
CHAPTER III

**SEASONAL DYNAMICS OF POPULATION GENETIC
STRUCTURE IN CRYPTIC TAXA OF THE *PELLIODITIS MARINA*
COMPLEX (NEMATODA: RHABDITIDA)**

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ABSTRACT

The distribution patterns and genetic structure of the *Pellioditis marina* species complex in Belgium and The Netherlands were compared between four consecutive seasons. Different types of habitats (coast, estuary, semi-estuary and lake) with different degrees of connectivity were sampled. In addition, each habitat type was characterized by either temporal or permanent algal deposits. We screened 426 bp of the mitochondrial cytochrome oxidase c (COI) gene with the single – strand conformation polymorphism (SSCP) method in 1615 individuals of *Pellioditis marina*. The 51 haplotypes were divided into four (sympatric) lineages, with divergences ranging from 0.25 % to 10.6 %. Our results show that the lineages have different temporal dynamics, which may be linked to abiotic factors. Analysis of Molecular Variance (AMOVA) indicated a significant structuring in the PmI lineage, which correlated with habitat characteristics and which changed over time (Mantel, $r = 0.51$; $p = 0.126$). Intrapopulational diversity was similar in all locations, and temporal changes in haplotype frequencies were not higher in temporary than in permanent algal deposits. Instead, the results of the temporal survey indicated that (some) *P. marina* populations are characterized by a metapopulation structure. It is emphasized that a complete and correct interpretation of processes causing genetic structuring within species and of the genetic structure itself can only be done when analyzes are performed at several time points.

INTRODUCTION

Increased interest in the population genetic structure of a wide range of organisms has yielded many new insights in patterns of genetic diversity, of migration and of dispersal abilities in species belonging to very different phyla. For instance, several marine organisms which were long thought to have a homogeneous gene pool over large geographical ranges, have recently been shown to exhibit a substantial degree of genetic differentiation (e.g. Schizas et al. 1999, Kirkendale & Meyer 2004, Ovenden et al. 2004, Remerie et al. 2006a). Moreover, studying genetic variation within species has revealed the existence of substantial intraspecific genetic diversity, indicating the existence of multiple cryptic species (Williams et al. 2001, McGovern & Hellberg 2003, Lee & O' Foighil 2004, Derycke et al. 2005).

Many spatial studies implicitly assume that the observed genetic structure and diversity are stable over time, which is at odds with the effects stochastic processes may have on allele frequencies (Heath et al. 2002, Arnaud & Laval 2004). Hence, accurate interpretation of population genetic structure requires assessment of temporal variability. In turn, information on temporal changes in genetic structure may reveal to what extent natural populations of species exist in dynamic metapopulations, or in stable populations at equilibrium between drift and gene flow (Hoffman et al. 2004). Next to these stochastic processes, selection and migration can also influence allele frequencies (Hartl & Clark 1989). Selection leads to microevolutionary changes as a result of the species' response to environmental changes in its habitat. Therefore, habitat characteristics and demography also play an important role in shaping genetic structure within species (Charbonnel et al. 2002, Bousset et al. 2004). Temporal surveys on population genetic structuring of invertebrate species have been few (e.g. Charbonnel et al. 2002, Bousset et al. 2004, Arnaud & Laval 2004) and have included only few marine species (Barcia et al. 2005, Martinez et al. 2005, Papetti et al. 2005, Remerie et al. 2006a).

*Pellioditis marina*¹⁴ (Nematoda; Rhabditida) is an obligate outcrosser and typically lives either on dead macrophytes washed ashore and/or on standing macroalgae in sheltered places along coasts and estuaries. Suitable macroalgal thalli are covered with microbial biofilms - bacteria being the main food source for

¹⁴ See General Introduction, p 9: *Pellioditis* is now considered a subgenus of *Rhabditis*

P. marina (Moens & Vincx 1997) - and predominantly belong to the genus *Fucus* in our sampling area. The highest densities of *P. marina* are, however, found in piles of decaying algae. While dispersal abilities of nematodes are generally considered to be limited for lack of pelagic stages, these temporal deposits are rapidly colonised. *P. marina* is an opportunistic species with mean and minimum generation times of 7.2 and < 3 days, respectively, under the climatic conditions typical of our study area. Females can produce up to 600 eggs (Vranken & Heip 1983), so even one or a few gravid females should be able to establish viable populations. Hence, metapopulation dynamics are likely to be pronounced in this species. In addition, earlier work showed that *P. marina* actually refers to a complex of at least four cryptic taxa (currently defined as mitochondrial DNA lineages). Until now, no ecological or morphological difference between them has been reported (Derycke et al. 2005). Obviously, this cryptic taxonomic diversity must be taken into consideration when exploring eventual metapopulation dynamics in *P. marina*.

In order to evaluate to what extent metapopulation dynamics govern population genetic structuring in the *P. marina* complex, we analysed the spatiotemporal patterning of mitochondrial DNA COI haplotype frequencies in Belgian and Dutch populations of *P. marina* from different habitats (coastal - estuarine - semi-estuarine - lake) with different degrees of connectivity (from complete isolation to supposedly free exchange between habitats). Furthermore, we compared 'temporary' and 'permanent' habitats. Temporary habitats are algae that have been randomly deposited in the littoral by tidal currents or wind, while permanent habitats are algae attached to dikes and stones.

Three main issues are addressed in this study: (1) mapping the spatiotemporal occurrence of the four cryptic mitochondrial DNA lineages described by Derycke et al. (2005); (2) assessing habitat- related population genetic patterns in the most widespread lineage (PmI), and (3) analyzing the temporal stability/variability of the population genetic structure in PmI.

With respect to the habitat-related population genetic patterns in PmI, we assumed that: (1) the potential for migration and colonisation is higher in coastal and estuarine habitats because of their higher connectivity, and (2) extinction and genetic drift should be more common in temporary than in permanent habitats (Bousset et al. 2004). On this basis we expected that: (1) estuarine populations should be genetically more diverse; (2) temporal variation in haplotype frequencies should be more

pronounced in habitats where algal debris is periodically washed ashore (coastal and lake samples) than in habitats with permanent *Fucus* stands (estuarine samples); (3) differentiation should be stronger between different types of habitats (estuarine – coastal – lake) than between the samples from the estuarine system as a result of different habitat characteristics.

