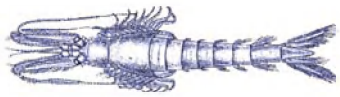
CHAPTER V



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.

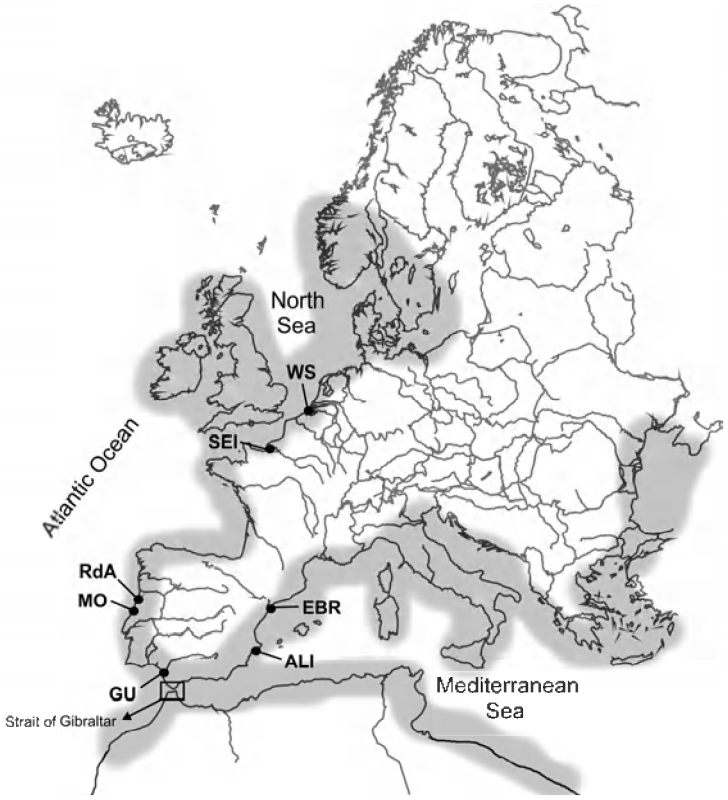

Fig. 5.1: Map of Europe showing the sample locations of *Mesopodopsis slabberi*. Shading represents the distribution range of *M. slabberi*. See Table 5.1 for sampling site codes.

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').

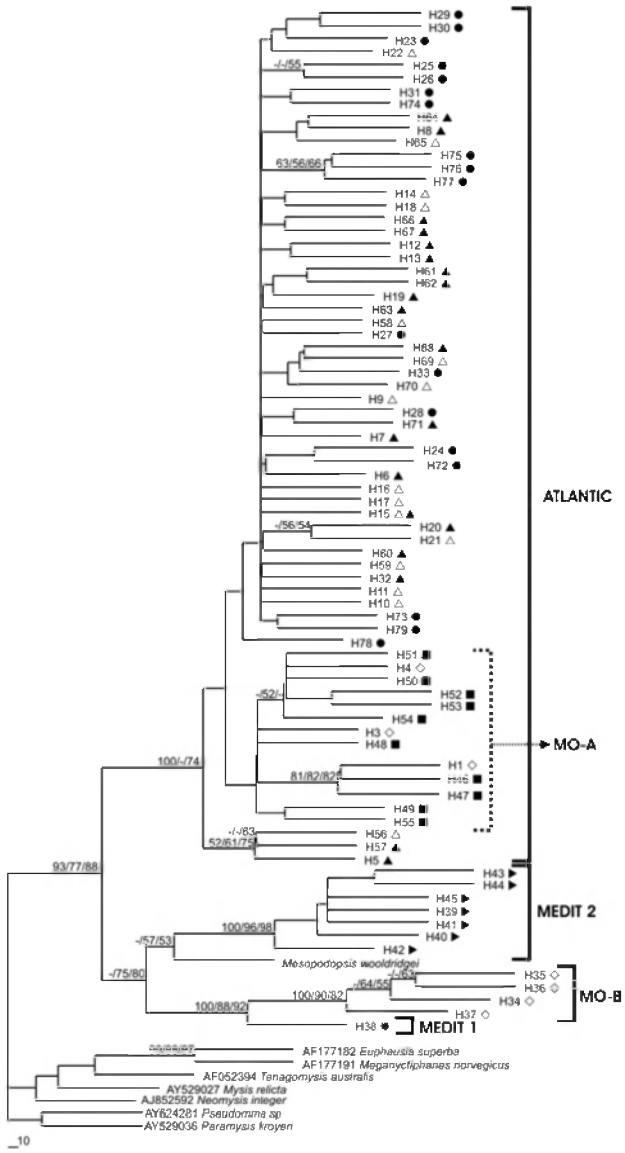

Fig. 5.2: Maximum parsimony consensus tree (895 steps) of the mitochondrial COI haplotypes of *Mesopodopsis slabberi* obtained after a heuristic search of 1,000 random sequence addition replicates. For each node the MP, ML and NJ bootstrap support is indicated, for clearness only bootstrap values > 50% 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**

