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Species status and population structure of mussels (Mollusca: Bivalvia: *Mytilus* spp.) in the Wadden Sea of Lower Saxony (Germany)

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Abstract Three species of mussel (genus *Mytilus*) occur in Europe: *M. edulis* (Linnaeus 1758), *M. galloprovincialis* (Lamarck 1819) and *M. trossulus* (Gould, Boston Society of Natural History 3: 343–348, 1850). Although these species are indigenous to the North Sea, the Mediterranean and the Baltic Sea, respectively, they form an extended patchy species complex along the coasts of Europe ("the *Mytilus edulis* complex") and are able to hybridize where their distributions overlap. Recent studies examining the taxonomic status and genetic composition of *Mytilus* populations in the Netherlands and the British Isles have revealed introgressive hybridization processes within this species complex, with hints of an invasion of nonindigenous *M. galloprovincialis* into the North Sea. Furthermore, an extensive international mussel fishery industry in Europe (i.e., Great Britain, the Netherlands, Denmark, and Germany) is also in discussion for a possibly anthropogenically induced bioinvasion of nonindigenous *Mytilus* traits into the Wadden Sea area. Although it is assumed that the Wadden Sea of Germany comprises *M. edulis* only, this has never been confirmed in a molecular genetic study. To assess the situation for the Wadden Sea of Lower Saxony, we conducted the first molecular study of the *Mytilus* genus in the region. Taxonomic identification of 504 mussels from 13 intertidal mussel banks using the nDNA marker Me15/16 revealed a population composition of 99% *M. edulis* and 1% *M. edulis X M. galloprovincialis* hybrids. Hence, the Wadden Sea population is unaffected by range expansion of nonindigenous *Mytilus* traits. The genetic structure of the *M. edulis* populations was investigated using the phylogenetic and population genetics analyses of the mitochondrial DNA cytochrome-*c*-oxidase subunit I (COI) and the first variable domain of the control region (VD1), which were sequenced for >120 female individuals. These results showed a heterogeneous, panmictic population due to unrestricted gene flow. This can be attributed to extensive larval dispersal linked to the tidal circulation system in the back barrier basins of the Wadden Sea.

Keywords Bioinvasion · Haplotype-networks · Hybridization · *Mytilus edulis*-complex · Panmixia · Phylogeny · Population structure

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Introduction

Species of the genus *Mytilus* are vital members of the intertidal communities. As important ecosystem engineers, intertidal mussel beds play a key role in the food web structure and diversity of such dynamic environments (Buttger et al. 2008; Nehls et al. 2009). They also serve as an important food resource for humans, with many mussel banks being maintained and harvested through commercial fishing industries. For instance, average yearly yields of the Blue Mussel, *Mytilus edulis* (Linnaeus 1758) in the Wadden Sea alone were 80×10^6 kg in the Netherlands, 30×10^6 kg in Germany, and 100×10^6 kg in Denmark by the year 2002 (Smaal 2002). Thus, it is perhaps not surprising that the genus is one of the most comprehensively examined mollusc taxa worldwide and also among the most sampled in the BOLD database (http://www.boldsystems.org).

Studies carried out to infer the phylogenetic relationship and taxonomic status among *Mytilus* species based on morphological and molecular data (e.g., Rawson 2005; Riginos and Henzler 2008; Smietanka et al. 2009; Stuckas et al. 2009) have revealed a complex history of introgression and recombination among the three very closely related species *M. edulis*, *Mytilus galloprovincialis* (Lamarck 1819) and *Mytilus trossulus* (Gould 1850). All three species are indigenous to Europe, with the coasts along the North Sea and Norway being the main habitat of *M. edulis*, that of the Mediterranean Sea of *M. galloprovincialis* and that of Baltic Sea of *M. trossulus* (Gosling 1992; Hilbish et al. 2000). Nevertheless, *M. edulis* and *M. galloprovincialis* are well known to form a patchy metapopulation along the Atlantic coasts of France, Ireland, and Great Britain (e.g., Daguin et al. 2001; Hilbish et al. 2002; Bierne et al. 2003; Smietanka et al. 2009; Kijewski et al. 2009). In addition, recent studies have extended (and further complicated) our knowledge of the molecular genetic distribution of the three species in Europe. For example, *M. trossulus* was found recently in parts of Scotland and the Netherlands (Smietanka et al. 2004; Beaumont et al. 2008; Kijewski et al. 2009). Another intriguing result was the discovery of two genetically distinct *M. galloprovincialis* populations in the Mediterranean Sea and the Atlantic (Smietanka et al. 2009).

An important genetic feature of the three species is their ability to hybridize in areas of sympatry. One example is the narrow hybrid zone between North Sea *M. edulis* and Baltic Sea *M. trossulus* (Väinölä and Hvilsom 1991; Riginos and Cunningham 2005; Stuckas et al. 2009). Many other hybrid zones have been discovered recently along the Atlantic coasts of France, Ireland and the coast of Great Britain (Hilbish et al. 2002; Gosling et al. 2008). These areas exist typically in the form of a patchy mosaic consisting of 'pure' populations with hybridization zones where they overlap. This long history of potential introgression and hybridization further complicates taxonomic classification among the three species, with diagnostic morphological traits, in particular the characters of the shell, being known to vary with different abiotic factors (e.g., food supply or salinity) and levels of hybridization (Beaumont et al. 2008; Gardner and Thompson 2009). However, it appears that the nuclear marker Me15/16 (Inoue et al. 1995) can reliably discriminate among the three species (Coghlan and Gosling 2007; Beaumont et al. 2008; Kijewski et al. 2009) and enables the frequency of parental (homozygous) and hybrid (heterozygous) mussel classes to be estimated. This marker has been applied successfully in recent studies of North Sea *Mytilus* populations (e.g., Luttikhuizen et al. 2002; Smietanka et al. 2004; Beaumont et al. 2008; Kijewski et al. 2009). Even so, it remains common to consider the blue mussel *Mytilus edulis* complex to be composed of these three hybridizing species.

The Wadden Sea—located in the southeastern part of the North Sea—is the world's largest soft-bottom intertidal ecosystem. This ecosystem reaches across the Netherlands, Germany and Denmark and hosts many endemic species in high numbers. In this tidal driven marine habitat, *M. edulis* assumes its characteristic roles as a main ecosystem engineer and important economic species for the European fishery industry (Smaal 2002; Nehls et al. 2009). An aspect crucial to the latter is the common practice of importing and exporting consumption mussels and also mussel seed across the borders of European countries (e.g., Ireland, Great Britain, the Netherlands, Germany) (Wijsman and Smaal 2006). Thus, there exists a distinct risk of accidentally importing nonindigenous *Mytilus* species into the Wadden Sea environment by the fishery industry—a topic of ongoing discussion and one underlying future monitoring activity (Kijewski et al. 2009). Changes in species composition are also possible through climate change or additional disruptions through other anthropogenic activities (e.g., transport of larvae via shipping), with a documented invasion of *M. galloprovincialis* in South Africa to count as one of the most invasive marine species worldwide (Lowe et al. 2000; Robinson et al. 2005).

Against this backdrop, our goal is to characterize the *Mytilus* population within the Wadden Sea of Lower Saxony in Germany, which is assumed to consist solely of individuals of *M. edulis* (Gosling 1992; Hilbish et al. 2000). Thus, using the Me15/16 marker, we sought initially to classify the mussel population throughout the Wadden Sea of Lower Saxony with respect to its allelic composition with the *M. edulis* complex. Although no evidence of *M. galloprovincialis* has been detected along the northern coasts of the Netherlands (Kijewski et al. 2009), the same study could not exclude its presence absolutely. In addition, the Wadden Sea of Germany remains uninvestigated in this regard and also faces a possible invasion from the east from the Baltic *M. trossulus* population. In addition, we analyzed population dynamics within the indigenous *Mytilus* species in Lower

Saxony via phylogenetic and population genetics analyses of the most conservative mitochondrial loci cytochrome-*c*-oxidase subunit I (COI) and the first variable domain of the control region (VD1) (Cao et al. 2004; Smietanka et al. 2010). By combining results at inter- and intraspecific levels, we hope to gain a novel and comprehensive view about the present molecular genetic status of one of the most ecologically and economically important taxa in the Wadden Sea. In addition, our results should provide insight into the potential for invasion of nonindigenous *Mytilus* species into the traditional *M. edulis* habitats of the Wadden Sea, a process of considerable importance and the subject of much current discussion (Kijewski et al. 2009; Nehring et al. 2009).