MATERIAL AND METHODS

SAMPLE LOCATIONS AND THEIR ECOLOGICAL CHARACTERISTICS

Sample locations of *Pellioiditis marina* were situated in Belgium and The Netherlands, all populations being separated by distances of 10-100 km (Fig. 3.1). Different degrees of connectivity are present in our sampling scheme: within the Westerschelde estuary (The Netherlands) five locations (Sl, Br, Pa, Ze, Kr) were sampled along a salinity and pollution gradient; these locations have no apparent physical barriers between them, nor with the two coastal locations in Belgium (Ni, Bl). The Oosterschelde (Os) is regularly separated from the sea by the storm surge barrier, while Lake Grevelingen (Gr) is isolated by permanent geographical and man-made barriers (The Grevelingendam). The history and ecology of the Westerschelde, Oosterschelde and Lake Grevelingen are described in Heip (1989). The nine locations also differ in (1) habitat type (coastal - estuarine – coastal lagoon - lake), (2) the availability of algae throughout the year (permanent vs. temporary), and (3) salinity (averages ranging from 16 to 31 in the Westerschelde, and from 28 to 35 elsewhere). These ecological characteristics are summarized in Table 3.1.

Pop	Locality	Geographical coordinates		Habitat characteristics			
		Latitude	Longitude	environment	salinity	type of algae	presence of algae
Sl	Sloehaven (Westerschelde)	51° 27' N	3° 36' E	estuary	31	<i>Fucus</i> sp.	permanent
Br	Breskens (Westerschelde)	51° 24' N	3° 33' E	estuary	30	<i>Fucus</i> sp.	permanent
Pa	Paulina (Westerschelde)	51° 21' N	3° 49' E	estuary	25	<i>Fucus</i> sp.	permanent
Ze	Zeedorp (Westerschelde)	51° 24' N	3° 58' E	estuary	18	<i>Fucus</i> sp.	permanent
Kr	Kruispolderhaven (Westerschelde)	51° 22' N	4° 3' E	estuary	16	<i>Fucus</i> sp.	permanent
Os	Oosterschelde	51° 36' N	3° 50' E	coastal lagoon	28	<i>Fucus</i> sp.	permanent
Gr	Lake Grevelingen	51° 44' N	3° 57' E	Lake	35	<i>Ulva</i> sp./ <i>Enteromorpha</i> sp.	temporary
Ni	Nieuwpoort	51° 9' N	2° 43' E	coast	33	<i>Fucus</i> sp.	temporary
Bl	Blankenberge	51° 19' N	3° 8' E	coast	31	<i>Fucus</i> sp.	temporary

Table 3.1. *Pellioiditis marina*. Geographical and ecological characteristics of the nine sampled populations. Average salinity values for the Westerschelde and Oosterschelde were calculated using values from www.waterbase.nl, coastal values were available from the MIDAS database on www.VLIZ.be. Pop = population.

All nine locations were sampled three-monthly in April, July and October 2003 and in January 2004 to evaluate the temporal (in)stability of the populations.

SAMPLE COLLECTION AND PROCESSING

Suitable fragments of *Fucus* sp. (*Ulva* sp. and *Enteromorpha* sp. at Lake Grevelingen, as *Fucus* sp. was not found here) were randomly collected from an area of approximately 50 * 50 m within the distribution range of the algae in each location, and pooled in buckets. In the lab, we again randomly took fragments from these buckets and divided them over 50 agar slants which were incubated for about two days. Adult *Pellioiditis marina* were then identified under a dissecting microscope using diagnostic morphological characters (Inglis & Coles, 1961), and handpicked with a fine needle from as many slants as possible. This would ideally lead to one nematode being picked from each slant. In practice, however, *P. marina* are patchily distributed on the algae and hence were found only on part of the agar slants. As a result, an overall average of four nematodes was collected per ‘successful’ slant. We consider this to be a representative subset of the local population because our sampling procedure ensures that nematodes were collected from a variety of patches within this local population. As such, ‘populations’ in this study conform to our sampling locations. All individuals were photographed digitally as a morphological reference and then stored in acetone (70 – 95 %) until processed.

GENERATING THE MOLECULAR DATA

On average 45 individuals were processed for each location and season. Exceptions were Lake Grevelingen in July 2003 (n = 18) and Nieuwpoort (n = 26) and Blankenberge (n = 34) in January 2004 (Table 3.2).

DNA extraction and characterisation of genetic variation were performed as described in Derycke et al. (2005). In short, after DNA extraction, 426 bp of the mitochondrial cytochrome oxidase c subunit 1 (COI) gene were amplified with primers JB3 and JB4.5 (Hu et al. 2002). Nematodes from Lake Grevelingen and a large number of individuals from the July campaign were amplified with primers JB3 and JB5 (Derycke et al. 2005). Variation in the COI fragment was detected using the Single Strand Conformation Polymorphism (SSCP) method (see Zhu & Gasser 1998 for a description of the method used). PCR and SSCP conditions were as in Derycke

et al. (2005). All samples with different SSCP-profiles were sequenced with both the forward and reverse primers mentioned above. Conformity between band mobility and sequence variability was checked by additionally sequencing 10 % of the samples in every location. Two haplotypes (X1, $n = 2$ in S1 and X2, $n = 1$ in Ni) were indistinguishable on SSCP from the common haplotype J and differed from it by 1 base substitution. These two haplotypes (Accession numbers: AM085439 and AM085440 for X1 and X2 respectively) were included for phylogenetic analyses, but omitted from the dataset for all other analyses.

PCR products were purified with shrimp alkaline phosphatase ($1 \text{ U } \mu\text{l}^{-1}$, Amersham) and exonuclease I ($10 \text{ U } \mu\text{l}^{-1}$, Amersham) and cycle sequenced using BigDye V3.1 Terminator Mix (PE Applied Biosystems). Electrophoresis was conducted on a Perkin Elmer ABI Prism 377 automated DNA sequencer. After trimming both ends of the sequences, we obtained a fragment of 396 bp.

New sequences that have not been reported by Derycke et al. (2005) were submitted to GenBank (accession numbers AM076731 – AM076741; AM076817 – AM076824, Table 3.2).

