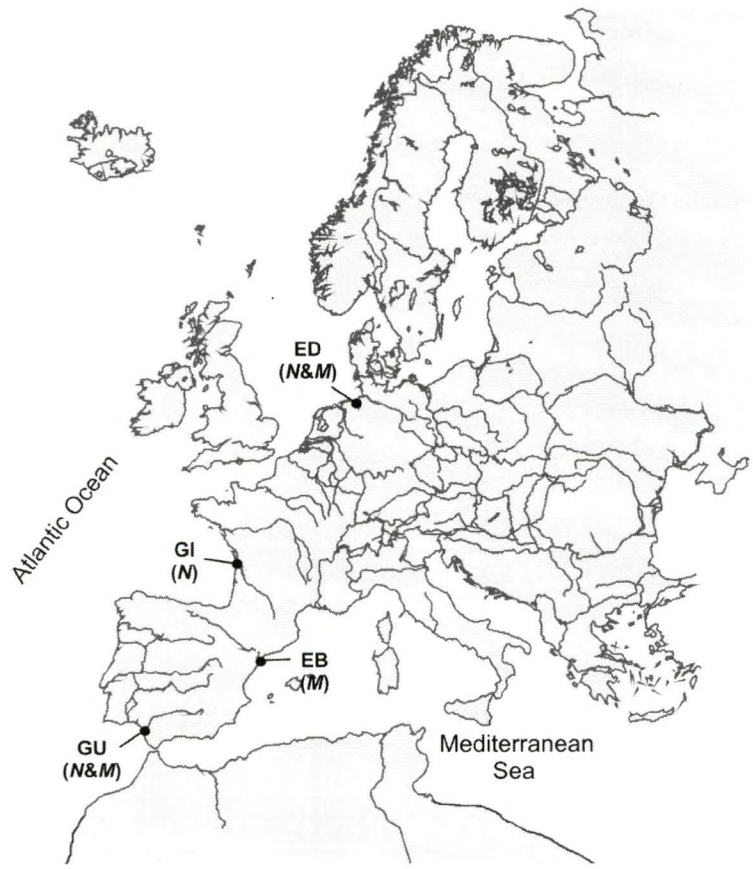

Fig. 7.1: Sampling locations (*N* = *Neomysis integer*, *M* = *Mesopodopsis slabberi*), sampling site abbreviations: ED = Eems-Dollard, GI = Gironde, GU = Guadalquivir, EB = Ebro

MATERIALS AND METHODS

Sampling

Samples of *Neomysis integer* were collected in three NE Atlantic estuaries covering most of the species' distribution range. *Mesopodopsis slabberi* was collected in two NE Atlantic and one Mediterranean estuary (see Fig. 7.1). Most samples were collected with a hyperbenthic sledge, with exception of the sample of the Ebro delta, which was collected with a hand net (mesh size 1 mm). All sampling was done during the summer months between 1991 and 2001. The samples were either stored in 7% formaldehyde (all *N. integer* samples and *M. slabberi* from the Eems - Dollard) or in 70% ethanol (*M. slabberi* samples from the Guadalquivir and Ebro). The ethanol-preserved samples of *M. slabberi* were also used for molecular analyses (see Chapter 5).

Measurements and statistical analyses

From each sample a random number of about 50 adult, and mostly gravid, females were examined morphologically. A total of 12 metric (Fig. 7.2) and two meristic characters. The metric measurements were related to the shape of the telson, antennale scale, eyes and uropods. The meristic counts included the number of spines on the lateral margin of the telson (only for *M. slabberi*) and on the inner margin of the uropod endopodite. Standard length was measured from whole animals under a binocular microscope. Other characters were measured from slide mounts of the appendages under a microscope and recorded with a digitizer.

All statistical analyses were performed using the STATISTICA 6.0 software package (STATSOFT 2001). The most conspicuous outliers were excluded when suspecting measurement error and missing data were case-wise deleted in the statistical analyses. To minimize size effects in all analyses, the continuous variables were divided by standard length followed by an arcsin transformation. Univariate analysis of variance (ANOVA) was performed, in case of homogeneity of the variances, to test whether the different populations showed significant differences in morphometric measurements and meristic characters. In those cases where homogeneity of variances was violated, even after transformations of the raw data, a non-parametric test was used (Kruskall-Wallis and Mann-Whitney). The data set (only metric measurements with exclusion of the standard length) was subjected to a backward stepwise Discriminant Function Analysis (DFA). DFA finds linear