Materials and methods

Sampling

Juvenile and adult blue mussels were collected from 13 intertidal mussel banks within the 'Nationalpark Niedersächsisches Wattenmeer' between March and July 2009 (Fig. 1 and Table 1). The majority of the sampled mussel banks are located on the mud flats of the local back-barrier basin of the East Frisian Islands. Exceptions were the three sampling sites WK, WK2 and Hoo, which are more exposed relative to the sites to the rear of the East Frisian Islands or the Jade Estuary. Mussels were sampled randomly using a 1×1 m sampling square that was thrown onto the mussel bank, with five mussels being sampled from predefined areas from the square. Additional samples were taken in the harbor areas of Helgoland (German Bight) and Kiel (Baltic Sea). The sex of each mussel was determined by microscopic examination of the gonads in the laboratory. Foot, gill and gonadal tissue were stored in ethanol (98%) at −20°C until DNA extraction.

DNA extraction and sequencing

DNA was extracted from gonadal tissue using either Chelex (BioRad, Richmond, CA) or the Maxwell 16 Mouse Tail DNA Purification Kit (Maxwell 16 System, Promega, Madison, WI) following the manufacturers' protocols in both cases. Because mussels present doubly uniparental inheritance of mtDNA and distinct male (M) and female (F) mtDNA haplotypes, gonadal tissue of female individuals was used preferentially because it is usually homoplasmic for the F-mtDNA (Zouros 2000) and therefore preferable for the analysis of *M. edulis* phylogeny and population genetics (Stewart et al. 1995). However, additional DNA were also extracted from 16 males and 7 individuals of unknown sex. The extracted DNA was stored at −20°C until further processing of the Me15/16 or mtDNA markers.

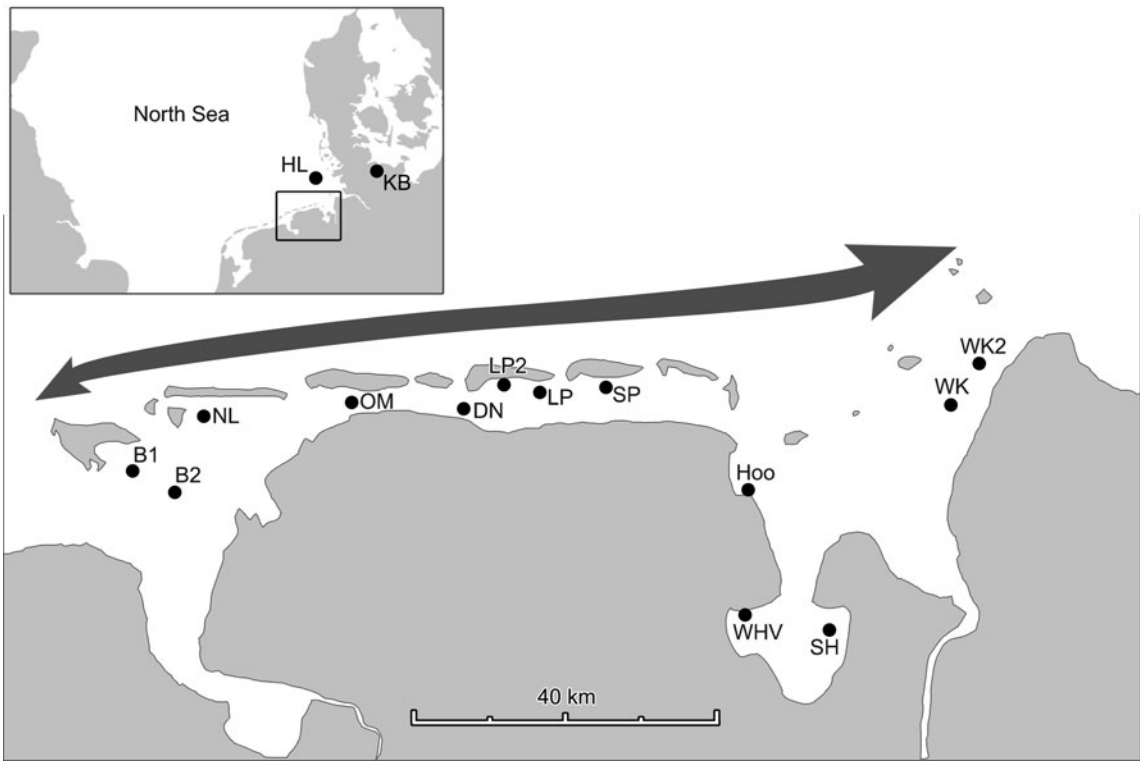

Fig. 1 Sampling locations within the Wadden Sea of Lower Saxony (Germany). For the population genetic analyses, the sampling area was subjectively separated into three areas: west, central and east. The *arrows* represent the average current direction and strength (Loewe et al. 2004)

Table 1 Location data of the sampling sites

Site	Acronym	Location	Northern latitude	Eastern longitude
Borkum	B1	Randzel	53°33.6'	6°48.5'
Borkum	B2	Randzel	53°32.6'	6°53.5'
Juist	NL	Juister Inselwatt	53°38.5'	6°56.3'
Norderney	OM	Hilgenrieder Watt	53°41.1'	7°14.4'
Langeooger Balje	DN	Dornumer Nacken	53°42.2'	7°28.1'
Langeoog	LP1	Langeooger Plate	53°44.1'	7°37.0'
Langeoog	LP2	Langeooger Plate	53°44.3'	7°32.6'
Spiekeroog	SP	Swienplatte	53°44.8'	7°44.8'
Hooksiel	Hoo	Jade	53°38.9'	8°04.2'
Wilhelmshaven	WHV	Jadebusen	53°30.2'	8°05.9'
Stollhamm	SH	Jadebusen	53°29.6'	8°16.5'
Wurster Küste	WK	Wurster Watt	53°47.0'	8°27.7'
Wurster Küste	WK2	Wurster Watt	53°50.3'	8°30.6'
Helgoland	HL	Westmole	54°10.2'	7°53.4'
Kiel	KB	Schwedenkai	54°19.8'	10°08.9'

PCR amplification of the Me15/16 marker was performed in 20 µl of a solution containing 1 µl DNA template extract, 1 µl dNTP mix (200 µM) (Jena Bioscience, Jena, Germany), 2 µl *Taq* buffer 10× (Jena Bioscience), 0.25 µl of each primer (0.5 µM), 0.25 µl *Taq*-Polymerase (1 U) (Jena Bioscience), and 15.25 µl water (Millipore, Bedford, MA). Thermal cycling used an Eppendorf Mastercycler ep gradient S (Eppendorf, Germany) with the following parameters: an initial denaturation for 5 min at 95°C; and then 30 cycles of 95°C for 30 s, 56°C for 30 s and 74°C for 30 s; all followed by a final extension period of 5 min at 74°C. The species-specific PCR product lengths (180 bp for *M. edulis*, 168 bp for *M. trossulus* and 126 bp for *M. galloprovincialis*; Inoue et al. 1995; Table 2) were identified using a 3% agarose gel and a 25 bp DNA ladder (Invitrogen, Karlsruhe, Germany). Individuals with a single band were considered to be homozygous, whereas heterozygous individuals with two bands were considered to be hybrids. In making this assumption, we should add that single-banded individuals can indeed also be of hybrid origin (as F2 backcrosses) if hybrid individuals are fertile (Coghlan and Gosling 2007).