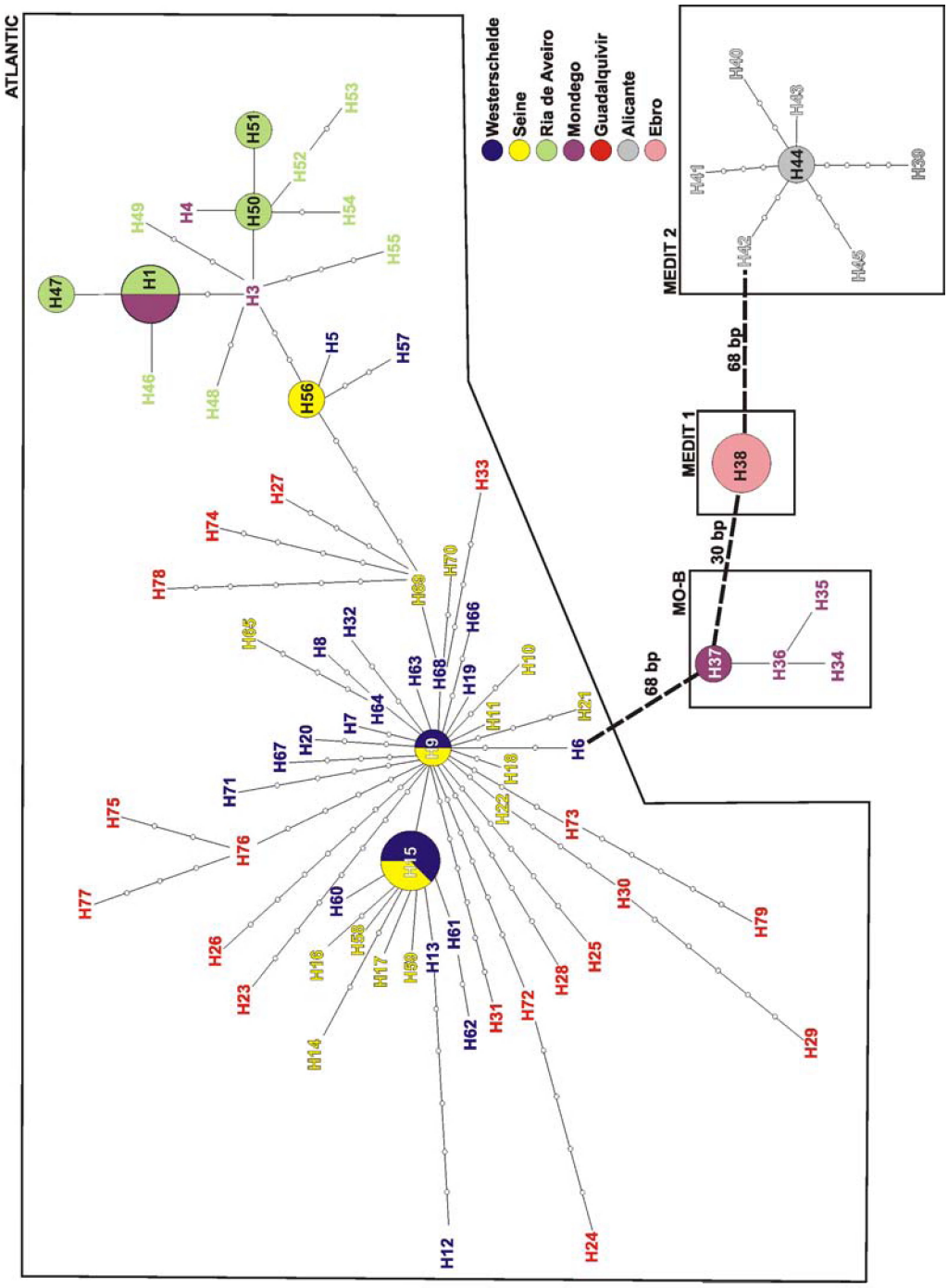

Fig. 5.3: Minimum spanning network showing the mutational relationships among the mitochondrial COI haplotypes of *Mesopodopsis slabberi*. Each line in the network represents a single mutational change and haplotypes are represented by a circle if the haplotype frequency > 1. The surface size of each 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%).