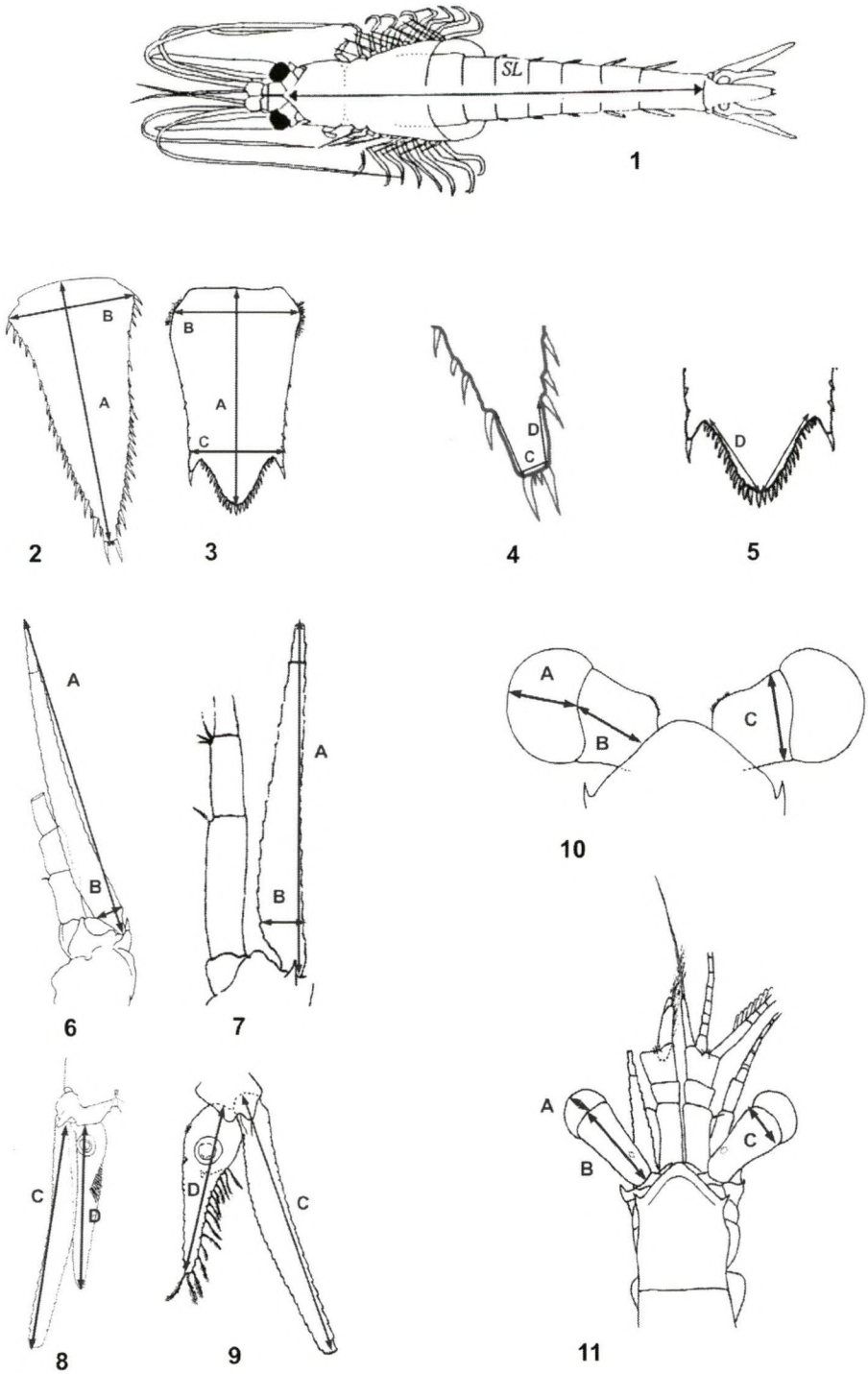

Fig. 7.2: Morphometric measurements: **1**: Standard length (SL); **2, 3, 4 & 5**: Telson of *Neomysis integer* (**2&4**) and *Mesopodopsis slabberi* (**3&5**), A = telson length (TELL), B = distal telson width (TELDW), C = caudal telson width (TELCW), D = caudal telson length (TELCL); **6 & 7**: Antennale scale of *Neomysis integer* (**6**) and *Mesopodopsis slabberi* (**7**), A= length of antennale scale (ANTL), B = width of antennale scale (ANTW); **8 & 9**: Uropode of *Neomysis integer* (**8**) and *Mesopodopsis slabberi* (**9**), C = exopodite length (EXOL), D = endopodite length (ENDOL); **10 & 11**: Eye of *Neomysis integer* (**10**) and *Mesopodopsis slabberi* (**11**), A = cornea length (CORNEA), B = length of eyestalk (EYESTL), C = width of eyestalk (EYESTW).

combinations of variables (roots), that maximize differences among a priori defined groups (in this case populations). The resultant discriminant functions were used to classify individuals into samples. The classification success rate (cross-validation test) was evaluated based on the percentage of individuals correctly classified in the original sample. Alternatively, a principal components analysis (PCA) was performed and in order to eliminate the size effect the first principal component (PC1) was eliminated. Subsequently the other PC scores (PC2–n) were subjected to a canonical variate analysis (see Väinölä *et al*, 2002). However, since a similar pattern was obtained as with the DFA, the results of the PCA-method are not presented.

Table 7.1: Mean and standard deviation (in parenthesis) of the different metric and meristic characters. Metric values are in mm. For the sampling site and metric measurement abbreviations see Figs. 7.1 and 7.2. Meristic character abbreviations: #SPENDO = number of spines on the inner margin of the uropod endopodite, #SPTEL = number of spines on the lateral margin of the telson.

	Neomysis integer				*Mesopodopsis slabberi*			
	OVERALL	**ED (N = 50)**	**GI (N = 54)**	**GU (N = 64)**	**OVERALL**	**ED (N = 50)**	**GU (N = 52)**	**EB (N = 30)**
STDL	10.29 (1.61)	10.60 (1.33)	10.30 (0.82)	10.03 (2.19)	8.45 (0.94)	8.49 (0.85)	8.87 (0.89)	7.64 (0.63)
EYESTW	0.46 (0.07)	0.53 (0.05)	0.39 (0.02)	0.46 (0.05)	0.31 (0.03)	0.33 (0.03)	0.28 (0.03)	0.30 (0.02)
CORNEA	0.26 (0.05)	0.31 (0.05)	0.21 (0.02)	0.26 (0.03)	0.21 (0.03)	0.23 (0.03)	0.21 (0.03)	0.19 (0.02)
EYESTL	0.43 (0.06)	0.48 (0.06)	0.41 (0.04)	0.42 (0.05)	0.72 (0.06)	0.73 (0.05)	0.74 (0.06)	0.68 (0.04)
TELL	1.64 (0.23)	1.73 (0.21)	1.66 (0.12)	1.53 (0.29)	0.78 (0.14)	0.88 (0.08)	0.81 (0.08)	0.58 (0.05)
TELDW	0.75 (0.08)	0.78 (0.08)	0.74 (0.05)	0.73 (0.10)	0.54 (0.06)	0.56 (0.04)	0.57 (0.05)	0.46 (0.04)
TELCW	0.10 (0.02)	0.10 (0.02)	0.10 (0.03)	0.09 (0.02)	0.37 (0.04)	0.39 (0.03)	0.38 (0.03)	0.32 (0.02)
TELCL	0.15 (0.04)	0.18 (0.04)	0.16 (0.03)	0.11 (0.02)	0.24 (0.05)	0.27 (0.03)	0.24 (0.03)	0.18 (0.02)
ANTW	0.30 (0.04)	0.33 (0.03)	0.29 (0.02)	0.28 (0.05)	0.20 (0.02)	0.20 (0.01)	0.21 (0.01)	0.16 (0.02)
ANTL	2.73 (0.39)	3.02 (0.34)	2.66 (0.20)	2.51 (0.38)	1.25 (0.17)	1.29 (0.10)	1.29 (0.21)	1.23 (0.09)
EXOL	2.18 (0.33)	2.39 (0.25)	2.17 (0.17)	2.02 (0.39)	1.69 (0.20)	1.75 (0.12)	1.80 (0.16)	1.45 (0.12)
ENDOL	1.52 (0.20)	1.64 (0.15)	1.51 (0.14)	1.43 (0.22)	1.12 (0.11)	1.17 (0.07)	1.16 (0.08)	0.96 (0.06)
#SPENDO	28.55 (4.07)	28.36 (4.82)	28.93 (5.05)	28.38 (1.89)	20.97 (1.24)	20.66 (1.68)	21.65 (0.48)	20.40 (0.56)
#SPTEL	-	-	-	-	6.56 (0.81)	6.96 (0.20)	7.00 (0.34)	5.57 (1.07)

RESULTS

Neomysis integer

The mean standard length of *Neomysis integer* across all populations amounted to 10.29 mm (SD 1.61). A significant difference in standard length was observed between the three populations (Kruskal-Wallis test: H (2, N= 168) = 8.55; P = 0.0139), with the mysids of the Eems-Dollard population having the largest length (mean = 10.60 mm; SD 1.33) and those of the Guadalquivir being the smallest (mean = 10.03 mm; SD 2.19) (see Table 7.1).

All morphometric characters could be used in the discriminant analysis since no multicollinearity was registered between the variables (for all correlations: R < 0.7). The backward stepwise Discriminant Function Analysis (DFA), using geographical origin of each population as separator factor, revealed that four of the 12 morphometric characters contributed significantly to the multivariate discrimination between the three *N. integer* populations (Table 7.2).

Table 7.2: Summary of the Discriminant Function Analysis.