Indeed, although various recent studies have shown the high reliability of the Me15/16 locus as a diagnostic marker (Coghlan and Gosling 2007; Beaumont et al. 2008; Kijewski et al. 2009), the observable Me15/16 allele frequency can be altered by the presence of fertile hybrids (Coghlan and Gosling 2007). Moreover, recent multi-locus analyses showed a more complex hybridization/introgression history that single markers are less able to detect (e.g., Kijewski et al. 2009; Stuckas et al. 2009). However, it appears that the Me15/16 allele is diagnostic enough to determine the abundance of homozygous and heterozygous genotype distributions within *M. edulis*-complex (Coghlan and Gosling 2007; Beaumont et al. 2008; Kijewski et al. 2009).

COI fragments of ≤450 bp were obtained using the primer pair *MytF* and *MytR* (Wares and Cunningham 2001; Table 2). To amplify the first variable domain of the mitochondrial control region, we designed a new pair of primers (VD1F and VD1R) using a promotional version of CLC Workbench 2009 (CLC bio; http://www.clcbio.com) (Table 2). The VD1 region is the first part of the main control region (i.e., D-Loop) of the mitochondrial genome. Cao et al. 2004 have divided the main control region into three domains [i.e., the first variable domain (VD1), the conserved domain (CD), and the second variable

Table 2 Primers used for DNA and mtDNA amplification

Gene	Taxa	Primer sequences (forward / reverse)	Length	Reference
Me15/16	*M. edulis* (Me) *M. galloprovincialis* (Mg)	5'–CCA GTA TAC AAA CCT GTG AAG A–3'	180 bp (Me) 168 bp (Mt)	Inoue et al. 1995
	M. trossulus (Mt)	5'–TGT TGT CTT AAT AGG TTT GTA AGA–3'	126 bp (Mg)	
COI, female	*M. edulis*	5'–GGT TTT ATA ATG TGG TTG TTA C–3' 5'–TCA AAA AAT GTT GTG TTA AAA T–3'	> 450 bp	Wares and Cunningham 2001
VD1, male and female mtDNA	*M. edulis* *M. galloprovincialis*	5'–CTG TTC GCC CTT TAA AAT C–3' 5'–CTC ACA TGA CTC TGA CAA A–3'	> 600 bp paternal > 900 bp maternal	present study

domain (VD2)]. While the conserved domain is one of the slowest evolving parts, the variable domain is one of the fastest evolving parts of the *Mytilus* mitochondrial genome (Cao et al. 2004). The annealing sites for the forward (VD1F) and reverse (VD1R) primers are located at the end of the 16S rRNA subunit and within the constant domain (CD) of the control region, respectively, thereby encompassing the entire VD1 region. It is therefore possible to distinguish VD1 sequences from the male and female mtDNA haplotypes based on their different lengths (ca. 600 vs ca. 900 bp, respectively; Cao et al. 2004). PCR reactions for both COI and VD1 were performed in 50 μl of a solution containing 4 μl DNA template extract, 2 μl dNTP mix 10 mMol (Finnzymes), 10 μl *Taq* buffer 10× (Finnzymes), 1 μl of each primer, 1.5 μl $MgCl_2$ (Finnzymes), 0.5 μl Phusion Hot Start Polymerase (Finnzymes) and 30 μl water (Millipore, Bedford, MA). The thermal cycling parameters were as follows: an initial denaturation for 4 min at 98°C; 25 cycles of 98°C for 30 s, 55°C for 30 s, and 70°C for 30 s; all followed by a termination step of 10 min at 74°C. PCR products were separated on a 1.2% agarose gel and purified with the JETQUICK Gel Extraction Spin Kit (Genomed, Löhne, Germany) before being sequenced using a BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 3130 × l Sequencer (Applied Biosystems).

All mtDNA sequences were aligned manually and edited to a consistent length (i.e., removal of flanking singletons) with BioEdit 7.1.3 (Hall 1999), resulting in alignments of 399 bp for COI, 859 bp for VD1 (comprising the partial 16S ribosomal RNA gene from site 1 to 134, the complete VD1 fragment from site 135 to 823, and the partial conserved domain from site 824 to 859), and 1,259 bp for the concatenated data set of the two linked loci. All sequences were deposited in GenBank under accession numbers: JF825556–JF825694 (COI) and JF902026–JF902159 (VD1). The molecular data sets were transformed into haplotype data sets with DnaSP v5.10.00 (Librado and Rozas 2009) and the Perl script seqConverter 1.1 (Bininda-Emonds 2010). GenBank COI and VD1 haplotypes from *M. galloprovincialis*, *M. trossulus*, *M. californianus* (Conrad 1837) and *M. coruscus* (Gould 1861) were added to the haplotype data sets as outgroup taxa (Supplementary Table 1) for the phylogenetic analysis. The concatenated COI and VD1 haplotype dataset was assembled without additional GenBank haplotypes (expect for the outgroup: *M. trossulus*, GenBank accession number: DQ198248.1). Additionally, DnaSP and PAUP* 4.0b10 (Swofford 2002) were used for sequence statistics (e.g., parsimony informative sites, GC content, haplotype diversity).

Bioinformatic analysis

Recombination between evolutionarily distinct paternal and maternal mtDNA lineages is well known within *Mytilus* (e.g., Ladoukakis and Zouros 2001; Burzynski et al. 2003, 2006; Rawson 2005; Filipowicz et al. 2008). To detect such events in our data, which would otherwise invalidate our phylogenetic or population genetics analyses, we used the Recombination Detection Program (RDP3; Martin et al. 2010) with search parameters as described in Riginos and Henzler (2008). Each mtDNA data set was analyzed separately, both with and without the additional GenBank haplotypes and outgroups.

Deviation from a random association of alleles (Hardy-Weinberg equilibrium, HWE) within the Me15/16 locus was calculated using ARLEQUIN v.3.5 (Excoffier and Lischer 2010). The hierarchical AMOVA (analysis of molecular variance) and the pairwise comparisons (pairwise Φ_{ST}) package from ARLEQUIN were used to assess the genetic differentiation for each of the COI, VD1 and the concatenated mtDNA data sets.

For the hierarchical AMOVA the sequence data were assigned to 12 populations according to their sampling site, which were then pooled into one of three hypothetical groups ("west", "center", and "east") (Fig. 1; Supplementary Table 2). Due to an average number of ten sequences per sampling site (Supplementary Table 2), and the high haplotype diversity, this step was conducted to ensure the minimum amount of samples within each group for an optimal AMOVA. Because of the small sample sizes (n=5 on average), the sequence information from Kiel and Helgoland was not included in the AMOVA and the pairwise comparisons. Significance was assessed by 1.023 permutations of the data matrices, with the nominal P value of 0.05 corrected for multiple comparisons using a standard Bonferroni correction (Φ_{CT}, P<0.017; Φ_{SC}, P<0.004; and Φ_{ST}, P<0.0004). Pairwise differentiation for each data set was analyzed by Φ_{ST} statistics. Significance was assessed by 10.100 permutations.

The Mantel test (Mantel 1967) from ARLEQUIN was used for the correlation analysis between genetic and geographical distances in the concatenated data set. GoogleEarth (http://www.google-earth.com) was used to calculate the shortest geographical distances between the sampling sites. Significance was assessed by 1,000 permutations. The demographic history based on the concatenated mtDNA sequence variation ("Group 1") was examined by mismatch distribution analysis with ARLEQUIN (Slatkin and Hudson 1991; Rogers and Harpending 1992). Significance was assessed by 1,000 permutations. To compare the observed distribution of pairwise differences between individuals to that expected under demographic expansion the sum-of-squared-difference statistic (SSD) was used.

The evolutionary relatedness of the Lower Saxony *Mytilus* haplotypes was inferred using both minimal-spanning networks as well as traditional phylogenetic analysis. Haplotype networks provide a more accurate display of intraspecific gene genealogies than bifurcating phylogenetic trees (Pleines et al. 2009). Nevertheless, like the latter, equally parsimonious pathways in network analyses (e.g., due to homoplasious mutations) means that often no single best network exists (Riginos and Henzler 2008).

