Population genetic connectivity of *Limecola balthica* between two locations in the Western Scheldt

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Contents

Summary

Two locations in the Western Scheldt were sampled for four age classes of the burrowing bivalve *Limecola balthica* (Linnaeus, 1758). The aim of the research was to determine whether sand nourishments and subsequent bivalve mortality may be expected to lead to extirpation or replenishment from nearby sources. The study locations were rich subtidal bivalve beds near 'De Kapellenbank' and 'De Suikerplaat'. The samples were examined for five genetic loci (microsatellites) and for the morphological character shell globosity. No genetic structure was observed, neither between the locations, nor among age groups or in some other, not previously defined way. Shell shape was found to show small statistical differences between locations. However, the distribution of the shape data was not uniform and therefore the biological relevance of these small potential differences cannot be stated. We conclude that genetic connectivity between the two locations is strong. This implies that, at evolutionary time scales, sufficient gene flow between the locations has occurred to maintain genetic and morphological similarity. The two locations may be connected by recruitment directly or indirectly. It is, however, possible that on ecological time scales gene flow is reduced or even absent ('Waples effect'). On the basis of these data there is no reason to assume that one location will not be recolonised from the other in case the population would be removed, e.g. because of dredging activities. The data cannot predict the time scale of recolonisation, which may theoretically be anything from years to millennia.

1. Introduction

The shipping lanes in the Western Scheldt are regularly dredged to ensure passage to the port of Antwerp. Dredged sediment in turn is used to create ecologically valuable sublitoral habitat in the Western Scheldt. To validate the assumption ecologically valuable habitat is indeed created, a biological monitoring campaign was designed to measure the effect of sediment nourishments on present and developing fauna.

During the first monitoring event (March 2017) on a sublitoral nourishment site ('de Suikerplaat'), a previously unrecognised shellfish bed of high densities of *Limecola balthica* (Linnaeus, 1758) (formerly *Macoma balthica*) on and in clay sediment was discovered. Because of this discovery the sediment nourishment on this site was relocated to avoid suffocation of this shellfish bed. During subsequent samplings, research on the contours and vitality of this shellfish bed was performed (Figure 1). Because of both this research and investigation of historic data, it was established that this bed is not temporary, but has probably been there since at least 2012; recruits in every year class are found and the bed is able to withstand winter storms.

The shellfish bed is habitat for many more species than locations outside the shellfish bed. It is therefore already ecologically valuable habitat in itself. The question arises whether nourishments from nearby sediment might do it harm. Harm could be caused by direct suffocation (which was avoided this time by relocation of the nourishment) or indirectly by deteriorating living conditions (reduced light and food, higher concentrations of indigestible dissolved particulate matter, etc.). If nourishment would indeed harm the shellfish bed, it could be that the harm is indefinite and irreversible, or the harm is temporary or marginal and the bed is able to replenish itself with recruits. If the shellfish bed were to be completely suffocated, the recolonization of the site would completely depend on recruitment from other shellfish beds. If the bed were to be covered locally, but not to the full extent, it could be able to self-recruit or use another shellfish bed to recruit.

During a monitoring event in 2016 another hotspot for *L. balthica* was discovered in the Western Scheldt on 'de Kapellenbank'. Although not as densely populated, this other shellfish bed could potentially replenish the bed on 'de Suikerplaat' after harm through coverage by nourishments. Whether replenishments from the other bed happened in the past, can be tested using genetic analysis.

This text reports on the genetic analysis of and genetic relationships between the two shellfish bed locations. In addition, shell shape is analysed because, as is known from the Wadden Sea and the adjacent North Sea coastal zone, the globosity of shells may differ between habitats and is likely a local adaptation in those areas (Luttikhuizen et al. 2003). From these analyses we formulate a policy advice on the potential of (partial) nourishment of these shellfish beds.

2. Materials and methods

2.1. Samples

Samples of *Limecola balthica* (Linnaeus, 1758) were taken from two subtidal locations, 'De Kapellenbank' (-11 m NAP) and 'De Suikerplaat' (-5 m NAP), in the Western Scheldt in March and October 2018 (Table 1). Age was determined by counting the number of growth rings on the shell. As the growing season of *L. balthica* is in spring and summer, samples taken in March are expected to have one ring fewer than those taken in October, given the same year of birth. Frequencies of years of birth were estimated for all samples and years of birth by taking random samples from size classes (Table 1); 2012-2015 were selected for genetic analysis. Per year per location, 20 individuals were analyzed except for Kapellenbank 2015, for which only 18 shells were available. Shell length, height and width were measured as in Luttikhuizen et al. (2003) to the nearest 0.01 mm with calipers (Supplement A).