	Wilks' Lambda	Partial Lambda	F-remove (2,139)	*P*-level	Toler.	1-Toler. (R-Sqr.)
EYESTW	0.2190	0.5953	47.2443	< 0.0001	0.4278	0.5722
CORNEA	0.1496	0.8715	10.2433	< 0.0001	0.7302	0.2698
TELDW	0.1837	0.7095	28.4490	< 0.0001	0.4599	0.5401
TELCL	0.2691	0.4845	739.581	< 0.0001	0.9495	0.0505

Wilks' lambda amounted to 0.1304 and was highly significant (appox. $F_{8,278}$ = 61.489; P < 0.001). The morphometric characters showed a low degree of overlap (maximal 57.22% in case of the eyestalk width (EYESTW), see Table 7.2). Squared Mahalonobis distances (D^2) between populations (i.e. a distance measure between the group centroids) are listed in Table 7.3. All distances were significant (P < 0.001) and the largest distance was observed between the Eems-Dollard (ED) and Gironde (GI) populations, while the distance between the Eems-

Table 7.3: Squared Mahalanobis Distances

	ED	GI	GU
ED	-	***	***
GI	14.3579	-	***
GU	6.0109	14.0135	-

Dollard and Guadalquivir (GU) populations seemed to be smaller. A scatterplot of the individual canonical scores is presented in Fig. 7.3. The relative importance of Root 1 in distinguishing the three populations was up to 3 times higher than Root 2 (Eigenvalue of Root 1 = 2.8666, Eigenvalue of Root 2 = 0.9836), and the first discriminant function accounted for 74.5% of the explained variance. A clear separation of the Gironde population could be observed along Root 1. In contrast, Root 2 separated the Eems-Dollard (ED) and Guadalquivir (GU) populations, although some overlap existed between both populations. The segregation along Root 1 was mainly caused by differences in the variables eyestalk width (EYESTW) and cornea length (CORNEA) (Gironde < Eems-Dollard & Guadalquivir mysids), as evidenced by the high correlation of these morphometric characters and the canonical Root (Table 7.4). The differences along Root 2 were almost exclusively related to the variable caudal telson length (TELCL) (Guadalquivir < Gironde < Eems-Dollard mysids). The cross-validation test using the discriminant functions derived from the morphometric characters showed that overall 87.34% of the *a priori* grouped cases were correctly classified, with the within-group correct classifications ranging from 78.18 (GU) to 96.23% (GI) (see Table 7.5).

Table 7.4: Structure matrix of discriminant loadings for each of morphometric variable selected by the backward stepwise Discriminant Function Analysis (DFA).

	Root 1	Root 2
EYESTW	-0.6785	0.1669
CORNEA	-0.6112	0.1503
TELDW	-0.1200	-0.0583
TELCL	0.2151	0.9306

Table 7.5: Results of the discriminant analysis classification, showing the numbers and percentage of specimens classified in each group (Rows: Observed classifications, Columns: Predicted classifications).

	% Correct	ED	GI	GU
ED	88	44	0	6
GI	96.23	0	51	2
GU	78.18	8	4	43
TOTAL	87.34	52	55	51

Analyses of the meristic characters (spines on the inner margin of the uropod endopodite) revealed no significant differences between the three populations (Kruskal-Wallis test: H (2, N = 163) = 5.0697 p = 0.0793). In addition, a total of 12 aberrant telsons were recorded (ED = 5, GI = 3, GU = 4); the morphology of these telsons were similar to those described in Mees *et al* (1995).

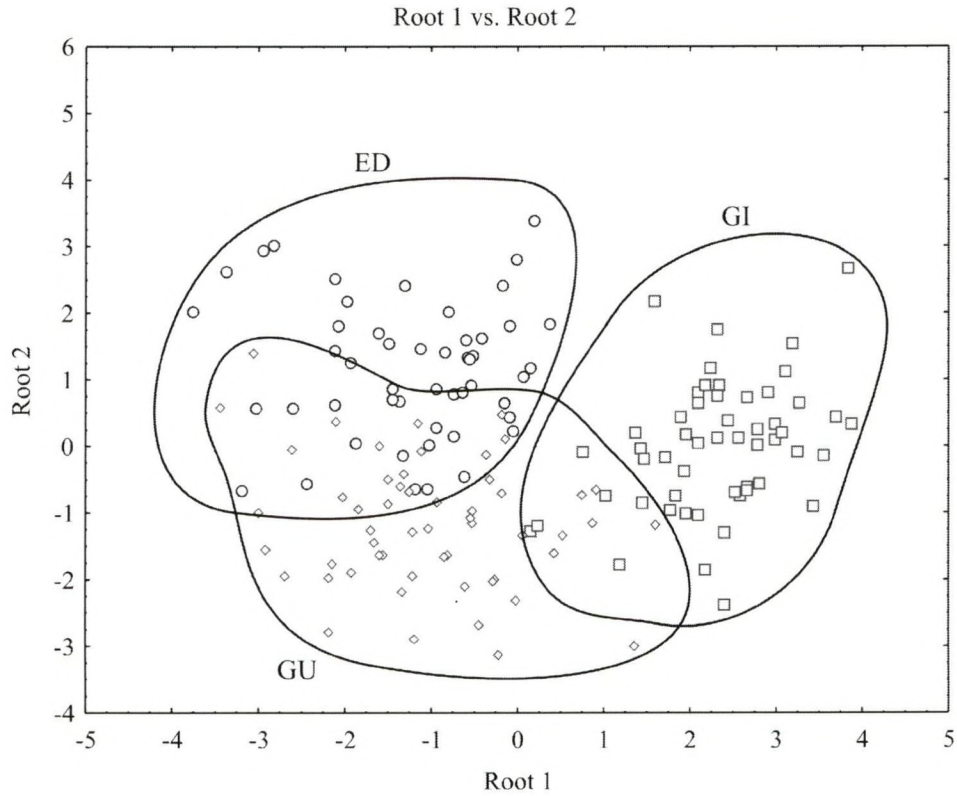

Fig. 7.3: *Neomysis integer*: Scatterplot of the DFA scores along the first and second root. For sampling site abbreviations see Fig. 7.1.

Mesopodopsis slabberi

Mean standard length of *Mesopodopsis slabberi* across all populations amounted to 8.45 mm (SD 0.94). A significant difference in standard length was observed between the three populations (ANOVA: $F_{2,193} = 23.91$; $P < 0.001$), with the mysids of the Mediterranean Ebro population having the lowest standard length (mean = 7.64 mm; SD 0.63) (see Table 7.1).

Again, no multicollinearity was registered between the variables and consequently all morphometric characters could be used in the discriminant analysis. The backward stepwise DFA revealed that only three out of the 12 morphometric

characters contributed significantly to the multivariate discrimination between the three *M. slabberi* populations (Table 7.6). The largest Mahalanobis (D^2) distances were observed between the Mediterranean Ebro population and both Atlantic populations (Table 7.7). The canonical analysis showed that most of the observed variance between the populations (83%) was observed along Root 1 (Eigenvalue = 3.44 vs. Eigenvalue Root 2 = 0.70), with a clear distinction between the Ebro (EB) and Eems-Dollard (ED) populations (Fig. 7.4). The differentiation along Root 1 mainly correlated with the variables telson length (TELL) and caudal telson length (TELCL) (ED > GU > EB mysids), while the differences along Root 2 were related to the variable eyestalk width (EYESTW) (ED > EB > GU mysids) (Table 7.8). The morphometric discriminant analysis correctly classified, on average, 83.85% of the individuals (Table 7.9). The highest classification success rate was obtained for the Ebro mysids with 93.33%, while a lower amount of individuals (74%) were correctly classified in case of the Guadalquivir mysids.

Table 7.6: Summary of the Discriminant Function Analysis.

N=114	Wilks' Lambda	Partial Lambda	F-remove (2,109)	*P*-level	Toler.	1-Toler. (R-Sqr.)
EYESTW	0.2458	0.5370	46.9902	< 0.0001	0.8585	0.1415
TELL	0.2310	0.5713	40.9025	< 0.0001	0.7854	0.2146
TELCL	0.1629	0.8102	12.7675	< 0.0001	0.9068	0.0932

Table 7.7: Squared Mahalanobis Distances between populations.