Network analyses used TCS v1.21 (Clement et al. 2000) with gaps being treated as a 5th character state. Hence, each indel, regardless of its size, was considered a single state to not loose important information about the relationships within the population. Gaps being treated as 5th character state and the fix connection limit was set at 99 steps to connect all haplotypes. Only haplotypes represented by two or more sequences were included to exclude multifurcating connections between single haplotypes (singletons) due to their low and therefore uncertain genealogical significance. Additionally, one network was generated from the concatenated haplotype data set that included all obtained sequences.

Maximum likelihood (ML) analyses of the unique haplotypes for each of the COI, VD1 (including outgroup and GenBank sequences), and the concatenated mtDNA fragments (including only outgroup sequences) were conducted using RAxML 7.0.4 (Stamatakis et al. 2005). Analyses employed a GTR + G model (using the GTRMIX function for COI and VD1, and GTRCAT for the concatenated sequences) using the rapid bootstrapping search function (Stamatakis et al. 2008) with 1,000 replicates. Otherwise, the analyses used the default parameters of the respective programs.

Results

Me15/16 locus distribution and summary sequence statistics

A total of 504 mussels from 13 sites in the Wadden Sea and two additional sites (Kiel and Helgoland) were analyzed successfully using the Me15/16 nuclear marker. The results reveal an almost universal presence of the *M. edulis* allele (Me) within the total sample, with 499 individuals being homozygous for the Me allele (Table 3). Five individuals are heterozygous (eg) for the *M. edulis* and *M. galloprovincialis* alleles and belong to the four intertidal mussel beds WHV, NL, LP, and SP of the Wadden Sea area (Fig. 1, Table 3). The frequencies of the homozygous Me and heterozygous eg genotypes (0.9908 and 0.0092, respectively) are in HWE (Table 3).

The mtDNA data sets comprised 139 sequences (= individuals) for COI and 134 for VD1. Both gene fragments were amplified successfully for 132 individuals (concatenated data set) and/or 84 sequences (concatenated haplotype data set). Each sampling site was represented by an average of about ten individuals (Supplementary Table 2; Fig. 2). Character and polymorphism information are shown in Table 4.

Population genetics

Hierarchical AMOVAs for each of the three mtDNA haplotype sequence sets revealed that by far the greatest amount of variation (around 99%) was found within the populations (= sampling sites; Table 5), with a contribution among the populations (Φ_{CT}) or groups (Φ_{SC}). Due the high variation within the populations and the low Φ_{ST} values (0.018, 0.012, and 0.006 for COI, VD1, and COI&VD1 respectively) no significant genetic structuring was detected at any level within our study system. The pairwise Φ_{ST} test between 12 sampling sites for the three mtDNA data sets showed almost no significant differences between most of

Table 3 Me15/16 allele distribution, frequencies and expected and observed heterozygosity. *Me* Homozygous for the *M. edulis* allele; *eg* heterozygous for the *M. edulis/M. galloprovincialis* allele; *he* heterozygosity expected; *ho* heterozygosity observed; *P* probability of agreement with the Hardy-Weinberg model

	Number of individuals		Percent of total				
Site	Me	eg	Me [%]	eg [%]	he	ho	*P*
Hoo	40	0	1	0	0	0	
Whv	35	1	0.97	0.03	0.02778	0.02778	1
Sh	40	0	1	0	0	0	
Om	38	0	1	0	0	0	
NL	34	2	0.94	0.06	0.05556	0.05477	1
Dn	39	0	1	0	0	0	
KB	5	0	1	0	0	0	
LP	37	1	0.97	0.03	0.02632	0.02632	1
SP	39	1	0.97	0.03	0.025	0.025	1
LP2	38	0	1	0	0	0	
WK	31	0	1	0	0	0	
WK	37	0	1	0	0	0	
HL	12	0	1	0	0	0	
B1	38	0	1	0	0	0	
B2	36	0	1	0	0	0	
Total	499	5	0.99	0.01	0.00992	0.00988	1

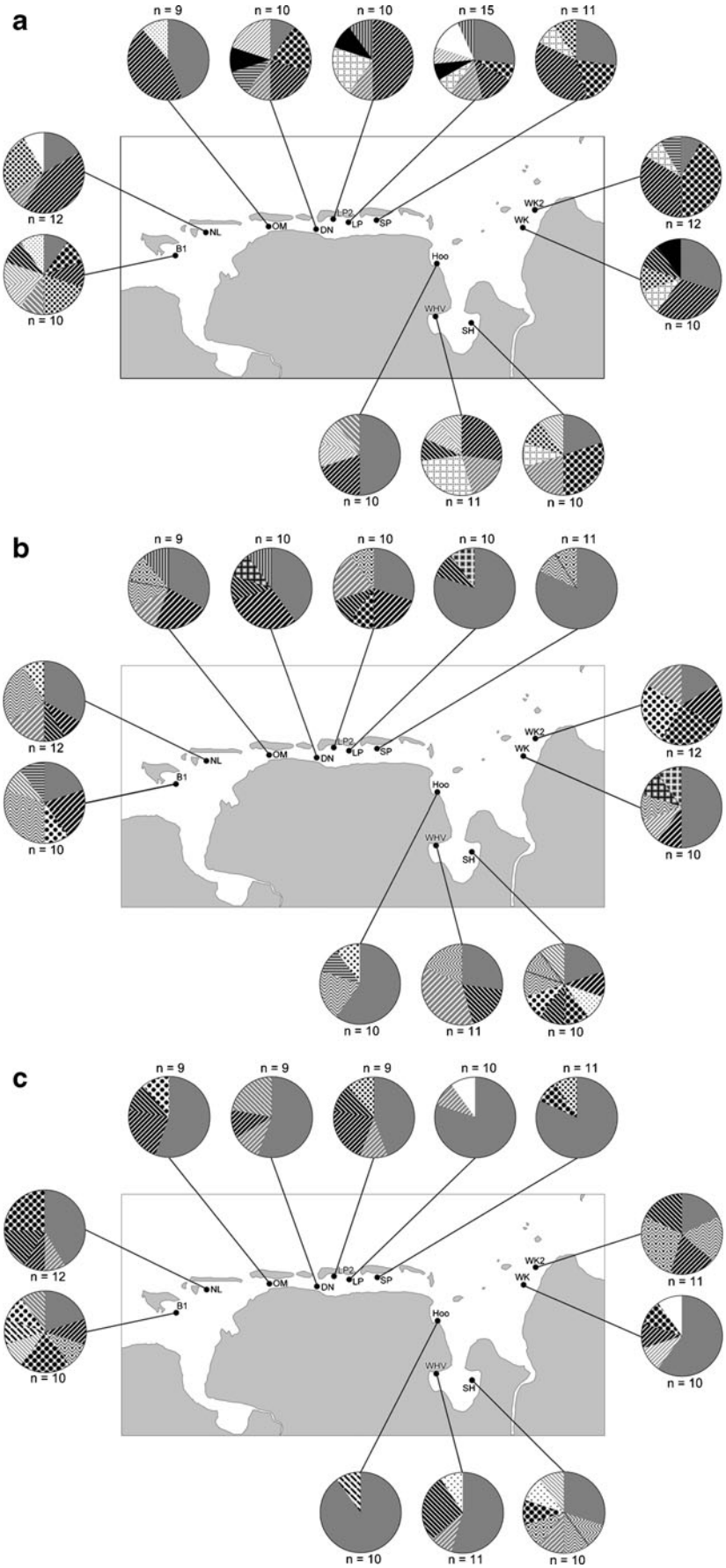

Fig. 2 Geographical distribution and abundance of the haplotypes for **a** COI (*n* haplotypes = 43; *n* sequences = 139), **b** VD1 (*n* haplotypes = 70; *n* sequences = 134), and **c** the combined data set (*n*=84 haplotypes; *n* sequences = 132). Unique haplotypes are pooled and indicated in *gray*; all other patterns represent haplotypes found in two or more individuals. The patterns for the latter match with those used for the corresponding minimum-spanning-networks (Fig. 3)