DATA ANALYSIS

Genetic diversity and phylogenetic analysis

Sequences were aligned with the Clustal X program v.1.74 (Thompson et al. 1997). Nucleotide (π , Nei 1987) and haplotype (h , Tajima 1983, Nei 1987) diversities were calculated with Arlequin v.2.0. (Schneider et al. 2000). Pairwise differences between sequences were calculated in Mega 3.0 (Kumar et al. 2004). To test if intrapopulation genetic diversity differed significantly among habitats, non-parametric Kruskal-Wallis tests were applied to mean h or π values over habitats, using the Statistica 5.5 program (Statsoft Inc. 2000). Changes in haplotype frequencies between temporal samples of a given population were evaluated using an exact test of population differentiation implemented in Arlequin v.2.0 (Schneider et al. 2000). Phylogenetic analyses of the haplotype sequences were performed with the Paup* 4.0 beta 10 program (Swofford 1998). MODELTEST 3.7 (Posada & Crandall 1998) was used to select the best substitution model for our data, using the corrected Akaike Information Criterion (AICc, Posada & Buckley 2004). The K81uf+G model (Kimura 1981), corrected for unequal base frequencies and unequal rate variation

among sites (G), best fitted our mitochondrial data (AICc score = 3188.2075, Akaike weight = 0.3178). Unrooted maximum parsimony (MP, 10000 rearrangements) and maximum likelihood (ML, 10000 rearrangements) trees were constructed via random stepwise addition (10 replicate trials) and a tree-bisection-reconnection branch swapping algorithm. One tree was held at each step. Bootstrap values for MP and NJ were calculated from 1000 replicates, and for ML from 100 replicates.

Spatiotemporal analysis of haplotype frequencies

Spatiotemporal variation in haplotype frequencies among and within populations was analysed with a hierarchical AMOVA, and by calculating pairwise F_{st} values between populations as implemented in Arlequin v.2.0 (Schneider et al. 2000).

AMOVA was performed at several levels, based on haplotype frequencies. We refer to ‘two-level AMOVA’ when seasons were nested within populations, and to ‘single-level AMOVA’ when no nesting was performed. Single - level AMOVA’s using conventional F-statistics were conducted to look at (1) the temporal differentiation within each location for each mitochondrial DNA lineage (Table 3.4); (2) the spatial differentiation in each season for the PmI lineage (Table 3.5). A two-level AMOVA was performed to compare the temporal and spatial differentiation (1) in the PmI, PmII and PmIII lineage (Table 3.3), and (2) in each habitat type for the PmI lineage (Table 3.6). The significance of the variance components was assessed by comparing the observed distribution with a ‘random distribution’ generated by permuting haplotypes (10000 times) among populations and among groups of populations (Excoffier et al. 1992). Differences between F_{st} values from temporary and permanent habitat types were assessed with a t-test for independent samples using Statistica 5.5 (Statsoft Inc. 2000).

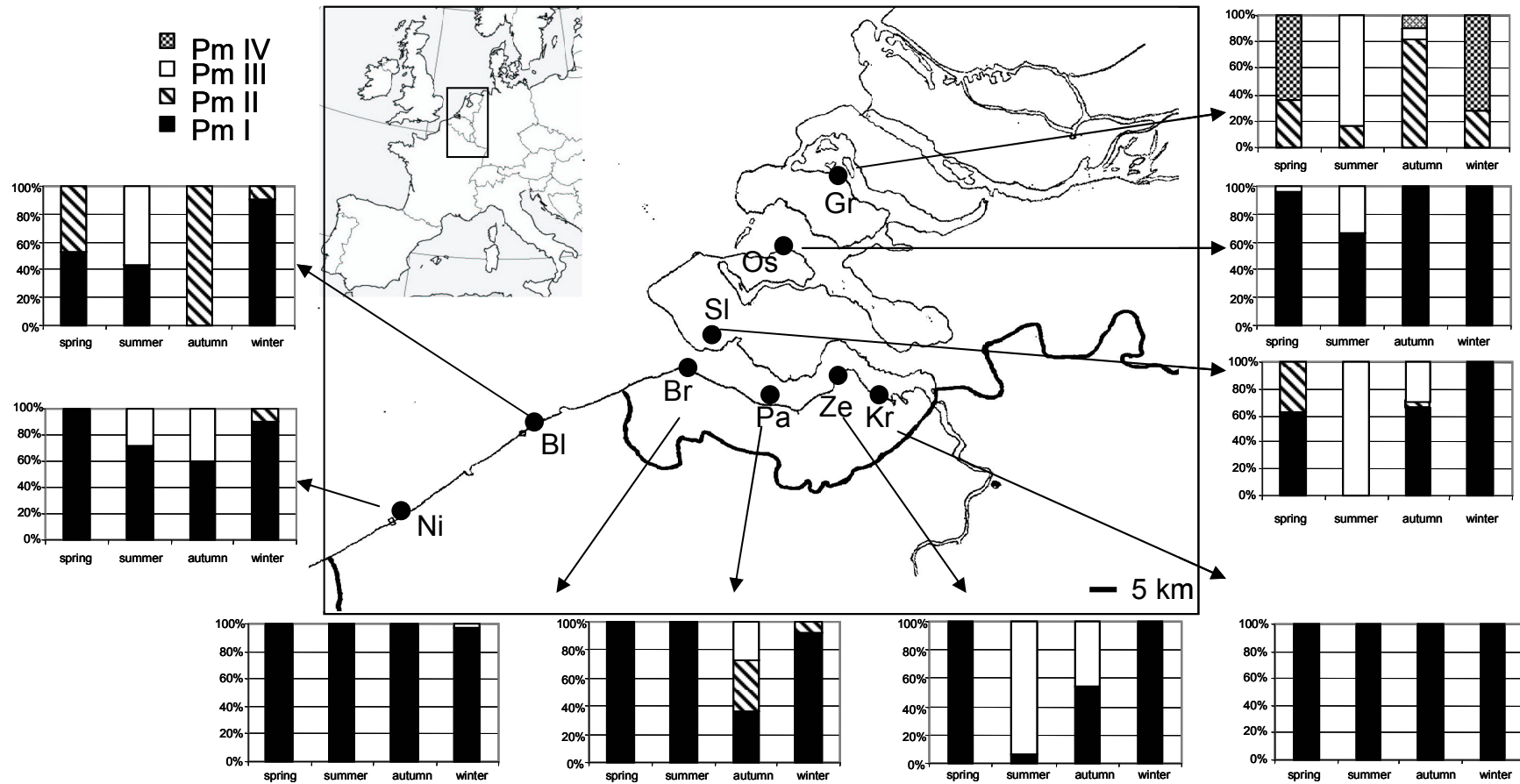
Cavalli-Sforza & Edwards’ (1967) (DCE) chord distances between all samples containing PmI haplotypes were calculated in Phylip 3.6 (Felsenstein 2004), and were visualized in a nonmetric multidimensional scaling-plot, superimposed by a minimum spanning tree, using NTSYS v 2.11 (Rohlf 2000).