	ED	GU	EB
ED	-	***	***
GU	5.1972	-	***
EB	21.0172	13.5901	-

Table 7.8: Structure matrix of discriminant loadings for each of morphometric variable selected by the backward stepwise Discriminant Function Analysis (DFA).

	Root 1	Root 2
EYESTW	-0.0733	-0.9403
TELL	0.8057	-0.2767
TELCL	0.6674	-0.3742

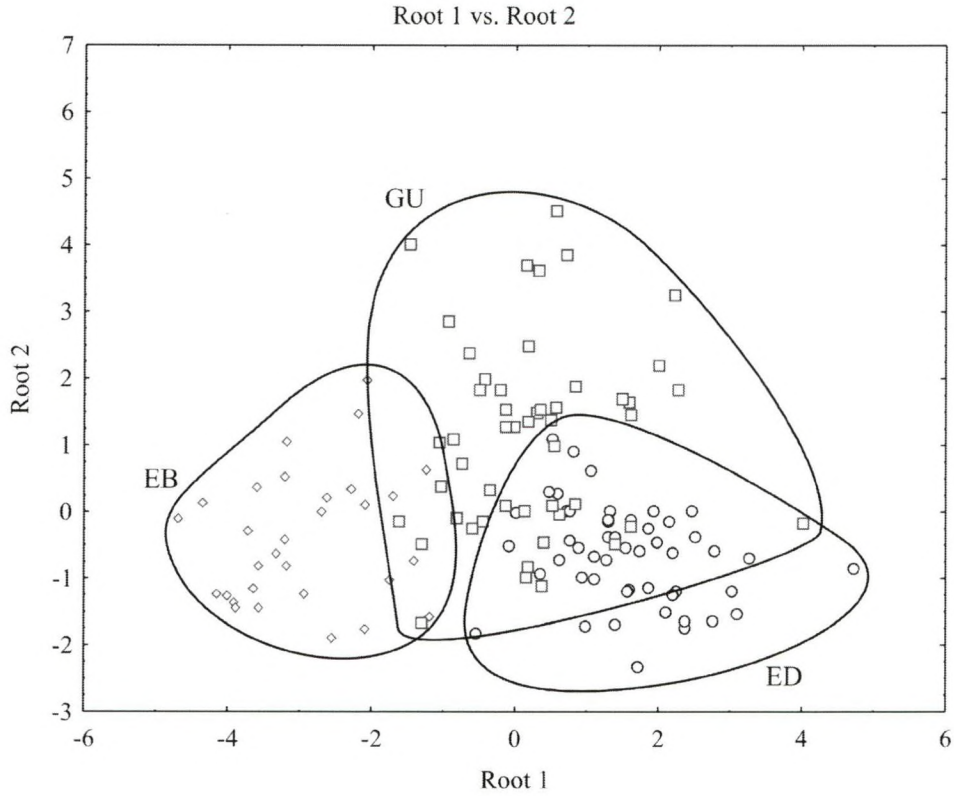

Fig. 7.4: *Mesopodopsis slabberi*: Scatterplot of the DFA scores along the first and second root. For sampling site abbreviations see Fig. 7.1.

Analysis of the meristic characters showed a significant difference in the number of spines on the inner margin of the uropod endopodite (#SPENDO) and on the lateral margin of the telson (#SPTEL) between the different populations (Kruskal-Wallis test for #SPENDO: H (2, N = 128) =36.013 $P < 0.001$; #SPTEL: H (2, N = 132) = 75.82 $P < 0.001$). Mysids of the Mediterranean Ebro populations had, on average, less spines on the lateral margin of the telson, while those of the Guadalquivir population possessed, on average, more spines on the inner margin of the uropod endopodite (Table 7.1). Contrary to *N. integer*, no aberrant telsons were observed in the samples of *M. slabberi*.

	% Correct	ED	GU	EB
ED	88	44	6	0
GU	74	10	37	3
EB	93.33	0	2	28
TOTAL	83.85	54	45	31

Table 7.9: Results of the discriminant analysis classification, showing the numbers and percentage of specimens classified in each group (Rows: Observed classifications, Columns: Predicted classifications).

DISCUSSION

The multivariate analyses of morphometric characters revealed a significant differentiation between populations of both *Neomysis integer* and *Mesopodopsis slabberi* throughout their distribution range. Very often, such differences are to a large extent related to sexual dimorphism, allometric growth and/or different cohort size (Thorpe, 1976; Mamuris *et al*, 1998; De Grave & Diaz, 2001). In order to minimize the variances caused by these parameters, the present study used only adult, (mostly gravid) female specimens from the summer generation. In addition, all measurements were size standardized and transformed prior to statistical analysis. The method used here to correct the measurements for size proved to be effective, since all correlation coefficients which were close to 1 decreased to lower values after data transformation. Moreover, the second method used to eliminate the size effect gave similar results (i.e. performing a PCA and subsequently performing a canonical variate analysis on the individual PC scores (PC2 – 12) with elimination of the first principal component, see Materials & Methods).

Both species showed significant latitudinal differences in standard length. In the case of *N. integer*, the mysids of the southern Guadalquivir population had, on average, a shorter length. For *M. slabberi*, the Mediterranean mysids were smaller than those of the Atlantic populations. Considerable variations in life history characteristics (e.g. length, growth rate, number of cohorts, brood size) of mysid species at different latitudes, including *N. integer* and *M. slabberi*, have been reported (Pezzack & Corey, 1979; Mauchline, 1980; Sorbe, 1984; Morgan, 1985; Greenwood *et al*, 1989; San Vicente & Sorbe, 1995; San Vicente, 1996; Delgado *et al*, 1997). Water temperature, light cycle and food conditions seem to be the principal environmental factors influencing the growth and reproductive cycle of crustaceans (Pezzack & Corey, 1979; Winkler & Greve, 2002). In general, there is a tendency towards an extended reproductive season with decreasing latitude in shallow-water mysid species (Delgado *et al*, 1997). In the case of *M. slabberi*, the Atlantic reproductive cycle with three generations (spring, summer and winter generation) shifts to a more or less continuous breeding throughout the whole year in Mediterranean populations (Delgado *et al*, 1997; Azeiteiro *et al*, 1999; Uppabullung, 1999). Hence, the present results corroborate the general observations of lower cohort-size in populations with an extended breeding season.

Phenotypic variation in populations of Neomysis integer and Mesopodopsis slabberi

Extensive variation in morphometric characters was apparent between all three populations of *N. integer* and *M. slabberi*. This was not only supported by the DFA scores along the first two roots, but also by the significant, large Mahalanobis distances between the populations of both mysids (see Tables 7.3 and 7.7) and the high percentage of correctly reclassified specimens in the original groups (populations) (see Tables 7.5 and 7.9). For *N. integer*, the variables of primary importance in separating the populations along Root 1 were related to eye morphology: eyestalk width (EYESTW) and cornea length (CORNEA). While the morphometric variable related to the caudal telson morphology, caudal telson length (TELCL), had the largest discriminatory power along Root 2.