2.2 Molecular procedures

From a piece of mantle tissue approximately 5 mm^3 in size, total genomic DNA was extracted using a CTAB (cetyltrimethylammonium bromide) protocol modified from (Hoarau et al. 2002). Before DNA extraction, as much ethanol as possible was removed from the sample by dabbing on a clean piece of paper tissue. The sample was then digested overnight in a 2.0 mL microcentrifuge tube at 60°C in 800 μL of CTAB buffer (100mM Tris HCl, 1.42 M NaCl, 20 mM EDTA, 2% CTAB) plus 20 μL proteinase K (20 mg/mL) and 2 μL of β-mercaptoethanol. Then 400 μL of chlorophorm/isoamyl alcohol (24:1) was added and mixed using a Bead Ruptor (Omni International) at 0.8 m/s for 10 min. After centrifuging at maximum speed for 10 min, 500 μL of the aqueous supernatent was transferred to a new 2.0 mL microcentrifuge tube and 400 μL of chlorophorm/isoamyl alcohol (24:1) was added. This was mixed using the Bead Ruptor at 0.8 m/s for 10 min and then centrifuged at maximum speed for 10 min. 400 μL of the aqueous supernatant was transferred to a new 1.5 mL Eppendorf tube and an equal volume of ice-cold isopropanol was added. This was mixed using the Bead Ruptor at 0.8 m/s for 5 min, incubated at -20°C for 45 min and centrifuged at maximum speed for 20 min at 4°C. The isoporanol was then poured off and the pellet washed with 80% cold ethanol. After centrifuging at maximum speed for 10 min at 4°C, the ethanol was poured off and the pellet washed with 500 μL of 70% cold ethanol. After centrifuging at maximum speed for 10 min at 4°C, the ethanol was poured off and the DNA pellet air-dried overnight at room temperature. The pellet was resuspended in 50 μL 10mM Tris buffer by letting it stand for 2 h at room temperature. The concentration and quality of the DNA extracts were measured on a Nanodrop (Thermo Fisher Scientific). DNA extracts were stored at 2-8°C for a few weeks and at -20°C long-term.

Five microsatellite loci were amplified from the DNA extracts: mbsat04, mbsat10, mbsat19, mbsat64 and mbsat84 (Becquet et al. 2009; Table 2). Each polymerase chain reaction (PCR) consisted of 2 μL 10X PCR buffer, 2 μM of each dNTP (2.5 μM), 0.4 μL bovine serum albumin (BSA), 0.2 μL forward primer (50 μM), 0.2 μL reverse primer (50 μM), 0.1 μL Biotherm+ DNA polymerase, 0.4 μL fluorescently labelled tail (50 μM, 5' end dye FAM or HEX with tail 5'-CACGACGTTGTAAAACGAC-3') and 1 μL 1:10 diluted DNA template in a final volume of 20 μL. PCR products were visualised on 2% TAE agarose gels. PCR reactions that failed to produce a visible band on the gel were repeated once

(N = 218). 1 μL of each succesful PCR products was mixed with 12 μL HiDi formamide and 0.4 μL Red 500 DNA size standard (Nimagen) and loaded onto 96-well sequencing plates. The plates were run on a capillary DNA sequencer (Applied Biosystems 3730 Genetic Analyzer) at Baseclear B.V. (Leiden, the Netherlands) for fragment analysis. Fragment lengths were scored from the electropherograms using the software Peak Scanner v1.0 (Applied Biosystems). Raw data are listed in Supplement A.

2.3 Data analysis

Frequency distributions of allele sizes were estimated and visualized using custom Python 3.7 code (Luttikhuizen 2019). Overall microsatellite variation was visualised in a principal coordinates analysis plot using GenAlEx 6.5 (Peakall & Smouse 2012). Descriptive genetic statistics were estimated in software package Arlequin version 3.5 (Excoffier & Lischer 2010). Analyses of MOlecular VAriance (AMOVA) were carried out, also in Arlequin, to test for differences among groups. This was done for one level of two groups (Kapellenbank versus Suikerplaat), one level of eight groups (four age groups for both locations) and in a two-level AMOVA (two locations with each four age groups nested).