The temporal stability of population structuring within lineage PmI was tested as follows: (1) differences in overall spatial genetic differentiation (F_{st}) between populations among the four seasons were tested with the randomisation procedure (10000 permutations) implemented in Fstat v 2.9.3 (Goudet 2001); (2) matrices of

DCE chord genetic distances between seasons were compared with a Mantel test (1000 permutations) as implemented in TFPGA 1.3 (Miller 1997) to evaluate whether genetic distances were similar between seasons (Hoffman et al 2004).

In addition, the isolation-by-distance model was tested within the PmI lineage with the program IBD 1.5 (Bohonak 2002). Geographic distance was measured as the shortest continuous water surface distance, and was compared with the DCE genetic distances. For IBD analysis, Os and Gr were omitted from the dataset: besides distance, they also show other habitat characteristics (differences in isolation, substrate, tides, ...) which could affect the IBD results. IBD was calculated for the PmI lineage in each season separately. The strength of the IBD relationship was determined with a Reduced Major Axis (RMA) regression (Bohonak 2002). Where applicable, significance levels of p-values were corrected for multiple comparisons according to the sequential Bonferroni method (Rice 1989).

Fig. 3.1: *Pellioditis marina*. Distribution of the 4 lineages (PmI, PmII, PmIII and PmIV). For each location, a stacked column graph indicates the percentage of each lineage occurring in spring 2003, summer 2003, autumn 2003 and winter 2004. For sample abbreviations see Table 3.1. Note the small proportion of PmIII during winter 2004 in Br.



RESULTS

GENETIC DIVERSITY AND PHYLOGENETIC ANALYSIS

Sequence variation in 396 bp of the mitochondrial COI gene was analysed in 1615 individuals of the morphospecies *Pellioditis marina*. There were 95 variable sites (23.7 %), 68 of which were parsimony informative. This yielded 51 different haplotypes, 20 of which were new compared to the April 2003 campaign (cf. the 19 haplotypes with an accession number beginning by AM in Table 3.2 and the haplotype X2) (Derycke et al. 2005). The evolutionary relationships among all haplotypes are shown in Fig. 3.2. The MP tree clearly differentiates four distinct haplotype groups (PmI, PmII, PmIII and PmIV), with divergences ranging from 0.25 – 2.3 % within, and from 5.3 – 10.6 % between groups (Table 3.7). In addition, three haplotypes (Z, Z2 and Z3) are highly divergent from the four haplotype groups as well as from each other. The lineages PmI, PmIII and PmIV are supported by high bootstrap values, while the PmII haplotypes received low bootstrap support. Of the 20 new haplotypes, eight belonged to lineage PmII. To evaluate whether taxon sampling could be the cause of the low bootstrap support of this lineage, the trees were recalculated with only PmII haplotypes from the April 2003 campaign and without the highly divergent Z haplotypes. This resulted in bootstrap values of 100, 99 and 100 (MP, NJ and ML resp.) for lineage PmII (data not shown). The Z haplotypes and the PmII haplotypes differ in one distinct amino acid change (Valine to Leucine) from the PmI, PmIII and PmIV lineages.

SPATIOTEMPORAL DISTRIBUTION OF THE FOUR CRYPTIC LINEAGES (PmI, PmII, PmIII AND PmIV)

From Fig. 3.1, it is clear that PmI is the most abundant lineage in every season (69.84 % of all individuals analysed belong to this lineage), even though this does not imply that it is always present or highly abundant in each location. Peak relative abundances were observed in spring (79.8 %) and winter (85.7 %). Despite this high overall dominance, PmI was not found at Gr, while it is the only lineage found at Kr and at Br (except one individual out of 186 for this last location). The PmII lineage was most frequent in autumn (23.2 %), and was mainly found at Bl and Gr. It was

relatively rare in the estuarine samples. The PmIII lineage had the highest relative abundance in summer (42.17 %), while it was sparse in spring (0.47 %) and even completely absent in winter. Finally, the PmIV lineage was restricted to Gr, where it reached a peak abundance during winter (72.34 % of the Gr individuals). Up to three lineages could be found jointly at the same site, but the timing and frequency of the different combinations varied over the course of a year and did not reveal any obvious consistent pattern.