In the case of *M. slabberi*, the DFA showed that again the morphometric variables related to telson (TELL: telson length, TELCL: caudal telson length) and eye morphology (EYESTW: eyestalk width) were the most important variables in differentiating the populations. Contrary to *N. integer*, a significant difference in meristic characters was observed between the Atlantic and Mediterranean populations. According to Mauchline (1980) the number of spines in the margins of telsons and both endopod and exopod of the uropods is correlated to the overall body size of several mysid species. However, in the present study the size effect on spine numbers between populations is thought to be minimal since we tried to uniform our samples by selecting only adult (gravid) females of the summer generation. The assumption that meristic characters are independent of mysid size was further confirmed by the absence of correlations between the meristic characters and standard length or uropod endopodite/telson length.

Causes of the phenotypic variation

The causes of morphological differences between populations are often quite difficult to explain. In general, changes in morphology are under control of environmental conditions or genetic background, or (most often) a combination of both. However, separating the effects of environmental induction from those under genetic control can be one of the most intricate problems in the analysis of geographic variation (Thorpe, 1976). Genetic differences and reproductive isolation between

populations can lead to local adaptation, which is reflected in morphology, behaviour, physiology and/or life history traits (Taylor, 1991). The alternative possibility is that morphological variation may result from phenotypic plasticity in response to varying environmental conditions (e.g. temperature, salinity, food availability, flow regime, predator/prey interactions, etc.) within different geographical areas (Scheiner, 1993).

Extensive genetic surveys of different mitochondrial loci revealed a significant differentiation of populations of both *N. integer* and *M. slabberi* (see chapters 3, 4 and 5). Although not yet supported with nuclear markers, a large phylogeographic break was observed between Atlantic and Mediterranean populations of *M. slabberi*, indicating the possible existence of cryptic species. On the other hand, the observed genetic distances between populations throughout the whole distribution range of *N. integer* were smaller. Still, an isolation of the Gironde population and a well-supported break at the southern distribution range (i.e. of the Guadalquivir population) could be observed. Concordance between the molecular data and the present morphometric analyses were noticed for *M. slabberi*, where the largest molecular and morphometric distances were found between the Mediterranean and Atlantic populations. Hence, the combination of the genetic differentiation (with possible reproductive isolation) and the adaptations to environmental conditions may have played a role in the Atlantic-Mediterranean separation and the morphological variability (mainly related to telson morphology) between both regions. In contrast, the patterns of genetic differentiation within *N. integer* do not correspond fully with the present morphometric results. Largest squared Mahalanobis distances were observed for the Gironde populations (Table 7.3), while the largest genetic divergence was found for the Guadalquivir and not the Gironde population (see Chapters 3 & 4). However, it must be noted that the patterns of genetic differentiation within *N. integer* were only based on a single mitochondrial marker and hence need further validation of other (unlinked) molecular markers in order to fully correlate them with the present morphometric results.

One of the morphometric characters of primary importance in separating the populations, both in *N. integer* and *M. slabberi*, was related to the eye morphology. It is not unlikely that this morphological character can vary in association with environmental conditions. Mysids have well-developed compound eyes, and are known to use vision in various situations, e.g. schooling behaviour and choice of specific habitats, diurnal migrations, feeding and predator avoidance behaviour

(Fulton, 1982; Nilsson & Modlin, 1994; Lindström, 2000; Lindén *et al*, 2003). A study on the eye function of mysids has shown that there may be functional intraspecific differences in the visual systems of mysids living in different photic environments (Lindström, 2000). Another study has shown differences in predator avoidance behaviour of mysids, and more specifically in the way of predator detection (chemical or visual signals) related to habitat characteristics (light vs. darker water) (Lindén *et al*, 2003). Hence, it is not unlikely that the higher turbidity of the water in the Gironde estuary (Castel, 1993) could lead to a slightly reduced development of the eye in the case of *N. integer* (e.g. narrow eyestalks and reduced cornea size). However, at this moment this hypothesis remains very speculative and additional morphological analyses, as well as breeding experiments under different environmental conditions could be useful to further elucidate these patterns and to disentangle the functional relationships.

Implications for species status and general conclusions

The final question which arises is whether the morphologically differentiated populations of *N. integer* and *M. slabberi* deserve a separate subspecies or species status. Although the discriminant analysis showed that the classification rate of individuals to correct populations was high (87.34% and 83.85% in case of *N. integer* and *M. slabberi* respectively), there is still morphological overlap of individual mysids. Thus, no individual mysid can be assigned unambiguously to a particular geographical area ('population') on the basis of linear measurements. In addition, the observed variation in meristic characters (e.g. number of spines on the lateral margin of the telson of *M. slabberi*), which generally is thought be a variable with more operational taxonomic utility than morphometric measurements (Spotte, 1997; De Grave & Diaz, 2001), did overlap between the populations despite the significant differences detected between their averages.

Intraspecific geographical variation within other mysids has been observed, such as variation in the numbers of spines on the lateral margins of the telson between populations of *Praunus flexuosus* and *P. neglectus* (Mauchline, 1971b), geographical differences in the proportions of the antennal scale of *N. americana* (Williams *et al*, 1974), and differences in the numbers of ommatidia in Atlantic and Mediterranean populations of *Eucopia hanseni* (Cassanova, 1977). However, these variations are

considered to be of a minor nature and could be consistent with the normal patterns of variation expected within species (Mauchline, 1980). In his review of the genus *Mesopodopsis*, Wittmann (1992) also reported (minor) morphological differences between Atlantic, Mediterranean and Black Sea populations of *M. slabberi*. However, the residual differences were found to be small and statistically overlapping and hence Wittmann (1992) noted that a reintroduction of the Czerniavsky's (1882) species (*goesi* and *cornuta*) and varieties (*major* and *minor*) was not appropriate.

In conclusion we can state that despite the limited number of populations analysed within both species and the selection of only adult female specimens which lowers the value of the present analyses in terms of general conclusions for both species, clear morphometric differences were observed between populations of *N. integer* and *M. slabberi*. These results corroborate the expectations for a species inhabiting a wide geographic range and possessing limited dispersal capacities. However, the present morphometric analysis in itself does not allow us to conclude that the present species status of both mysids is in need of a revision. Hence, the observed morphological variation should be interpreted as geographical variation. On the other hand, the strong concordance of the morphometric results with the mitochondrial DNA data in the case of the Atlantic-Mediterranean separation of *M. slabberi* probably indicates that these populations are approaching the species stage in the evolutionary continuum of speciation. This aspect definitely deserves more attention. Consequently, future research should focus on a larger number of populations and morphological characters, preferably using geometric morphometric techniques since these 'new' morphometric techniques are regarded as more powerful in analysing the external morphology and shape differences among organisms (Rohlf & Marcus, 1993; O'Reilly & Horn, 2004).

ACKNOWLEDGEMENTS

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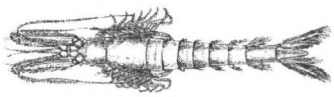
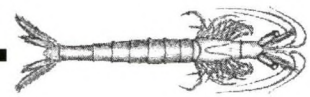
CHAPTER VIII



General conclusions and perspectives



1. Phylogeography of the brackish water mysid Neomysis integer: *restricted gene flow, multiple glacial refugia and complex postglacial recolonisation.*

Because of its typical life history characteristics (brooder and absence of free-living larvae) and the particular habitat preferences (brackish part of estuaries, brackish lagoons), the mysid *Neomysis integer* was selected as a potential model organism for inferring the impact of the Pleistocene glaciations on low dispersal marine taxa along the northeast Atlantic coast. The results of the mitochondrial DNA analyses (of both the cytochrome *b*, cyt *b,* and cytochrome *c* oxidase 1, COI, genes) clearly corroborated these expectations (see Chapters 3 & 4). A clear phylogeographic structure was observed with a very high proportion of population-specific haplotypes (up to 88% in the case of COI). The cyt *b* gene turned out to be more conserved, since one dominant haplotype was distributed throughout the whole distribution range with exception of the Gironde and Guadalquivir populations.