Table 4 Mitochondrial sequence and polymorphism information. *n* Number of total sequences, *nt* number of nucleotides with gaps, *vc* variable characters, *pi* parsimony informative sites, *h* number of haplotypes, *mh* number of haplotypes with n≥2 sequences, *sh* number of haplotypes with *n*=1 sequences, *hd* haplotype diversity, *gc* gc content

Fragment	Number of sequences	Aligned length	Number of variable characters	Number of parsimony informative characters	Number of distinct haplotypes	Number of shared haplotypes (n≥2)	Number of unique haplotypes	Haplotype diversity
COI	139	399	54	29	43	15	28	0.902
VD1	134	859	122	71	70	17	53	0.965
CO1 & VD1	132	1259	174	77	84	16	68	0.981

the sites (Supplementary Tables 3, 4 and 5), although significant differences between the sites WHV/WK2 and OM/WK2 appeared in each result. Exceptions were the additional significant differences between LP2/WK2 (COI) and WK/WK2 (VD1). The Mantel test showed no significant correlation (P=0.177) between genetic and geographic distances among populations; with low correlation (<0.19) and determination (<0.035%) values for each data set. The mismatch distribution among the 132 sequences is shown in Supplementary Fig. 1. The pairwise differences range from 0 to 37. Although the multimodal curve shape implies a population in equilibrium (Rogers and Harpending 1992), the mismatch distribution of mtDNA sequence differences among individuals did not differ from that expected under an expansion model. The result of the sum-of-squared-differences (SSD) statistic was 0.006 (P_{SSD} = 0.591).

Recombination analyses

No evidence for recombination was found in either of the primary single data sets (i.e., COI and VD1) using a step-down correction for significance (with a nominal alpha of 0.05). However, with no step-down correction, a potential recombination event was detected between the flanks of the sequence "80-4" (Langeooger Plate) and the sequence "55-3" (Nordland) for the VD1 data set. Similarly, the extension of the analyses to include the GenBank sequences also detected two potential recombination events (with no step-down correction) between each of the *M. edulis* sequence "48-3" (Nordland) for COI and the "80-4" sequence for VD1 with two GenBank *M. trossulus* sequences. The concatenated data set showed no evidence for recombination, either with step-down correction or without step-down correction.

Table 5 Hierarchical AMOVA for COI (n=130 individuals), VD1 (n=125 individuals) and the concatenated data set (n=123 individuals) for three groups (west, center, east). 1.023 permutations

Fragment	V_b(%)	Φ_{SC}	P-value	V_c(%)	Φ_{ST}	P-value
COI	1.55	0.015	0.212	98.83	0.018	0.222
VD1	1.36	0.014	0.233	98.81	0.012	0.225
COI&VD1	0.8	0.008	0.321	99.44	0.006	0.323

V variance components: V_b among populations within groups; V_c within populations; Φ_{SC}, Φ_{ST}, fixation indices

Haplotype networks

The COI haplotype network consisted of 15 haplotypes from a total of 111 individuals (Fig. 3a). The dominant structure in the network is a cluster comprising most haplotypes and centered on *hap4* (37 individuals). More distantly related haplotypes to this cluster are *hap29*, *hap15*, *hap3*, and potentially also *hap10* and *hap38*. Of these latter haplotypes, only *hap3* is represented by a large number of individuals (16). The VD1 haplotype network consisted of 17 haplotypes from 81 individuals (Fig. 3b), with *hap2* (15 individuals) presenting the central haplotype for the major cluster. Again, more distantly related haplotypes were apparent, including two minor clusters (*hap15* and *hap18* as well as that centered on *hap33*). Also conspicuous was a large reticulation in the network linking the major cluster with some of the individual haplotypes. Overall, genetic distances within the VD1 network were larger compared to those within the COI network. The combined COI and VD1 haplotype network consisted of 16 haplotypes from 64 individuals (Fig. 3c) and is again dominated by a large cluster (centered on *hap9*, which is represented by ten individuals), with numerous smaller clusters or individual haplotypes diverging from it. With one exception, all individuals within the minor cluster formed by *hap3*, *hap7* and *hap10* were distributed mainly within the eastern sampling locations (Fig. 2c). Otherwise, however, there was no apparent pattern between the geographical distribution of the individuals and the topologies of the haplotype networks. Finally, the overall haplotype network including singleton (84) sequences based on the concatenated haplotype data set showed the same domination by a large cluster (Supplementary Fig. 2). Furthermore, minor clusters were located within or at the end of branches. Multiple reticular connections between haplotypes and/or minor haplotype clusters and a more distinct star-shape clustering around the main haplotype characterized the main structure of

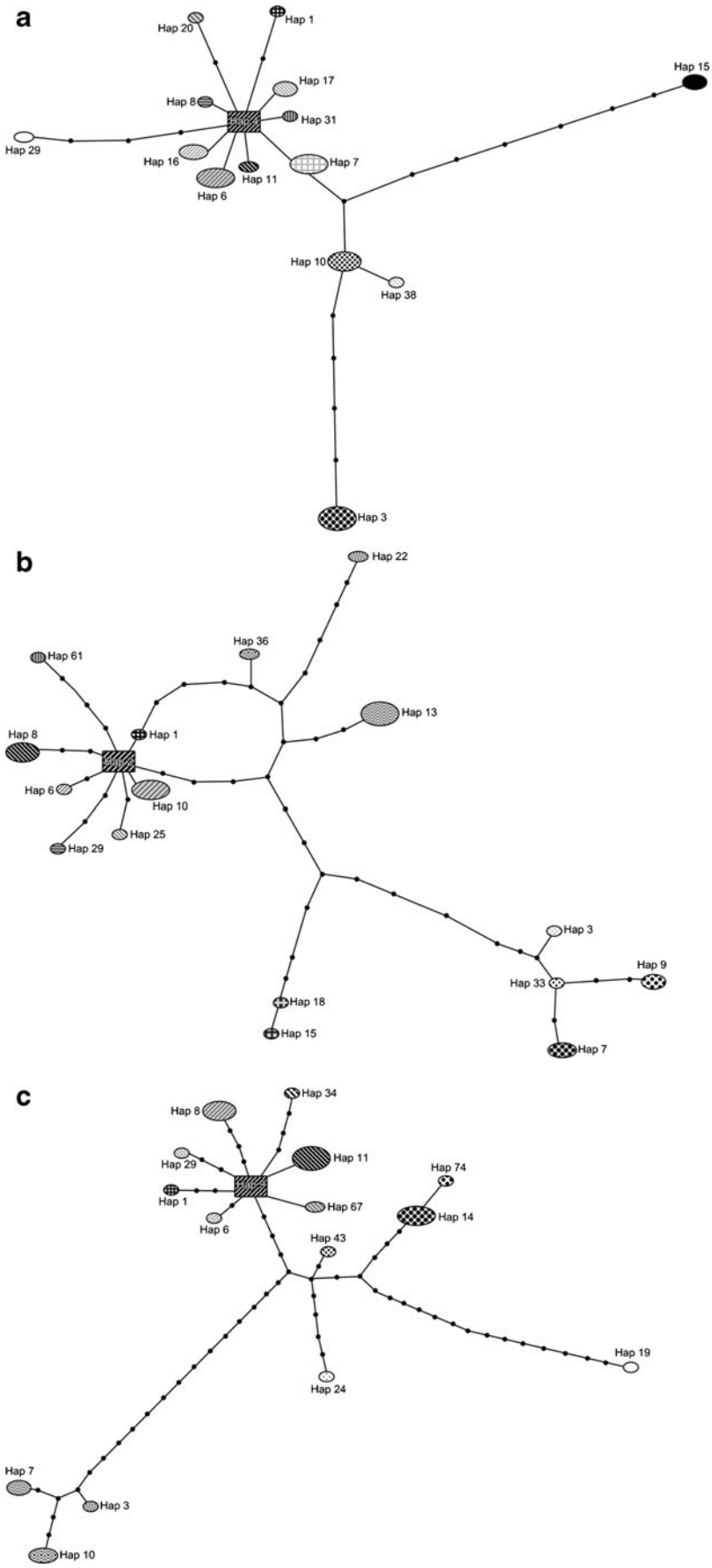

Fig. 3 Haplotype-networks for **a** COI (*n* haplotypes = 15; *n* sequences = 111), **b** VD1 (*n* haplotypes = 17; *n* sequences = 81), and **c** the combined data set (*n* haplotypes = 16; *n* sequences = 64). The sizes of the symbols are proportional to the number of individuals sharing that haplotypes (unique haplotypes are not included), with the rectangular haplotype having had the largest outgroup weight. Each node corresponds to one mutation step. The patterns used for the symbols match those used in the geographical distribution maps (Fig. 2)

this network. The long branches to haplotype 55 (KB) and haplotype 64 (LP) were also remarkable, and the large secondary cluster consisted of 13 haplotypes (11, 13, 15, 27, 39, 43, 46, 48, 56, 71, 76, 84) (Supplementary Fig. 2).