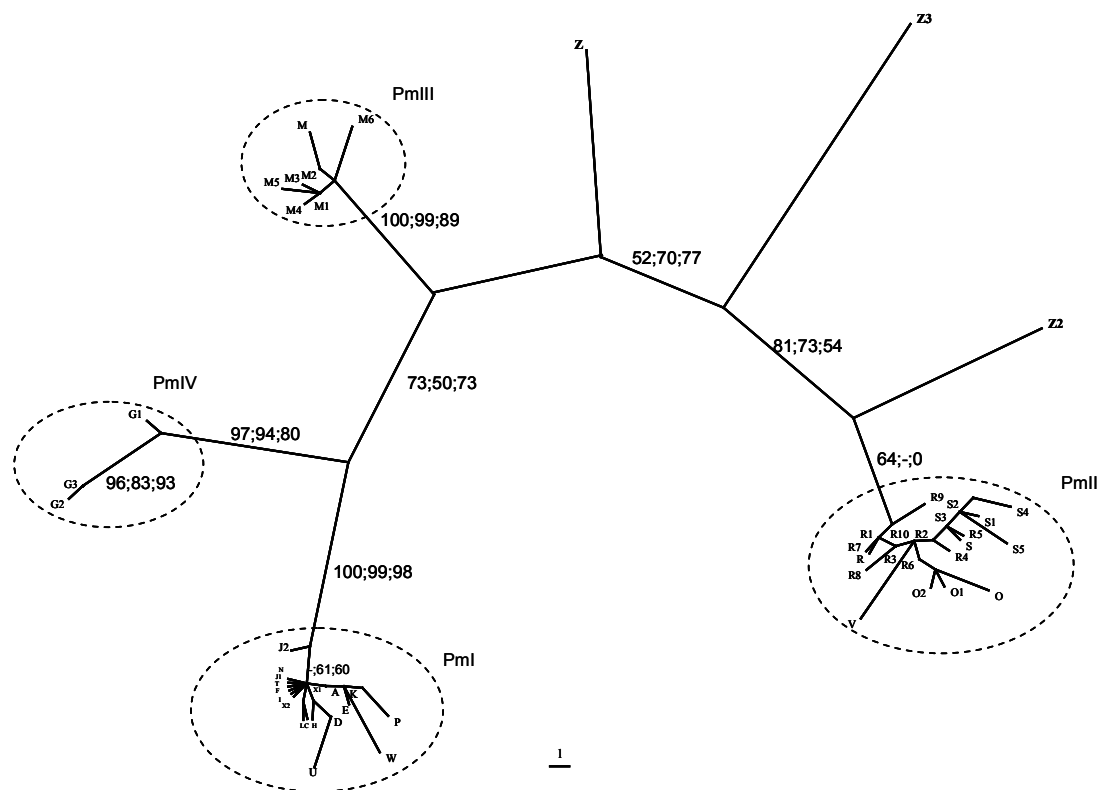


Fig. 3.2: *Pellioiditis marina*. Unrooted heuristic maximum parsimony phylogram of the 53 mitochondrial COI haplotypes. Bootstrap values above branches are from maximum parsimony, neighbour joining and maximum likelihood analyses resp. ‘-’ indicates bootstrap values lower than 50 and ‘0’ indicates that the branch was not present in the analyses. The genotypic clusters PmI, PmII, PmIII and PmIV are indicated with dashed lines.

SPATIAL VERSUS TEMPORAL VARIATION IN HAPLOTYPE FREQUENCIES WITHIN THE FOUR MITOCHONDRIAL LINEAGES

Because PmIV was only found at Gr, geographical differentiation within this lineage could not be assessed.

	source of variation	%	statistics	p
Lineage PmI	Among populations	10.70	Fct = 0.11	< 0.0001
	Among seasons within populations	9.54	Fsc = 0.11	< 0.0001
	Within populations	79.76	Fst = 0.20	< 0.0001
Lineage PmII	Among populations	5.50	Fct = 0.05	ns
	Among seasons within populations	21.15	Fsc = 0.22	< 0.0001
	Within populations	73.35	Fst = 0.27	< 0.0001
Lineage PmIII	Among populations	2.23	Fct = 0.02	ns
	Among seasons within populations	21.27	Fsc = 0.22	< 0.001
	Within populations	73.50	Fst = 0.23	< 0.0001

The two-level AMOVA's for each lineage separately indicate that geographical differentiation is prominent only within the PmI lineage (see next section and Table 3.3). This geographical component explains a similar amount of the observed variation as the temporal component (10.70 % vs. 9.54 %, Table 3.3). Differentiation due to temporal variation was large and highly

Table 3.3. *Pellioditis marina*. Two-level AMOVA for lineage PmI, PmII and PmIII. F-statistics were calculated for each mitochondrial DNA lineage separately. (%) percentage variance explained; (p) significance level, (ns) not significant.

significant in the PmII and PmIII lineages, explaining, respectively, 21.15 % and 21.27 % of the total variation (Table 3.3). Temporal differentiation occurs in both temporary deposits and permanent algal stands for each mitochondrial DNA lineage (Table 3.4). When Fst values from Table 3.4 were pooled by lineage according to habitat type (permanent vs. temporary), no significant differences were obtained with a t-test, indicating that temporal differentiation was not significantly higher in temporary algal deposits than in the permanent algal stands.

Habitat type	Pop	Lineage	#	n	%	Fst	p	Lineage	#	n	%	Fst	p	Lineage	#	n	%	Fst	p	Lineage	#	n	%	Fst	p
Coast	Ni	Pm I	4	130	14.26	0.14	***	Pm II	1	3	-	-	-	Pm III	2	30	1.25	0.01	ns	Pm IV	-	-	-	-	-
		Pm I	3	70	13.12	0.13	**	Pm II	3	66	25.46	0.25	***	Pm III	1	24	-	-	-	Pm IV	-	-	-	-	-
Estuary	SI	Pm I	3	110	12.80	0.13	***	Pm II	2	19	58.89	0.59	ns	Pm III	2	71	34.94	0.35	***	Pm IV	-	-	-	-	-
		Pm I	4	185	7.83	0.08	***	Pm II	-	-	-	-	-	Pm III	1	1	-	-	-	Pm IV	-	-	-	-	-
Estuary	Pa	Pm I	4	154	3.86	0.04	**	Pm II	2	20	26.02	0.26	**	Pm III	2	12	-	-	-	Pm IV	-	-	-	-	-
		Pm I	4	124	7.51	0.08	***	Pm II	-	-	-	-	-	Pm III	2	62	13.53	0.14	**	Pm IV	-	-	-	-	-
Estuary	Kr	Pm I	4	185	24.82	0.25	***	Pm II	-	-	-	-	-	Pm III	-	-	-	-	-	Pm IV	-	-	-	-	-
		Pm I	4	170	1.22	0.01	ns	Pm II	-	-	-	-	-	Pm III	2	19	4.95	0.05	ns	Pm IV	-	-	-	-	-
Lake	Gr	Pm I	-	-	-	-	-	Pm II	3	64	14.43	0.14	***	Pm III	2	18	39.69	0.40	**	Pm IV	4	67	7.92	0.08	*

Table 3.4: *Pellioditis marina*. Single - level AMOVA for the mitochondrial DNA lineages PmI, PmII, PmIII and PmIV. (Pop) population: abbreviations as in Table 3.1. (#) number of temporal samples; (n) number of individuals analysed for each location; (%) percentage of the variance explained by temporal differences in each location; (p) significance-level of the F – statistic: ns = not significant, * < 0.05, ** < 0.01, *** < 0.001.