These results were interpreted in relation to historical patterns and processes using paleoclimatic and paleobiogeographic knowledge. This lead to some striking patterns which contradicted the general expectations according to the current paleoclimatological models:

→ **no trend of declining haplotype diversity was detected at higher latitudes**, the levels of genetic diversity were relatively uniform throughout the whole distribution range, even in glaciated areas, with exception of a decline at the northern and southern edge of the natural distribution range.

→ **the Iberian Peninsula did not act as a single glacial refugium for *N. integer*,** and according to the COI data these **southern refugial populations did not participate in the most recent range expansion** after the last glacial maximum (LGM).

→ there are **multiple northern refugia**, probably located in the southern North Sea or English Channel, around the British Isles, and an additional refugium in the Bay of Biscay, leading to **a complicated recolonisation history.**

These observations are supported by several facts, such as the relatively high heterogeneity of populations in glaciated areas, the apparent lack of a (postglacial) demographic expansion of the populations in these areas, levels of divergence

between northern mitochondrial lineages pointing to a pre-LGM differentiation, the detection of an isolation-by-distance pattern in glaciated areas and the lack of any southern haplotype in these areas. Moreover, similar patterns (e.g. northern refugia) have been observed in other marine vertebrates, as well as invertebrates (see references in Chapters 3 & 4).

However, due to the lack of mysid fossil data along the NE Atlantic, the absence of a species-specific molecular clock and the use of a single (mitochondrial) locus in the present research, alternative scenarios cannot be discarded. Hence, **future research** should focus on the use of several (unlinked) molecular markers combined with a more intensive sampling on the Iberian Peninsula, the Bay of Biscay and the coasts of Bretagne. In addition, new studies on the phylogeographic patterns within mysid species should also consider **the impact of Holocene warming** on the genetic composition of the southern populations (see Dahlgren *et al*, 2000; Consuegra *et al*, 2002; Coyer *et al*, 2003). At this moment it still remains unclear whether the observed **divergence of the southern Guadalquivir population** and the **genetic diversity decline** at these latitudes is linked to enhanced selective pressure at the distribution edge of *N. integer* related to increased Holocene temperatures. Extensive geographic sampling within the Gulf of Cadiz (and north African coasts) combined with detailed molecular analysis might generate complementary information. It would also provide insights in the **sustainability of these southern *N. integer* populations**.

2. Phylogeography of the mysid Mesopodopsis slabberi: *strong genetic divergence between Atlantic and Mediterranean populations with complex patterns of cryptic speciation.*

The mitochondrial DNA analyses of both the COI and 16S rRNA genes of the mysid *Mesopodopsis slabberi* revealed an **extraordinary degree of phylogeographic structuring** throughout its distribution range. **Four monophyletic clades** were apparent in the COI and 16S phylogenies: a large Atlantic clade, two Mediterranean clades corresponding to the haplotypes observed in the Ebro and the Alicante samples, and a fourth clade comprising a subset of the haplotypes of the Atlantic Mondego sample. In general, the levels of divergence between the different clades obtained from the 16S fragment were lower than those from the COI fragment, probably

caused by the higher conservation, and slower evolution of the mitochondrial 16S rRNA gene (Simon *et al*, 1994).

As mentioned in chapter 5, unravelling the evolutionary history that lead to the contemporary distribution of the different mitochondrial lineages in the populations of *M. slabberi* remains challenging. When putting the observed **divergences between the Atlantic and Mediterranean populations (16%)** in a broader perspective, they seemed to be **amongst the largest** thus far reported for Atlanto-Mediterranean marine taxa (see Table 4.7). The estimates of divergence time date back to the late Miocene/ early Pliocene (9.8 - 6.3 Mya), pointing to a **vicariant event during the Messinian salinity crisis** when sea-level dropped 115-120 m below the present-day level (Nilsson, 1982; Maldonado, 1985). The **two divergent mitochondrial clades** within the Atlantic **Mondego** estuary further complicate the phylogeographic patterns within *M. slabberi*. However, the lower genetic distances, at least for the 16S fragment, between this clade and the haplotypes of the Ebro population suggest **a Mediterranean origin** of this divergent Mondego clade. **Ship's ballast water transport** may have played a role in the transportation of these mysids to Atlantic waters. Analysis of the major ship routes from Mediterranean to Portuguese ports, as well as a more detailed sampling within the Mediterranean Sea (in potential 'source regions') are needed to resolve the identity and evolutionary origin of these haplotypes. Moreover, detailed analysis of Mediterranean *M. slabberi* populations inhabiting different habitats (estuaries, brackish lagoons, coasts) will also clarify the underlying evolution of the disjunct Mediterranean populations of *M. slabberi* (allopatric, parapatric divergence or ecological diversification between populations in marine and brackish environments).

Finally, the question remains whether the **different mitochondrial clades** should be considered **cryptic species?** The answer largely depends on the species concept that is favoured. If Cracraft's (1989) **phylogenetic species concept** (i.e. species are defined as minimum diagnosable units) is used, the answer is yes, since a high number of fixed differences is present between the different mitochondrial clades. However, purely applying this species concept could lead to the recognition of trivially divergent taxa at the species level. In addition, it is also greatly dependent on the polymorphic level (variability) of the selected marker system (Knowlton, 2000; Müller, 2000). According to Avise & Wollenberg (1997), a better criterion for recognizing species boundaries would be the existence of multiple concordant

differences at several (unlinked) loci. This approach also resembles that of the **biological species concept** (i.e. a species can be defined as a group of actually or potentially interbreeding individuals, with boundaries between species defined by intrinsic barriers to gene flow that have a genetic basis; Mayr, 1963), because reproductive barriers will emerge during the long-lasting geographic isolation that is required for many (unlinked) loci to acquire fixed (diagnostic) differences (Avise & Ball, 1990; Avise & Wollenberg, 1997).

The difficulty in defining species boundaries is further evidenced by the results of the morphometric analyses (Chapter 7). Although multivariate analyses clearly separated the Atlantic and Mediterranean populations based on telson and eye morphology and meristic characters, some (small) overlap existed between both populations. Hence, no individual mysid could be assigned unambiguously to a particular geographical area ('population') on the basis of these linear measurements alone. Moreover, phenotype-environment interactions ('phenotypic plasticity') could further confound the species division based purely on morphometric grounds.

In conclusion, our results (and especially the mitochondrial data) largely suggest the existence of different cryptic species within *M. slabberi*, but **further evidence from unlinked genetic markers (e.g. nuclear genes) are needed to confirm these patterns.** Future research should preferably make use of an integrative approach, using molecular (joint analysis of mitochondrial and nuclear loci), extended morphometrical (using geometric morphometric techniques) and environmental information (e.g. Rocha-Olivares *et al,* 2001; Pfenninger *et al,* 2003).

3. Are the differences in molecular diversity and genetic population structure between Neomysis integer and Mesopodopsis slabberi related to species-specific characteristics?