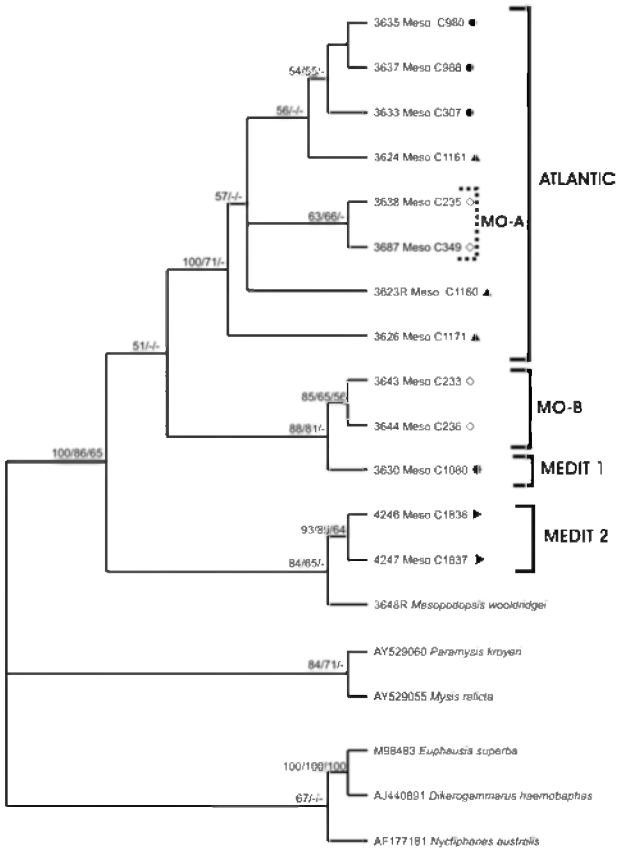

Fig. 5.4: Maximum parsimony consensus tree (682 steps) of the mitochondrial 16S rRNA haplotypes of *Mesopodopsis slabberi* obtained after a heuristic search of 1,000 random sequence addition replicates. For each node the MP, ML and NJ bootstrap support is indicated, only bootstrap values > 50% are indicated.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