Haplotype phylogeny

The phylogenetic trees for both COI and VD1 each consisted of a main clade consisting largely of the Wadden Sea *M. edulis* sequences, but also some of the GenBank sequences (Fig. 4; Supplementary Table 1). For instance, all *M. galloprovincialis* COI haplotypes were located within the *M. edulis* clade, with several being identical to haplotypes within the clade (Supplementary Table 1). Similarly, haplotypes obtained from individuals of *M. trossulus* also clustered within the Wadden Sea *M. edulis* clade for both the COI (one haplotype) and VD1 (two haplotypes) trees. All remaining GenBank sequences, however, existed as single branches or clades outside of the *M. edulis* clade.

The *Mytilus* Wadden Sea clade was otherwise conspicuous by being subtended by an extremely long branch in both trees as well as enjoying strong bootstrap support. By contrast, branch lengths within the Wadden Sea clade were extremely short in general, with individual clades typically not showing strong bootstrap support. Similar to the haplotype network analyses, there was no clear association between the degree of phylogenetic relatedness among the haplotypes and their geographical distribution. The concatenated haplotype ML tree showed also a poorly supported topology, where the different branches and internal clades reveal no geographical distinction between the sampling sites in the western part and the eastern part of the Wadden Sea (Fig. 5). Thus the heterogeneous distribution of haplotypes within the sampling area was also present within this data set.

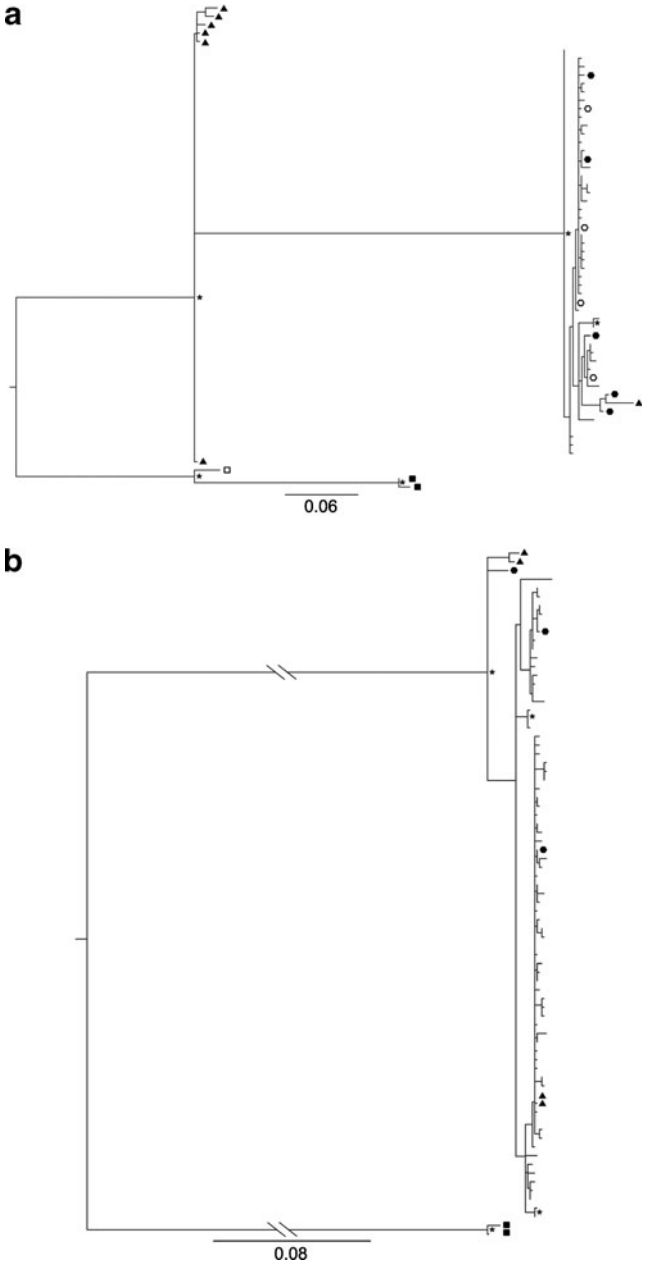

Fig. 4 Phylogenetic trees of **a** the COI and **b** VD1 haplotypes based on maximum likelihood estimates as constructed using RAxML. Symbols indicate either bootstrap values ≥95% (asterisks) or species affinity of the outgroup sequences obtained from GenBank: *triangle M. trossulus*, *filled hexagon M. galloprovincialis*, *empty hexagon M. galloprovincialis* sharing a haplotype with a Wadden Sea *M. edulis*, *filled square M. californianus*, *empty square M. coruscus*

Discussion

The complexity of mtDNA inheritance in *Mytilus* and the potential for hybridization within the *M. edulis* complex makes the taxonomic classification and phylogenetic reconstruction for this group extremely challenging, using either morphological or molecular data. Introgression and recombination, which are frequent and well studied events among sympatric *Mytilus* populations (e.g., Hilbish et al. 2002; Rawson 2005), can veil the genetic genealogy in phylogenetic analyses (e.g., due to a possible, but rarely, recombination of maternal and paternal inherited mitochondrial lineages). Furthermore, the morphological characters of the shell, even the diagnostic ones, can vary with different environmental factors and/or hybridization rates (Inoue et al. 1995; Beaumont et al. 2008).

In recent years, numerous genetic studies of *Mytilus* populations along the coasts of Western Europe and the North Sea have been performed using a large number of nuclear and mitochondrial markers (e.g., Coghlan and Gosling 2007; Stuckas et al. 2009; Kijewski et al. 2009). Our study contributes to the overall pattern by examining the genetic composition of the *Mytilus* population of the Wadden Sea for