Both *Neomysis integer* and *Mesopodopsis slabberi* lack free-living larvae resulting in a low dispersal potential, which is reflected by a high phylogeographic structuring. But on the other hand, both species show some marked differences in their habitat preferences and physiological tolerance. ***N. integer*** is a true brackish water species, occurring in relatively discrete ('natural fragmented') habitats such as estuaries and brackish lagoons (= **'closed' populations**). In contrast, ***M. slabberi*** lives in marine (coastal, surfzone) and estuarine habitats, and hence may have a more

continuous distribution (= **'open' populations**). The geographical distribution of both species along the European coasts shows some differences, *N. integer* is restricted to Atlantic waters, while *M. slabberi* is also distributed throughout the whole Mediterranean and Black Sea. Along the NE Atlantic the distribution of both species largely overlaps, but *N. integer* seems to occur far further north (whole Baltic Sea, and even the White Sea, although recent observations are lacking) than *M. slabberi*. The evolutionary history of both genera, as well as the temperature tolerance (*N. integer* restricted by higher temperatures, *M. slabberi* restricted by colder temperatures) may have largely affected the contemporary distribution of both species.

A comparison of the genetic diversity patterns in both species may be useful for recognizing the effects of intrinsic (= biological, ecological, physiological or behavioural) differences on phylogenetic and phylogeographical patterns. Several studies in various marine taxa have shown that relatively small difference in species-specific intrinsic factors may result in the development of quite disparate patterns of population genetic structure and phylogeography for sympatric species (e.g. Wilke & Davis, 2000; Dawson *et al,* 2002; Bargelloni *et al*, 2003; McMillen-Jackson & Bert, 2003).

The standard diversity values (number of haplotypes, haplotype and nucleotide diversity) showed large differences between *N. integer* and *M. slabberi* (Table 8.1). **Haplotype diversity of almost all *M. slabberi* populations was more than twice the values for *N. integer*.** In addition, the levels of nucleotide diversity were much higher in the *M. slabberi* populations. The AMOVA's in both species further corroborate these patterns: in *N. integer* the highest percentage of variance was observed among populations while for *M. slabberi* the within population variance component was the largest (Table 8.2). These discrepancies in genetic diversity levels between both species may not be surprising. **High levels of within population haplotype diversity** have been considered a typical phenomenon of many **marine species**, as evidenced for both vertebrates and invertebrates (Baldwin *et al*, 1998; Grant & Waples, 2000; Benzie *et al*, 2002; McMillen-Jackson & Bert 2003; Karaiskou *et al*, 2004), while **low within-population variability** is a common characteristic for **brackish-water species** (Maltagliati 1999; Cognetti & Maltagliati, 2000;Bilton *et al*, 2002; Maltagliati, 2002). A common explanation for the high haplotype diversity and for the large numbers of low frequency haplotypes may lie in the enormous population sizes of marine organisms, which could cause a retention of

numerous haplotypes and result in an undersampling of the populations (Bucklin & Wiebe, 1998). However, given the sometimes astonishing densities of *N. integer* in the Westerschelde estuary (peaks of 100s of thousands mysids per 1000 m² and yearly averages up to 6500 mysids per 1000 m², Mees *et al*, 1993a, 1995; see also census population size estimations of *N. integer* in chapter 6), other ecological and evolutionary processes may have been involved in the reduction of genetic diversity (e.g. environmental interactions, natural selection, a population bottleneck, different age of both species, small or historical variable effective population sizes; see Bucklin & Wiebe, 1998 & discussion in Chapter 5).

Table 8.1: Standard diversity values for the overlapping sampling locations of *Neomysis integer* and *Mesopodopsis slabberi*. N_h = number of haplotypes, h = haplotype diversity, π = nucleotide diversity. Standard deviations of h and π are indicated between brackets. All values were calculated from the mitochondrial COI data presented in Chapters 4 & 5.

Sampling location	Sample Size	N_h	h (SD)	π (SD)
Neomysis integer				
Westerschelde	60	6	0.4689 (0.0652)	0.00335 (0.00227)
Seine	48	4	0.4193 (0.0810)	0.00329 (0.00225)
Ria de Aveiro	30	5	0.6115 (0.0510)	0.00272 (0.00198)
Guadalquivir	40	5	0.2359 (0.0880)	0.00128 (0.00118)
Mesopodopsis slabberi				
Westerschelde	25	21	0.9667 (0.0292)	0.010888 (0.006104)
Seine	19	16	0.9766 (0.0267)	0.010483 (0.005985)
Ria de Aveiro	16	11	0,9500 (0,0364)	0,008461 (0,005022)
Guadalquivir	18	18	1.0000 (0.0185)	0.019993 (0.010789)

Table 8.2: Comparison between the results of the hierarchical analysis of molecular variance (AMOVA). **Top:** *Neomysis integer*, AMOVA on all the Atlantic samples and a separate AMOVA excluding the Guadalquivir samples (GU); **Below:** *Mesopodopsis slabberi*, AMOVA on all the Atlantic samples, with exclusion of the divergent haplotypes in the Mondego sample (MO-B, see Chapter 5).

	Source of variation	% Total variance	Fixation indices	*P*
Neomysis integer				
All samples	Among populations	78.67	$\Phi_{ST} = 0.7867$	< 0.001
	Within populations	21.33		
without GU sample	Among populations	71.39	$\Phi_{ST} = 0.7139$	< 0.001
	Within populations	28.61		
Mesopodopsis slabberi				
Atlantic samples	Among populations	40.08	$\Phi_{ST} = 0.4001$	< 0.001
	Within populations	59.92		

A comparison of the pairwise genetic distances between populations of both species revealed a **clear difference of the genetic structure at a meso-geographic scale** (i.e. between the Westerschelde and Seine populations). In the case of *N. integer* both populations were significantly differentiated, while for *M. slabberi* no differentiation was observed. This could imply high levels of contemporary gene flow between these *M. slabberi* populations, or recent common ancestry (which seems not unlikely for populations inhabiting areas that have been severely affected by glaciations) (Avise *et al*, 1987). **At a macro-geographic scale (> 500 km) both species showed similar trends**, with exception of the higher differentiaton of the *N. integer* population in the Guadalquivir estuary. The peculiar pattern of the *M. slabberi* Guadalquivir population (= higher similarity with the northern Westerschelde & Seine populations than with the geographically closer Ria de Aveiro populations) remains unexplained and will need further examination.

Table 8.3: Pairwise genetic distances (Tamura & Nei, 1993) based on the mitochondrial COI data. *Above diagonal*: genetic distances of *Mesopodopsis slabberi*. *Below diagonal*: genetic distances of *Neomysis integer*. ^{ns} = value not significant at the 95% level. Population abbreviations: WS, Westerschelde; SEI, Seine; Rda, Ria de Aveiro; GU, Guadalquivir. All data compiled from Chapters 3 and 4.