To explore the possibility of group structure without a priori group definitions, model-based clustering was performed using the software Structure version 2.3.4 (Pritchard et al. 2000). Simulations were run with a burnin time of 10,000 and 100,000 MCMC replications for 1 to 8 groups (K).

Globosity of shells was compared between samples by taking the natural log of maximum shell length ('lnlen') and of shell width ('lnwid') and fitting a linear model to the lnwid data with origin as a categorical variable and lnlen as a covariate using the Python package Statsmodels version 0.9.0 (Statsmodels Development Team 2019). Globosity is defined as shell width relative to shell length (Luttikhuizen et al. 2003).

3. Results

During DNA extraction it was in many samples hard to get rid of all mucopolysaccharides present in the tissue, which is a well known issue in several marine organisms, including molluscs (Maeda et al. 2013, Jaksch et al. 2016). This led to difficulties with PCR amplification in some cases, also after repeated DNA extraction with a different piece of tissue. This problem was most prominant in Suikerplaat samples from 2013 and 2014. From the total of 158 bivalves selected for analysis, 144 were successfully genotyped for at least one microsatellite locus. The bivalves that failed to be sequenced are distributed randomly over all age groups and both locations, and therefore these missing data are not expected to influence the results.

3.1 Genetic variation

Allelic variation for the five microsatellite loci ranged from 7 alleles for mbsat10 to 22 alleles for mbsat19 (Table 2, Figure 2). Variability and allelic size ranges observed were similar to what was

originally reported by Becquet et al. (2009). Significant deviations from Hardy-Weinberg equilibrium in the form of shortages of heterozygotes were observed within both locations for all five loci (Table 2). The same was observed within year classes for both locations, with the exception of 2012, 2013 and 2014 at Kapellenbank for locus mbsat64, which displayed a heterozygote deficiency which was non-significant (Table 3).

3.2 Analysis of genetic structure

The principal coordinates analysis (PCoA) plot shows that the genetic variation found among the eight samples does not show a clear subgrouping, neither among the samples, nor between the locations, nor do the individuals form other clear groups (Figure 3). Similarly, the analyses of molecular variance (AMOVA) all show small and non-significant values for F_{ST}, F_{CT} or F_{SC}, the statistics for population structure (Table 4). No population subdivision is detected, when two locations are compared with age classes within locations are lumped (Table 4A: F_{ST} = 0.00214, n.s.), when the eight samples are compared among eachother (Table 4B: F_{ST} = -0.0011, n.s.), nor when two locations are compared with four nested age classes (Table 4C: between-group F_{CT} = 0.00278, n.s.; among samples within groups F_{SC} = -0.0027, n.s.)The large and significant inbreeding coefficients point once more to strong overall heterozygote deficits.

The simulations run with Structure suggest that no group structure is present in the data, as the posterior probability of the data given the model and value of K (number of groups assumed) is highest when one group is assumed (Table 5).

3.3 Analysis of shell shape

Statistical analysis of globosity as a measure of shell shape shows that log shell width is strongly correlated with log shell length (Table 6, Figure 4), as expected. Furthermore, marginally significant effects can be seen of origin (additive effect, Suikerplaat versus Kapellenbank, P = 0.048) and of the interaction between log shell length and origin (interaction effect, P = 0.045) (Table 6). It can, however, be seen (Figure 4) that the size distributions of shells in the samples are not equal; shells sampled at Kapellenbank were on average smaller. In addition, the relationship between log length and log width does not appear to be fully linear (Figure 4). The statistical differences estimated would mean that shells are more globose at Suikerplaat when they are smaller, while they would become more globose at Kapellenbank as they grow larger. Because of the different size distributions of the samples, the biological significance of this effect cannot be inferred without more data, especially because the statistical significance is only marginal.

4. Discussion

The data presented here suggest that there is no population subdivision present at the two locations studied in the Western Scheldt: Kapellenbank and Suikerplaat. There is no genetic difference between the locations nor among age groups. In addition, there is no population structure in a manner that is not related to either age or location. Also, the small statistical difference in shell

shape between the two locations is small and more likely to be the result of sampling effects than to have biological significance.