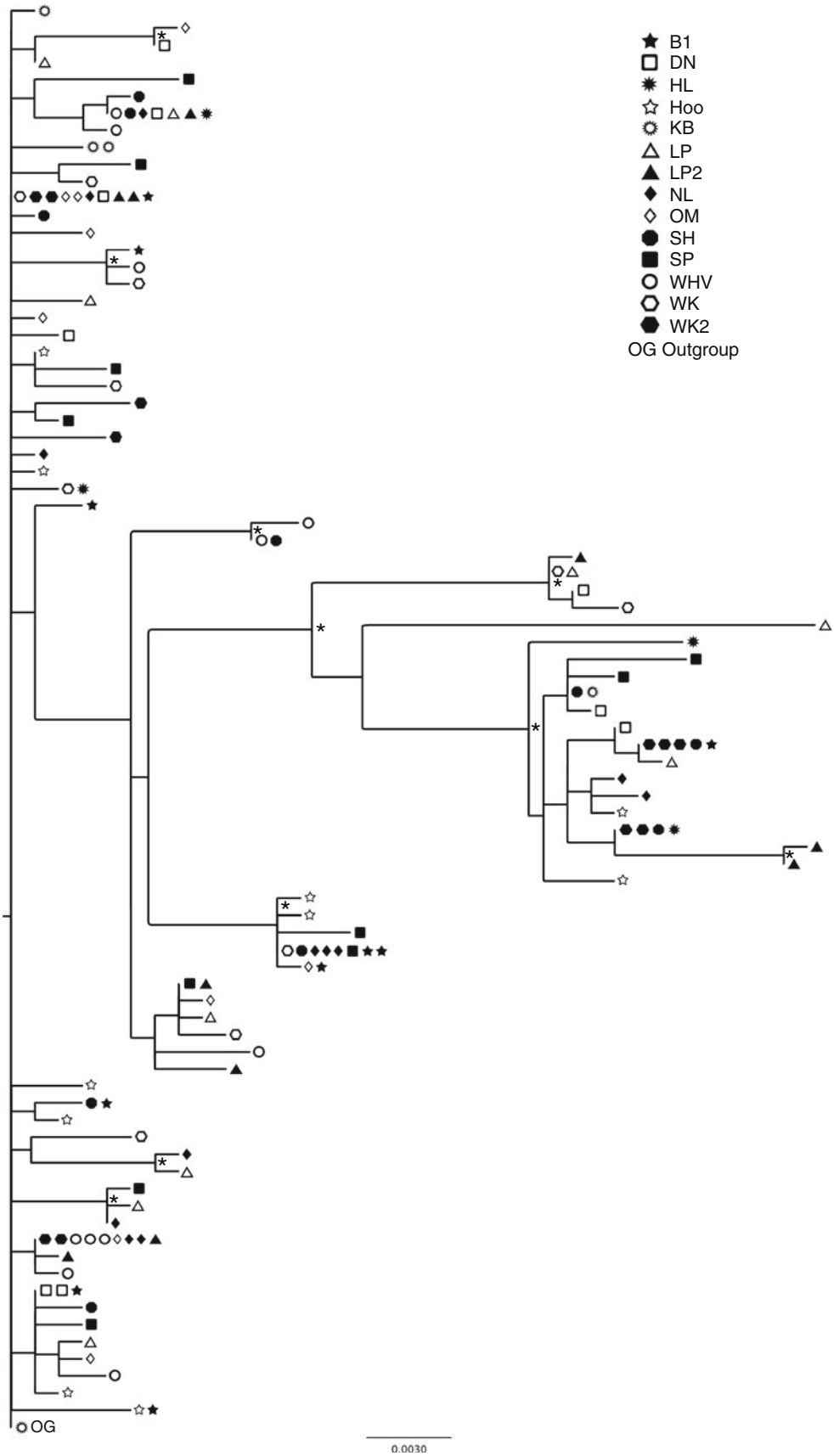

Fig. 5 Phylogenetic tree of the concatenated COI and VD1 haplotypes (n=84) based on maximum likelihood estimates as constructed using RAxML. Asterisks indicate bootstrap values ≥95%

the first time, both with respect to its species composition as well as evolutionary relationships within the species.

Taxonomic status of *Mytilus* spp. in the Wadden Sea of Germany

Analysis of the allele composition and frequency of the Me15/16 locus revealed a dominant *M. edulis*-type genotype frequency (0.9908), with an extremely minor contribution by *M. galloprovincialis*/*M. edulis* hybrid genotypes (0.0092). Otherwise, no other genotypes, including pure *M. galloprovincialis*, were found. Our observed genotype frequencies are almost identical to those measured by Luttikhuizen et al. (2002) (0.9907 and 0.0093, respectively) in their samples from the Dutch part of the Wadden Sea, which flanks our study area to the west. Similar values for Me15/16 derived genotypes were also obtained more recently by Kijewski et al. (2009) for the *Mytilus* population in Oosterschelde, the Netherlands (0.9915 Me and 0.0585 eg). Although Smietanka et al. (2004) did find genotypes that were homozygous or heterozygous for *M. trossulus* in Oosterschelde, this observation could not be replicated by Kijewski et al. (2009).

Drawing on recent studies of *Mytilus* hybrid zones in Western Europe, The North Sea, and The Baltic Sea (e.g., Smietanka et al. 2009; Väinölä and Strelkov 2011), several evolutionary and/or ecological explanations exist for observed dominance of the *M. edulis* allele, if not genotype, among adult individuals in the Lower Saxony Wadden Sea population. The question of genotypic distributions becomes especially interesting given the contradiction between the apparent highly invasive nature of the *M. galloprovincialis*, populations of which are to be found in England, and the virtual absence of the associated allele in the Wadden Sea.

In the first instance, the source of the *M. galloprovincialis* alleles could derive from (1) an introgression region formed by the hybrid zones between *M. edulis* and *M. galloprovincialis* (in parts also by *M. trossulus*) along the coasts of the British Isles and France and (2) the result of a recent invasion of *M. galloprovincialis* (i.e., their genetic traits) into the Wadden Sea area (Luttikhuizen et al. 2002). However, the potential for invasion of non-indigenous *Mytilus* individuals from hybrid zones or pure *M. galloprovincialis* populations through natural larval dispersal into the Wadden Sea area of Lower Saxony is small. Observed frequencies for *M. galloprovincialis* alleles in the immediate vicinity of the present research area are very low and coastal circulation patterns could provide strong gene-flow barriers for larval dispersal along the coast (Hilbish et al. 2002; Kijewski et al. 2009). Instead, anthropogenic influences like the translocation of mussel spat by bilge water or mussel-seed import represent a more likely source. Indeed, the import and export of blue mussel seed and the consumption of mussels in Europe is extensive (Wijsman and Smaal 2006) such that the import of seed mussels from *M. galloprovincialis* or hybrid populations cannot be ruled out (Nehring et al. 2009).

In the second instance, the virtual absence of *M. galloprovincialis* in the study area might derive from habitat specialization involving a post-settlement selection among genetically distinct, sympatric *Mytilus* populations. For instance, Gilg et al. (2009) provided evidence of selection against spat during the 1st year after settlement from an *M. edulis-galloprovincialis* hybrid zone in Cornwall (Great Britain) that settled at sites within a predominantly heterozygous *M. galloprovincialis* population. An analogous scenario cannot be ruled out here given that only mussels >1 cm in length were genotyped. Therefore, to test whether or not *M. galloprovincialis*-specific alleles are more abundant in juvenile blue mussels before being selected out, the Me15/16 allele composition should be recorded in the future for individuals <1 cm in length as well, if not for different size classes (which roughly represent age classes; Gardner and Skibinski 1991) in general. A similar sampling bias involves our focus on the intertidal zone, with genotype-specific habitat specialization (Bierne et al. 2002b) and associated reduced hybrid fitness and assortative fertilization (Bierne et al. 2002a) possibly also extending to the intertidal versus subtidal environments.

Nevertheless, as noted by Luttikhuizen et al. (2002), the extremely low frequency of the *M. galloprovincialis* allele means that homozygous *M. galloprovincialis* individuals are expected to be very rare under a scenario of random mating, especially if the effects of selection as described above are absent or negligible. Furthermore, Luttikhuizen et al. (2002) also genotyped samples from the subtidal zone and found no deviation in the total abundance of heterozygotes within this habitat. The general similarity in our results with those of Luttikhuizen et al. (2002), together with the very close geographic connection within the same ecosystem of the sampling areas means that our inference of a largely "pure" *M. edulis* population in the Wadden Sea of Lower Saxony is probably the most parsimonious explanation for the observed allele composition. Moreover, the very low frequencies of the *M. galloprovincialis* allele would indicate that the threat of an introduction of non-indigenous *Mytilus* populations at the genetic level, whether by larval dispersal and/or anthropogenic influences, is negligible (Kijewski et al. 2009).

Genetic structure among the *Mytilus edulis* complex

ML phylogenetic analysis with the additional GenBank sequences confirms previous observations of close mitochondrial relationships among members of the *M. edulis* complex (Rawson and Hilbish 1998; Burzynski et al. 2006; Smietanka et al. 2010), with several mtDNA haplotypes from individuals identified as *M. galloprovincialis* or

M. trossulus, often from highly distant geographical locations (e.g., Australia) nesting within the Wadden Sea *M. edulis* clade. Although erroneous taxonomic assignment cannot be ruled out, either for the GenBank sequences or our own, this potential source of error cannot explain the apparent close relationship between individuals obtained from opposite sides of the world.