	WS	**SEI**	**Rda**	**GU**
WS	-	0^{ns}	0.590	0.219
SEI	0.624	-	0.604	0.215
Rda	0.641	0.551	-	0.471
GU	0.901	0.879	0.911	-

Since both species occur sympatrically along the NE Atlantic, it can be assumed that they must have been subjected to the same paleoclimatological events (e.g. Pleistocene glaciations). Hence, a comparison of the phylogeographic patterns along the NE Atlantic within both species could reveal something about the species-specific responses to these historical climate events. However, the lopsided sampling regime in both studies (461 mysids from 11 sampling sites for *N. integer*, 78 mysids from 5 Atlantic sampling sites for *M. slabberi*) might hamper a clear comparison of the phylogeographic patterns, and hence, the conclusions for *M. slabberi* must be considered provisional. Nevertheless, some remarkable differences were apparent between both species, probably pointing to **a different response of *M. slabberi* to changing climatological conditions.**

When comparing the mismatch distributions of different geographical samples for both species (Fig. 8.1) the situation for the **northern populations** (North Sea &

English Channel) was clearly different. The distribution was unimodal for ***M. slabberi***, which is consistent with a model of **rapid population growth** from a small number of mysids, while a fit to the sudden expansion model was significantly rejected for ***N. integer***, pointing to **a more stable population structure** (see also mismatch distribution parameters in Chapters 4 & 5). These differences are also visible in the haplotype networks, with a star-like network for *M. slabberi* (see Chapters 4 & 5). The **mismatch distributions for the Portuguese samples seemed concordant for both species**; a fit to the sudden expansion model was rejected. Compression of the distribution range of *M. slabberi* to southern Europe (in the Bay of Biscay or maybe the northern Iberian Peninsula) during glacial periods caused by **lower temperatures and absence of suitable habitats**, followed by a postglacial range expansion to northern Europe, which is a common pattern in many European biota (see Hewitt, 1996, 2000), could have produced the unimodal mismatch distribution of the northern populations. In contrast, *N. integer* seemed to be able to withstand the glacial conditions in northern Europe and could have survived in isolated northern refugia (see previous discussions).

In conclusion, the present phylogeographic study of *M. slabberi* has **opened some interesting research perspectives**. Especially the large phylogeographic breaks (signals of cryptic speciation?) between *M. slabberi* populations and the disparate phylogeographic patterns of the sympatric mysids *N. integer* and *M. slabberi*, probably triggered by differences in eco-physiological tolerances, deserve detailed future research.

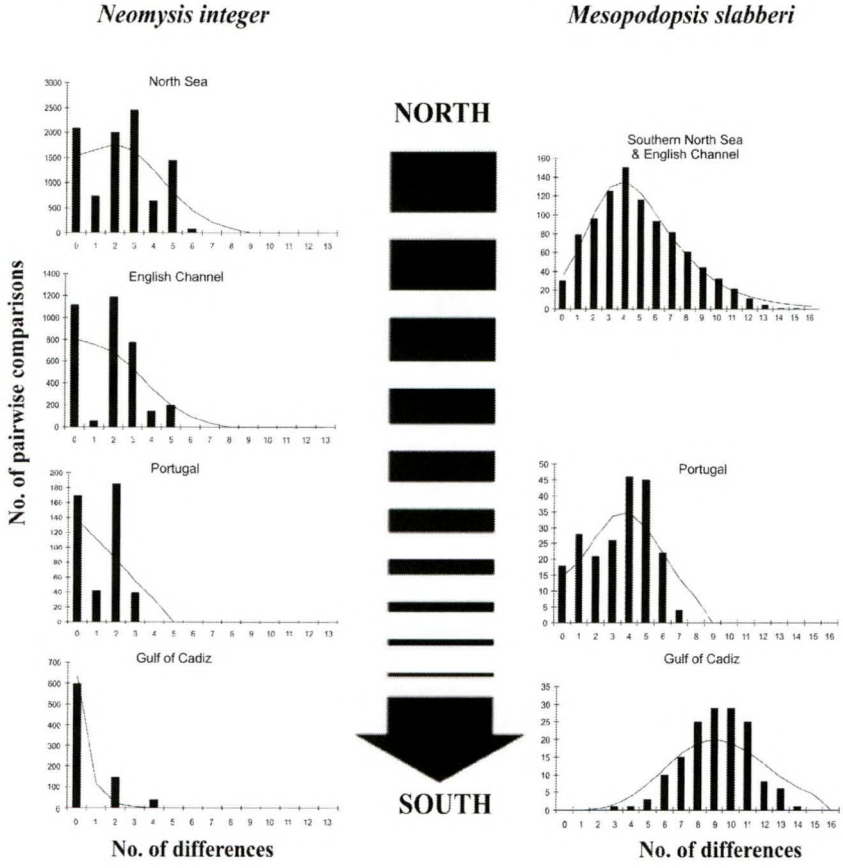


Fig. 8.1: Comparison of the mismatch distributions of different geographical samples for *Neomysis integer* and *Mesopodopsis slabberi*. In each case the bar represents the observed frequency of the pairwise differences among haplotypes, while the solid line shows the distribution expected under the model of a sudden demographic expansion (Rogers, 1995).

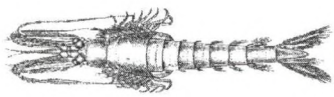
4. Small-scale and temporal patterns of genetic differentiation within Neomysis integer.

Most studies on the genetic structuring within species, including the present study of both mysid species, have focused on geographical patterns of genetic variation **regardless of the temporal variation**. However, the assessment of both spatial and temporal components of the genetic structure of a species is necessary to thoroughly understand the microevolutionary processes that influence the genetic variability and relationships among its populations (Maltagliati & Camilli, 2000). Therefore we conducted a temporal, as well as a fine-scale (intra-estuarine) genetic study of the Westerschelde population of *N. integer*. Although **(small) intra-estuarine differentiation** was detected within two of the three analysed years, and there seemed to be **no evidence for temporal stability** of this structure, the (single locus) molecular marker used in this study has **several limitations in terms of interpretation** of these contemporary genetic patterns (Allendorf & Seeb, 2000; Nevo, 2001; Wan *et al*, 2004). Hence, future research on the temporal and small-scale variation within mysids should preferably make use of a multilocus approach (e.g. microsatellites). In addition, the observations that *N. integer* has migrated further upstream, to more polluted sites, within the Westerschelde during the last decade warrants future investigation by continuing genetic monitoring. Bearing in mind the strong genetic differentiation of populations of *N. integer* (linked to the natural fragmented habitat and low dispersal capacities), the **very low (female) effective population size** estimations (see chapter 6) and the high potential of bioaccumulating endocrine disrupters and other toxicant compounds (Roast *et al*, 1999, 2000, 2002; Verslycke, 2003), these populations may be especially prone to rapid loss of genetic diversity.

5. The family Mysidae is in need for a taxonomical revision, as evidenced by the 18S rRNA phylogeny.

A phylogenetic study of the Mysidae, the largest family within the order Mysida, based on 18S rRNA sequences was conducted in order to test the morphology-based classification within this family. The molecular analysis did not support the monophyly of two of the three subfamilies included in the study. The

subfamily Gastrosaccinae was clearly resolved in two groups: "*Gastrosaccus*-group" and "*Anchialina*-group", which was further supported by morphological evidence. The paraphyly of the large subfamily Mysinae (comprising 91% of the genera and 80% of all species classified within the family Mysidae) highlights the problematic division into tribes, once introduced to permit an 'easier' structuring of this large subfamily. Hence, a revision of the tribes within this subfamily is suggested in order to tune taxonomy to phylogenetic relationships based on morphological and molecular data. In addition, representatives of the subfamilies Boreomysinae, Rhopalophtalmidae and Mysidellinae, which were not analysed in the present study, should be included in future research to evaluate the taxonomical rigidity of the whole Mysidae family.



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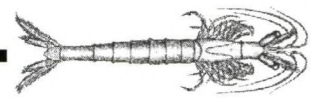

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Shrimp having a beer ...