SPATIOTEMPORAL VARIATION AMONG HAPLOTYPES OF THE *PMI* LINEAGE

source of variation	%	Fst	p
Spring			
Among populations	18.51	0.19	***
Within populations	81.49		
Summer			
Among populations	26.94	0.27	***
Within populations	73.06		
Autumn			
Among populations	25.45	0.25	***
Within populations	74.55		
Winter			
Among populations	14.71	0.15	***
Within populations	85.29		

Table 3.5: *Pellioiditis marina*. Single-level AMOVA for calculating the genetic structure of lineage *PmI* in each season. (%) percentage of the variance explained by; (p) significance-level of the F – statistic (Fst); *** < 0.001

seasons showed that this level of spatial differentiation did not change significantly among seasons (Fstat, $p = 0.47$). A detailed analysis of the spatial structure within *PmI* revealed a significant differentiation even within the Westerschelde and in every season (data not shown). *Bl* was differentiated from two locations within the Westerschelde: *Pa* on the one hand (Fct = 0.134, $p < 0.05$) and *Sl* on the other (Fct = 0.0523, $p < 0.001$), leading to a low but significant differentiation between the *Bl* and Westerschelde populations (Fct = 0.06, $p < 0.05$, Table 3.6). For comparison, the spatial differentiation between *Bl* and

The genetic diversity indices (h and π , Table 3.2) did not differ significantly among the eight *PmI* populations (Kruskal – Wallis test: $p = 0.0645$ and $p = 0.1225$ for h and π respectively). On average, the highest diversity was observed at *Pa* ($h = 0.7861$ and $\pi = 0.0074$) and the lowest at *Bl* ($h = 0.4335$ and $\pi = 0.0033$). Spatial differentiation for *PmI* was apparent in every season (Table 3.5), and permuting the spatial differentiation among

source of variation	%	statistics	p
Among Br, Sl, Pa, Ze, Kr	1.40	Fct = 0.01	ns
Among seasons			
within populations	11.40	Fsc = 0.11	***
Within populations	87.20	Fst = 0.13	***
Among Ws / Os	11.29	Fct = 0.11	**
Among seasons			
within populations	9.99	Fsc = 0.11	***
Within populations	78.72	Fst = 0.21	***
Among Ni / Bl	29.59	Fct = 0.30	*
Among seasons			
within populations	9.93	Fsc = 0.14	***
Within populations	60.48	Fst = 0.40	***
Among Ws / Bl	6.00	Fct = 0.06	*
Among seasons			
within populations	11.95	Fsc = 0.13	***
Within populations	82.05	Fst = 0.18	***
Among Ni / Ws	6.81	Fct = 0.07	**
Among seasons			
within populations	11.24	Fsc = 0.12	***
Within populations	81.95	Fst = 0.18	***
Among Bl / Os	36.39	Fct = 0.36	*
Among seasons			
within populations	2.31	Fsc = 0.04	***
Within populations	61.29	Fst = 0.39	***

Table 3.6: *Pellioiditis marina*. Two-level AMOVA for calculating genetic differentiation within lineage *PmI* between the different habitat types. (%) percentage of the variance explained by; (p) significance level of the F – statistic (Fst); (ns) not significant, * < 0.05, ** < 0.01, *** < 0.001.

Ni or between Bl and Os was much higher: 29.59 % ($p < 0.05$) and 36.39 % ($p < 0.05$), respectively. Overall, differentiation between the different habitat types (estuarine – semi-estuarine – coastal) was moderate, though significant (Table 3.6). The distribution and frequency of the PmI haplotypes in the eight locations are presented in Appendix 3.1. To test whether the observed spatial differentiation was correlated with geographic distance, IBD was tested in every season (omitting Os). Geographic and DCE genetic distances were only correlated in autumn ($p = 0.01$), and this correlation only just remained significant after Bonferroni correction. When Bl and Ni were omitted from the analysis, no IBD pattern was found within the Westerschelde ($p = 0.12$). In addition, DCE genetic distances were not correlated with each other among seasons, indicating that the genetic structure was not stable over time.

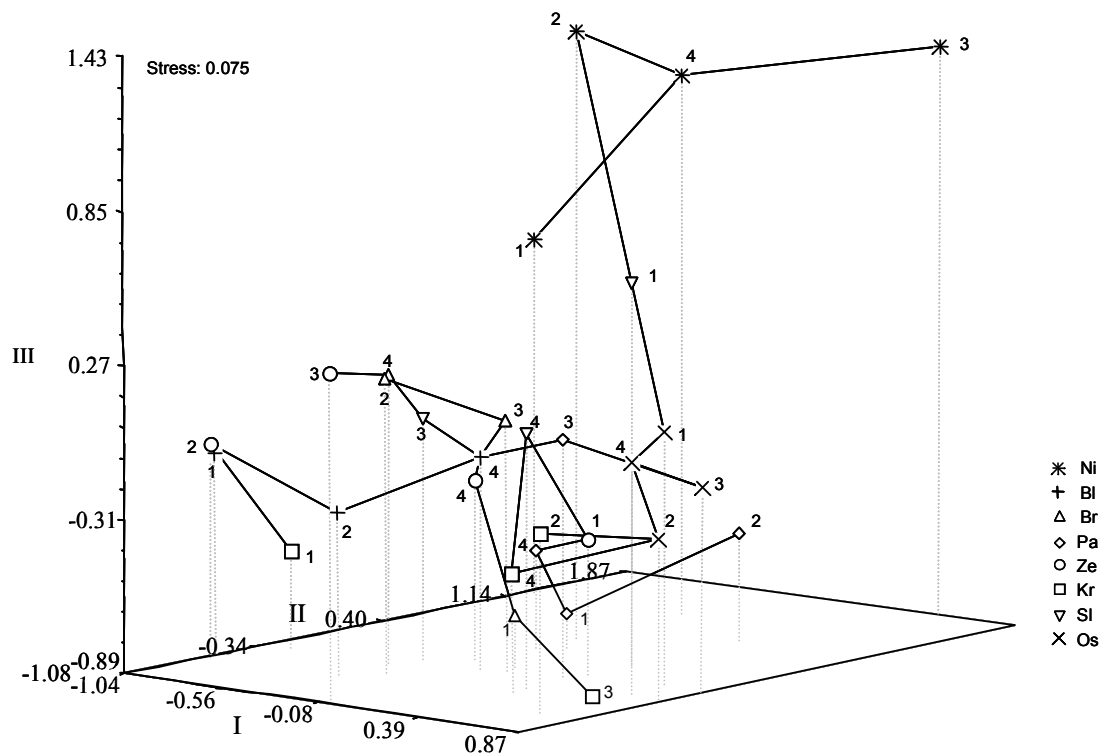


Fig. 3.3: *Pellioditis marina*. Lineage PmI. Minimum spanning tree (MST) superimposed on a nonmetric multi – dimensional scaling (nMDS) of the DCE distances between all temporal and spatial samples. Sample abbreviations are as in Table 3.1. Sl from summer and Bl from autumn are missing, as PmI was not found in these locations at that time. 1 = spring; 2 = summer; 3 = autumn; 4 = winter

The nonmetric multidimensional scaling (nMDS) ordination of all temporal samples of the PmI lineage did not group the populations according to season or algal availability, but illustrated the outlier position of the Ni samples and the high similarity between the Os samples (Fig. 3.3). Exact tests of population differentiation

indicated that haplotype frequencies changed with time for every population ($p < 0.0001$), except for Os ($p = 0.33$).