For *M. trossulus*, the high degree of similarity *with M. edulis* can be explained in some instances by both the maternally and paternally inherited mitochondrial *M. trossulus* lineages having been almost completely replaced through introgression by maternally inherited *M. edulis* mtDNA (Quesada et al. 1999; Burzynski et al. 2006; Zbawicka et al. 2007; Stuckas et al. 2009). Introgression has in fact long been documented among European *Mytilus* populations (e.g., Rawson and Hilbish 1998; Bierne et al. 2002b). This mechanism would account for three of five *M. trossulus* individuals obtained from the Polish coast of the Baltic Sea nesting within the Wadden Sea clade. The two remaining Baltic Sea individuals possess highly recombinant control region haplotypes, which were described recently by Burzynski et al. (2006). Due to their high level of recombination, these two haplotypes are located outside of the Wadden Sea clade on their own small branch next to a *M. galloprovincialis* haplotype from Puget Sound (WA) obtained by Cao et al. (2004).

The COI haplotypes derived from individuals of *M. galloprovincialis* sampled from the Atlantic coasts, Australia and New Zealand nest within the Wadden Sea clade (i.e., individuals from Australia or New Zealand) or even share identical haplotypes with individuals out of our *M. edulis* sample (i.e., individuals from Atlantic and South Africa). Although well studied among representatives of the *M. edulis* complex (e.g., Rawson 2005; Riginos and Henzler 2008; Burzynski and Smietanka 2009; Ladoukakis et al. 2011), mitochondrial recombination between possible North Sea endemic *M. galloprovincialis* populations and Wadden Sea *M. edulis* populations can be excluded as an explanation for the observed high degree of sequence homology seen in the present study. The recombination detection test detected the possible effects of recombination for only two GenBank individuals, with these two sequences actually belonging to the comparative few located outside of the *M. edulis* clade.

Moreover, the grouping of GenBank *M. galloprovincialis* COI haplotypes within the present *M. edulis* clade supports the distinction between Northern and Southern Hemisphere haplotypes (Hilbish et al. 2000; Gerard et al. 2008; Westfall et al. 2010). Individuals from the Northern Hemisphere share haplotypes, whereas haplotypes from the Southern Hemisphere, albeit related strongly to *M. edulis* haplotypes from the Wadden Sea, are not shared. This geographically related level of haplotype divergence supports the predicted scenario of a transequatorial migration from the Northern to the Southern Hemisphere as discussed by Hilbish et al. (2000) and Gerard et al. (2008).

To this end, the high degree of sequence similarities and the phylogenetic positions of *M. edulis* from the North Sea and *M. galloprovincialis* individuals from the Northern and Southern Hemisphere (e.g., Australia) within the same clade also supports the report of a recent probable introduction of mussels from the Northern Hemisphere to Australia (Gerard et al. 2008). Regarding their low genetic divergence, the suggested anthropogenic explanation such as unintentional transport through bilge water seems plausible, especially over the geographical distances involved.

The phylogenetic ML topology of the concatenated data set of the mtDNA fragments also shows the same heterogeneous distribution of haplotypes in the Wadden Sea area as the two single data sets with the additional GenBank haplotypes. Due to the clonal inheritance of the mitochondrial genome this result was to be expected. Although, the mtDNA and Me1516 analysis are not necessarily reliable for an accurate distinction between the closely related and interbreeding members of the *Mytilus edulis* complex, the fact that the two unlinked loci (Me1516 and mtDNA) show the same pattern of *Mytilus* identity strengthens the overall assumption for the support of a single *Mytilus* species in the sampling area.

Population genetic structure

A reasonable expectation was that the *Mytilus* population in the Lower Saxon Wadden Sea would show a geographic structuring along a west–east gradient, possibly with the west serving as the center of dispersal. Such a hypothetical scenario agrees with both the prevalent water circulation dynamics in the tidal basins in the research area (Loewe et al. 2004; Staneva et al. 2009; Stanev et al. 2009) as well as a population dynamics model by Brandt et al. (2008) that indicates a west to east larval dispersal pattern for the *Crassostrea gigas* (Thunberg 1793) spat across the research area over the course of only 3 years.

However, a series of different analyses conducted in this present study, each of which makes slightly different assumptions and manipulations of the mtDNA sequence data, all reach the same conclusion of a single panmictic *M. edulis* population in the Wadden Sea of Lower Saxony. In all cases, there was a general lack of correspondence between the pattern of relationships among the sequence haplotypes (whether for COI or VD1 alone or combined) and their geographical distribution, with identical haplotypes being found across the research area and single sites often containing genetically diverse haplotypes. In addition, haplotype diversity was found to be very high (COI, 0.902; VD1, 0.965) with AMOVAs also indicating that by far the greatest degree of variation was to be found within each of

the sampling sites with no significant structuring above this level. The general result of a panmictic population is also reflected by the low bootstrap values within the Wadden Sea clade for the ML analyses and is comparable to those found by Kijewski et al. (2009) for the neighboring population of *M. edulis* in Oosterschelde in the Netherlands.

Moreover, the star-shaped structure of each haplotype network hints at a recent expansion from a small number of individuals, for instance after a founder event or a genetic bottleneck (Zachos 2009). The remarkable decline of the mussel bed stock in the research area after the ice winter 1995/1996 (Herlyn et al. 2008) represents a good candidate underlying such a scenario. Additionally, this scenario gains some support by the mismatch distribution analysis, which does not differ from the distribution expected under an expansion model. Natural shifts in dominant haplotype allele frequencies (e.g., through larval dispersal in combination with pre- and post larval viability) do not appear to be a major force driving the structure of the networks given that significant larval dispersal by mussel spat from non-indigenous genotypes would probably attenuate the distinct haplotype structures observed. On the other hand, the long branches (except the branch leading to haplotype 55, which is originated in the Baltic Sea) and the few minor clusters within the haplotype network based on the concatenated data set could be fragments generated by the migration of individuals from outside the sampling area (i.e., the Netherlands, Great Britain). In spite of that, regarding the overall results, a significant migration of larvae from the Netherlands seems unlikely.

On the same subject, the tidal currents in the backbarrier basins appear to be having a larger effect on larval dispersal than initially expected. The average length of stay of the planktonic Veliger-larvae within the water column before settlement of 22 days (Widdows 1991) in combination with a possible second planktonic phase (Gosling 1992) are two factors that could be promoting the panmictic population structure. Nevertheless our sampling protocol, in which mtDNA data from individuals from all adult size classes was pooled, may be obscuring any larval dispersal related population structure on a genetic level. If the larval dispersal of *M. edulis* does follow the west to east pattern modeled for the Pacific oyster (*C. gigas*) (Brandt et al. 2008), then it might be possible to observe the migration of a dominant western haplotype into the eastern parts of the Wadden Sea using size-structured data. Nevertheless, the complete lack of structuring indicated in all our analyses makes such a situation appear unlikely. However, considering the high haplotype diversity and the large number of singletons in the data set, there is still a general need for more samples regarding the results in parts of the present population genetics analyses (i.e., AMOVA, pairwise distribution, and Mantel test).

Conclusions

Our results revealed a mainly homozygous *M. edulis* population in the intertidal zone of the Wadden Sea of Lower Saxony. In addition, phylogenetic and population genetic analyses showed a heterogeneous, panmictic population in our study area due to apparent unrestricted gene flow. Together with similar findings for the neighboring Dutch Wadden Sea, we conclude that the Wadden Sea population is unaffected by the current range expansion of *M. galloprovincialis*—the latter being driven either naturally and/or through the activities of the mussel fishery industry. Nevertheless, a future introduction of nonindigenous individuals or genetic traits by larval dispersal or seed mussel import remains distinctly possible, although perhaps not an immediate threat (Kijewski et al. 2009). The impact of an unintentional introduction of a small number of nonindigenous individuals would be presumably attenuated by a range dispersal of these traits combined with their dilution over a wide geographic range by the prevailing tidal circulation system of the Wadden Sea system.

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