From the absence of genetic and clear morphological differences we can infer that genetic connectivity between the two locations is strong. This means that, at the scale of evolutionary time scales, sufficient gene flow between the locations (directly or indirectly) has occurred to maintain genetic and morphological similarity. The two locations may be directly connected in the sense that recruits originate from parents at the other location, or indirectly if recruitment of both locations is from a common source or via stepping-stones. While it is possible that on ecological time scales gene flow is reduced or even absent (Waples 1998), it is nevertheless likely that gene flow between these locations is ongoing. This means that, with regard to population genetics, there is no reason to assume that one location will not be recolonised from the other in case the population would be removed, e.g. because of dredging activities. The data can, however, not predict the time scale of recolonisation, which may theoretically be anything from years to millennia.

The variability in the microsatellite loci is high and displays a strong shortage of heterozygotes (Tables 2 and 3). These phenomena are both typical for marine molluscs and may be related to the presence of null alleles (Panova et al. 2008), which would not change the conclusion drawn of no population structure. Alternatively, population mixing might underlie the heterozygote deficit; if two or more non-panmictic populations are mixed into a sample, more heterozygotes would be seen than under random mating (the 'Wahlund effect'). A Wahlund effect is not likely in this case, for two reasons. First, the heterozygote deficit is present independent of the AMOVA design. If, for example, different age classes would constitute different populations, then F_{IS} as a measure of heterozygote deficit would differ between AMOVA designs, which it does not (Table 4). Second, if population structure were present in the data in some other, unknown, way, this would be apparent from the Structure analysis. The Structure analysis, however, indicates that the most likely number of populations present given the data is a single one (Table 5).

For the Wadden Sea and nearby North Sea region, it has been shown that *L. balthica* shells are more globose in the Wadden Sea (both subtidal and intertidal) than in the nearshore North Sea locations (only subtidal) where the species is also found (Luttikhuizen et al. 2003). The shells differ in globosity: the width of the shell relative to its length. North Sea shells are less globose than Wadden Sea shells. This difference has a genetic basis as demonstrated with a common garden experiment. The data presented here for two locations in the Western Scheldt, which differ in depth by 7 m but are both subtidal, do not show a clear globosity difference. The overall shell shape similarity thus adds to the inference from the microsatellite data that it seems likely that gene flow between the two locations is ongoing.

5. Acknowledgements

6. Literature

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7. Tables

Table 1 – Sampling scheme of *Limecola balthica* in the Western Scheldt. In bold are the samples used for genetic analyses. Age class = number of growing seasons based on number of growth rings seen on the shell; N_{est} = estimated total number in sample; N_{DNA} = number user for DNA extractions; N_{gen} = number genotyped for at least one locus; year = inferred year of birth.

Age class	Suikerplaat March 21 2018		Kapellenbank March 21 2018			Suikerplaat October 10 2018		
	N_{est}	year	N_{est}	$N_{DNA}(N_{gen})$	year	N_{est}	$N_{DNA}(N_{gen})$	year
1	6	2017	0			0		
2	10	2016	5		2016	18		2017
3	45	2015	**16**	**18(17)**	**2015**	29		2016
4	93	2014	**195**	**20(20)**	**2014**	**75**	**20(20)**	**2015**
5	74	2013	**284**	**20(19)**	**2013**	**737**	**20(15)**	**2014**
6	0		**96**	**20(20)**	**2012**	**275**	**20(15)**	**2013**
7	0		0			**32**	**20(18)**	**2012**

Table 2 – Microsatellite loci analysed for *Limecola balthica*. Each forward primer was preceeded at the 5' end by tail CACGACGTTGTAAAACGAC for fluorescent dye attachment. F = forward primer, R = reverse primer, N_{all} = total number of alleles observed; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{IS} = inbreeding coefficient; ** $p < 0.00001$; * $p < 0.05$.