DISCUSSION

GENETIC DIVERSITY AND PHYLOGENETIC ANALYSIS

A previous study of the genetic structure of *P. marina* in the same locations already uncovered four sympatric mitochondrial DNA lineages (PmI, PmII, PmIII and PmIV), which are considered phylogenetic and genealogical species¹⁵. The cyto-nuclear disequilibrium between COI and ITS data further indicates that they are also reproductively isolated (Derycke et al. 2005). The present study adds 20 new haplotypes, the majority of which belong to the PmII and PmIII lineages (resp 8 and 6). Such high intraspecific variability has also been observed in plant- and animal-parasitic nematode species (Picard et al. 2004; Høglund et al. 2004, Nieberding et al. 2005). Our results support the idea that free-living nematodes are even more speciose than previously thought. In contrast, analysis of population genetic structure in the free-living soil nematode *Caenorhabditis elegans* revealed low levels of intraspecific diversity, despite a high level of outcrossing (Sivasundar & Hey 2005).

The low bootstrap support for the PmII lineage is likely the result of a taxon sampling effect (see e.g. Nylander 2001), in view of the high support obtained when only haplotypes from the April campaign are used. Additionally, the phylogenetic signal of PmII can be reduced by the higher intraspecific variation (2.3 %) observed within this lineage. As ITS sequences gave concordant tree topologies (Derycke et al. 2005) and MP and ML analyses of the COI data were consistent, we do not consider long-branch attraction to be important (Omilian & Taylor 2001, Anderson & Swofford 2004). A proper appraisal of the taxonomic status of the three Z-haplotypes also requires analysis of nuclear DNA sequence information¹⁶. While the cryptic lineages Pm I-IV were already uncovered by sampling in a single season, the addition of the divergent Z-haplotypes by our seasonal sampling campaign shows that sampling with substantial spatial (including different habitats) and temporal resolution

¹⁵ See Chapter 2

¹⁶ See Chapter 6

is required to adequately map and understand cryptic diversity within the *P. marina* species complex.

SPATIOTEMPORAL DISTRIBUTION OF FOUR CRYPTIC LINEAGES

Each cryptic mitochondrial DNA lineage reaches its highest relative abundance in a different season (Fig. 3.1). This suggests that the lineages at least have different temporal dynamics - whether these dynamics are in fact linked to seasonality can not be inferred from our data- which may reflect temporal changes in environmental and / or biotic factors. Regarding to abiotic conditions, salinities above 10 seem to have little effect on the overall fitness of *P. marina* (most likely PmI; Moens, pers. comm.), while temperature effects are more important (Tietjen et al. 1970, Moens & Vincx 2000a,b). Some *P. marina* populations thrive well at temperatures that are lethal for other populations. Besides local adaptation and phenotypic plasticity, this could be related to different temperature preferences among the cryptic taxa within *P. marina* (Moens & Vincx 2000a). Differential survival of cryptic lineages in other invertebrates to different types of pollutants has also been described (Sturmbauer et al. 1999, Kovatch et al. 2000, Schizas et al. 2001, Rocha-Olivares et al. 2004), but it is doubtful whether this could account for the predominance of the PmI lineage, the other lineages being no less abundant in the polluted Westerschelde estuary than elsewhere. Differential distribution patterns of the Pm lineages could also be influenced by biotic components: PmIV for instance was hitherto only found on the single location where we sampled green instead of brown algae, hence it is possible that lineage PmIV has been overlooked in the other locations because of our choice of sampling substrate. Additional sampling of these *Ulva/ Enteromorpha* deposits outside Lake Grevelingen is required to elucidate the role of substrate (and possible ecological speciation) in the distribution of these lineages.

The restriction of lineage PmIV to Gr and the absence there of lineage PmI could theoretically also reflect allopatric speciation. While possible in view of the isolation of Lake Grevelingen by geographical and man-made barriers since 1964-

1971 (Heip 1989), we consider allopatric speciation¹⁷ unlikely: a mean generation time of 6 days (Vranken & Heip 1983), a substitution rate of 9.7×10^{-8} site⁻¹generation⁻¹ (Denver et al. 2000, based on a nematode of the same family as *P. marina*) and 396 sites, yield ~0.095 substitutions since the isolation of Lake Grevelingen, whereas the true number of substitutions between PmI and PmIV ranges from 21-28 (Table 3.7).

	Pm I	Pm II	Pm III	Pm IV	Z	Z2	Z3
Pm I	0.25 - 1.5						
Pm II	7.3 - 10.6	0.25 - 2.3					
Pm III	6.3 - 8.3	7.8 - 10.3	0.25 - 1.3				
Pm IV	5.3 - 7.1	7.8 - 9.6	6.6 - 7.3	0.25 - 1.3			
Z	9.1 - 10.6	6.6 - 8.1	6.8 - 7.1	8.1 - 8.3	-		
Z2	9.3 - 9.8	4.5 - 5.5	8.3 - 9.1	9.1 - 9.6	7.3	-	
Z3	8.8 - 9.8	6.3 - 8.6	9.1 - 9.8	9.3 - 9.6	8.3	8.6	-

Table 3.7. *Pellioditis marina*. Divergence ranges (%) for the 396 bp COI fragment. Below diagonal are values between mitochondrial DNA lineages and the divergent Z haplotypes, values in bold are divergence ranges within each mitochondrial DNA lineage.