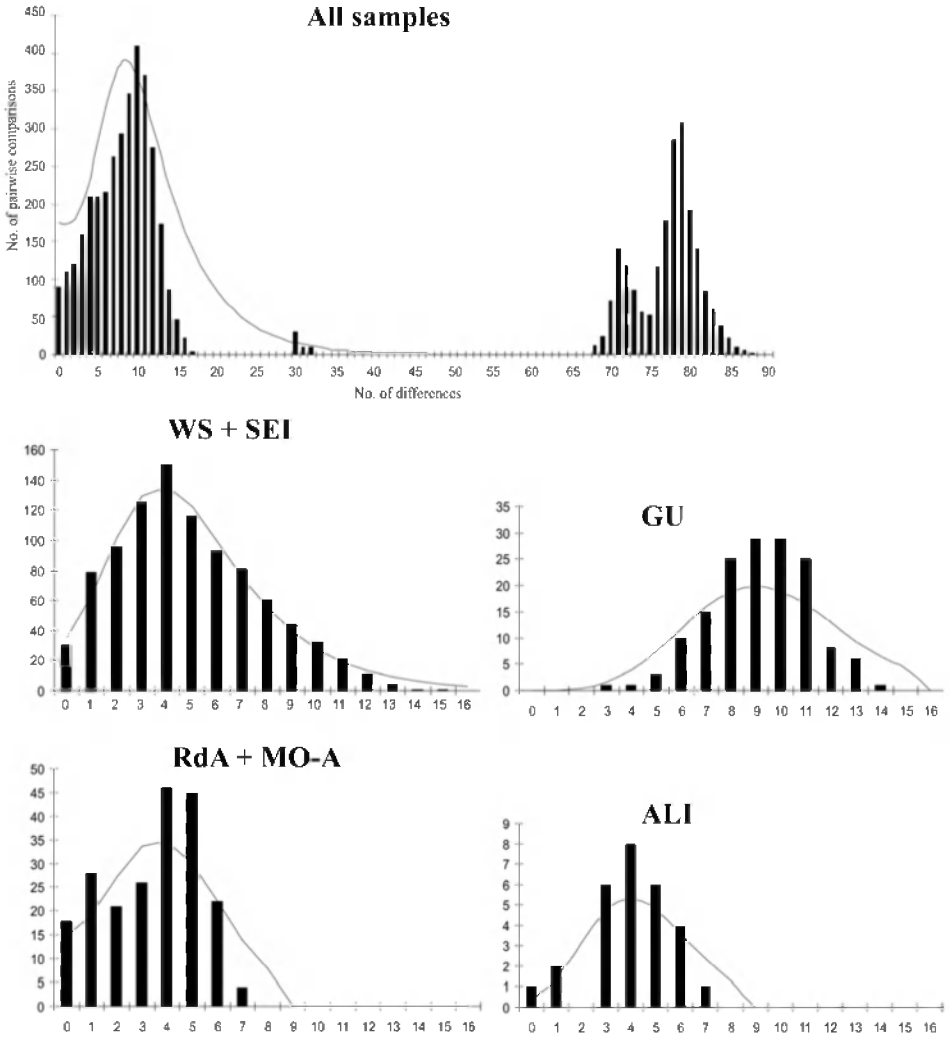

Fig. 5.5: Mismatch distribution of the samples; for sampling site abbreviations see Table 5.1. In each case the bar represents the observed frequency of the pairwise differences among haplotypes, while the solid line shows the distribution expected under a model of sudden demographic expansion (Rogers, 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 - cuphausiid	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-Iosada 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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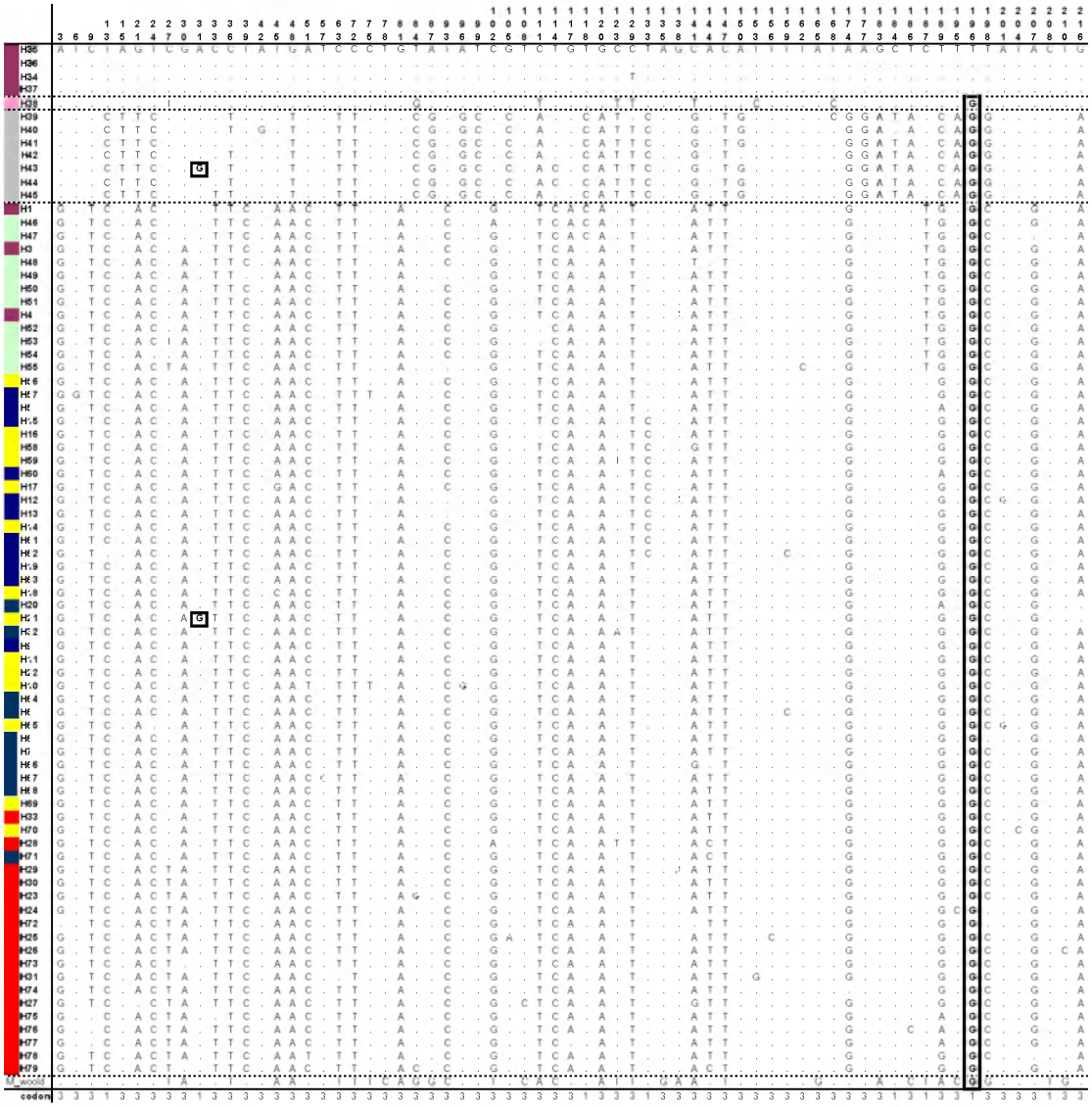
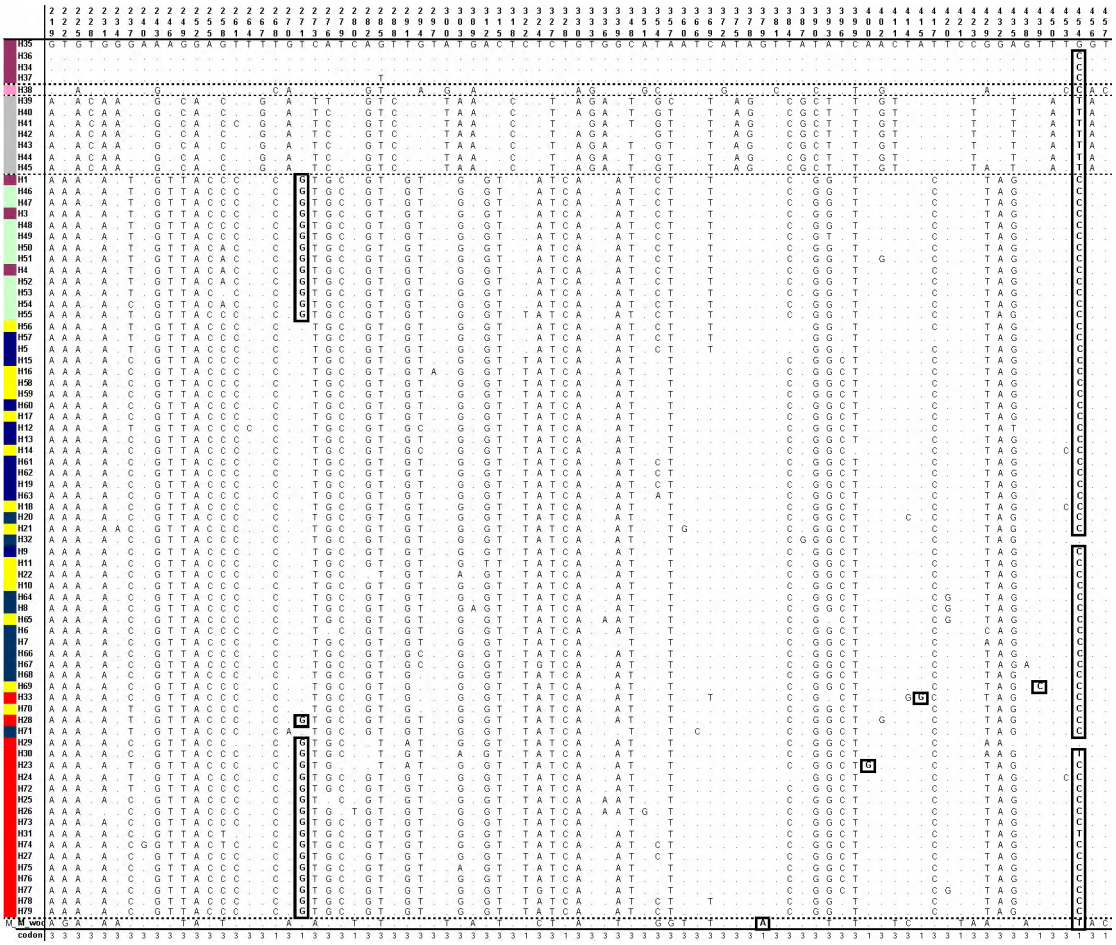
APPENDIX 1: Variable nucleotide positions observed in the 458 bp fragment of the mitochondrial COI gene of the *Mesopodopsis slabberi* and *M. wooldridgei* (M_woold) haplotypes. Colors in the first column indicate geographical origin of the haplotypes (same colour scheme as in Fig. 5.3), dots indicate 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	169	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	274	276	278	291	294	296	299	308	312	323	334	345	356	367	373	383	386	388	391	401	412	429	430	432	433	434	435	436	437	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487
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		