POPULATION GENETIC STRUCTURE OF PmI

Genetic structuring between populations of lineage PmI is observed in every season, and although the degree of spatial differentiation among populations remains constant, the genetic structure as such does not. First, the among-seasons component of the haplotypic variance is similar to the among-populations component (AMOVA, 9.54 % vs. 10.70 %, respectively). Such similarity has also been observed in steelhead trout (Heath et al. 2002), but is at variance with results for the estuarine copepod *Acartia tonsa*, in which geographical structuring clearly exceeded temporal differentiation (Caudill & Bucklin 2004). However, this result may be caused by differences in the geographical and temporal sampling scales. Secondly, pairwise DCE distances across seasons are not correlated. Such a correlation has been taken as evidence for temporal stability of population genetic structure (Hoffman et al. 2004). Finally, in our study, genetic structuring could only be explained by the IBD model in one season. This result indicates that factors other than geographic distance are important, e.g. evolutionary forces (e.g. Jorde & Ryman 1996, Heath et al. 2002) and/or environmental characteristics (Perrin et al. 2004, Bousset et al. 2004, Barcia et

¹⁷ The phylogeographic study in Chapter 9 does, however, point to an allopatric speciation at large geographical scales.

al. 2005). A geographical survey at one time point is clearly insufficient to adequately describe the spatial structuring in *P. marina* (and see Heath et al. 2002). The instability of spatial differentiation in *P. marina* is most clearly reflected in the differentiation between the Oosterschelde and Westerschelde. Haplotype frequencies within lineage PmI change over time in each population, except for Os (Table 3.4). Although such changes do not necessarily imply changes in population genetic structure (Arnaud & Laval 2004), they clearly do so in lineage PmI. In addition, the lower genetic diversity in the most upstream location within the Westerschelde (Derycke et al. 2005, and also observed in *L. littorea*, De Wolf et al. 2004) is consistent in all seasons. This location lies at the limits of the estuarine distribution of *P. marina* (Moens & Vincx 2000a) and is characterized by high fluctuations in salinity and by strong pollution (Baeyens 1998, Baeyens et al. 1998). These conditions can lead to lower genetic diversity as a result of density reductions in the population (De Wolf et al. 2004). When *P. marina* is exposed to cadmium concentrations similar to those at Kr, population densities are clearly lower than when exposed to cadmium concentrations present at Pa (Derycke et al. 2007b).

A second aim of this study was to assess whether the genetic composition of the populations would correlate with general habitat characteristics. Even a preliminary environmental characterisation can sometimes predict population variability (Bousset et al. 2004 Perrin et al. 2004). First of all, we expected a larger number of haplotypes in the estuarine samples because of higher exchange possibilities and because of the permanent availability of suitable substrata. However, the diversity was not significantly different between any of our sampling locations, suggesting large local population sizes and rendering any effects of genetic drift minimal. In contrast, genetic diversity in two freshwater snail species did correlate with habitat openness (Charbonnel et al. 2002, Bousset et al. 2004). However, these studies used microsatellite data, which because of the larger effective sequence information is probably more powerful in detecting (minor) population genetic variability than mitochondrial DNA (Kanda & Allendorf 2001).

Secondly, we also expected a higher temporal variability in the coastal locations which are characterized by only temporary substratum availability (algal deposits). This was again not supported by our data: F_{st} values from temporal vs. permanent populations were not significantly different, a result also found in the freshwater snail *Physa acuta* (Bousset et al. 2004). The lower than expected temporal

differentiation suggests that temporary populations never go extinct. *P. marina* forms metabolically less active dauer larvae when conditions turn unfavourable (e.g. when microbial activity declines, Bongers & Bongers 1998). If these dauer larvae (or eggs and other resting stages) are present and survive in the sediment, they may constitute some kind of *P. marina* stock, from which new algal deposits can be colonized. However, data of colonisation experiments suggest that such a ‘sediment stock’ is rather unlikely, as we repeatedly failed to isolate *P. marina* after inoculation of cleaned (with all fauna removed) fresh and decomposing algae on sediment collected at the Pa sampling site (Derycke et al. 2007b). The absence of any temporal differences in Os is in line with what we expected: this location is more stable due to reduced tidal effects, is ‘clean’ (Heip 1989), and contains permanent algal stands.

Thirdly, we expected a stronger differentiation between the different habitat types than between the different locations within the Westerschelde. This was confirmed by our data (Table 3.6), suggesting that genetic structuring within lineage PmI is correlated with environmental characteristics. However, the genetic structuring even within the Westerschelde estuary is remarkable, considering the life history characteristics of *P. marina*: its high reproductive output, short generation time and low juvenile mortality (Vranken & Heip 1983) make it a potentially good colonizer (Bongers & Bongers 1998). Furthermore, passive dispersal via drifting algae occurs (Derycke pers. obser.). *P. marina* is therefore able to quickly colonize new (empty) patches, which are prominently available in estuarine and coastal habitats. Results of a field experiment in the Westerschelde indeed indicate that empty algal deposits are rapidly colonized at substantial densities by PmI (Derycke et al. 2007c). Large census and effective sizes are therefore expected in the field, rendering the importance of genetic drift rather small. If changes in allele frequencies are the result of population turnover, rather than of genetic drift, than metapopulation dynamics are likely to occur (Hanski 1991, Ostergaard et al. 2003, Mc Elroy et al. 2003). We therefore conclude that populations of *P. marina*, and more specific lineage PmI, shows a metapopulation structure (except in Os), with colonisation of new patches of algae and subsequent extinction of older, completely decomposed patches.

CONCLUSION

In conclusion, habitat type and the ecological factors addressed here seem to be of minor importance for predicting the intrapopulation genetic diversity (h , π) within *P. marina*, indicating that large population sizes are maintained at every location (except in the most upstream location in the Westerschelde). More specifically, the temporal/permanent character of algae and connectivity between populations¹⁸ correlate with the genetic structure of *P. marina*. Moreover, this study shows that population genetic structure within species is not necessarily stable over time and that underlying, fine-scale patterns and processes can clearly be misinterpreted when only one time point is analysed. On the basis of our temporal survey, we conclude that lineage PmI most likely exhibits a metapopulation structure, except at Os.

Furthermore, our data also indicate that sampling all possible (micro)habitats, including different substrates, and broadening the geographical and temporal scale is necessary to fully understand the effects of micro-evolutionary forces on genetic structuring and speciation. In addition, the temporal dynamics of the four lineages within *P. marina* raise questions as to what extent their abundances are correlated with seasons and what the role is of temporal isolation in this taxonomic differentiation.

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¹⁸ We here mean that the unstable character and the patchy distribution of the algae together with abiotic differences between coastal, estuarine and lake locations best explain the population genetic structuring in PmI

Appendix 3.1: *Pellioditis marina*. Distribution of the PmI haplotypes among the eight sampling locations: the eight most abundant haplotypes are shown separately, while the other nine rare haplotypes are pooled together (grey). Ni: Nieuwpoort; Bl: Blankenberge, Br: Breskens, Pa: Paulina, Ze: Zeedorp, Kr: Kruispolderhaven, Sl: Sloehaven, Os: Oosterschelde