				Kapellenbank				Suikerplaat			
	Primer sequences (5' to 3')	Allelic size range (bp)	N_{all}	H_o	H_e	F_{IS}	p	H_o	H_e	F_{IS}	p
mbsat04	F: CTCATATCTTCACCCTAGA R: CCATTTCCTGTCATTAGCA	410-452	21	0.41	0.91	0.55	**	0.31	0.9	0.66	**
mbsat10	F: GGGTGTTGATGGGATAATA R: TGGGGGCTACGAATAAGT	401-417	7	0.18	0.68	0.74	**	0.13	0.61	0.79	**
mbsat19	F: TCTTCTTTATGTAGCGTGTT R: CCAGGGCGAGTTTTTCTT	347-390	22	0.57	0.91	0.37	**	0.5	0.91	0.45	**
mbsat64	F: ATAATTTGTGGGGTTGAGGT R: GTTTCGAGTTTCGCAGTCA	183-216	9	0.33	0.43	0.23	*	0.22	0.45	0.51	**
mbsat84	F: TATATCCCTTGATCGGTTT R: ACGTATGTTTTTGTCCATGT	267-289	8	0.16	0.69	0.77	**	0.1	0.68	0.85	**

Table 3 - Genetic variation per sample for *Limecola balthica* from two locations in the Western Scheldt.
N_{all} = number of alleles observed; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{IS} = inbreeding coefficient; probability that $H_O < H_E$ ** $P < 0.00001$; * $P < 0.05$.

Location	Year of birth	Locus	N_{all}	H_O	H_E	F_{IS}	*P*
Kapellenbank	2012	mbsat04	11	0.40	0.90	0.56	**
		mbsat10	6	0.050	0.76	0.93	**
		mbsat19	15	0.40	0.92	0.57	**
		mbsat64	5	0.25	0.24	-0.042	n.s.
		mbsat84	4	0.050	0.69	0.93	**
	2013	mbsat04	11	0.37	0.85	0.56	**
		mbsat10	4	0.053	0.68	0.92	**
		mbsat19	11	0.58	0.87	0.33	**
		mbsat64	4	0.42	0.44	0.045	n.s.
		mbsat84	7	0.32	0.75	0.57	**
	2014	mbsat04	14	0.40	0.91	0.56	**
		mbsat10	6	0.30	0.65	0.54	**
		mbsat19	15	0.60	0.91	0.34	**
		mbsat64	8	0.45	0.51	0.12	n.s.
		mbsat84	3	0.15	0.68	0.78	**
	2015	mbsat04	11	0.47	0.91	0.48	**
		mbsat10	5	0.35	0.61	0.43	*
		mbsat19	14	0.71	0.94	0.24	*
		mbsat64	5	0.18	0.50	0.64	**
		mbsat84	3	0.12	0.68	0.82	**
Suikerplaat	2012	mbsat04	11	0.22	0.87	0.75	**
		mbsat10	4	0.17	0.66	0.74	**
		mbsat19	15	0.44	0.91	0.52	**
		mbsat64	4	0.22	0.46	0.52	*
		mbsat84	5	0.11	0.72	0.85	**
	2013	mbsat04	11	0.33	0.89	0.63	**
		mbsat10	4	0.067	0.64	0.90	**
		mbsat19	10	0.40	0.86	0.53	**
		mbsat64	5	0.20	0.40	0.50	*
		mbsat84	3	0.067	0.51	0.87	**
	2014	mbsat04	9	0.40	0.85	0.53	**
		mbsat10	4	0.13	0.58	0.78	**
		mbsat19	12	0.40	0.91	0.56	**
		mbsat64	5	0.27	0.45	0.40	*
		mbsat84	4	0.13	0.68	0.81	**
	2015	mbsat04	12	0.30	0.91	0.67	**
		mbsat10	5	0.15	0.59	0.75	**
		mbsat19	15	0.70	0.94	0.26	**
		mbsat64	6	0.20	0.49	0.59	**
		mbsat84	3	0.10	0.60	0.83	**

Table 4 – Analyses of molecular variance for five microsatellite loci in *Limecola balthica* from two locations in the Western Scheldt in four year classes. Population subdivision as estimated with F_{ST} is not significant in any of the models.

A: One-level AMOVA, Kapellenbank versus Suikerplaat					
Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation index
Among samples	1	3.42	0.00386	0.21	**F_{ST} = 0.00214 (n.s.)**
Among individuals within samples	142	407.35	1.068	59.19	F_{IS} = 0.593 (p < 0.05)
Within individuals	144	105.5	0.7326	40.6	
Total	*287*	*516.27*	*1.805*		
B: One-level AMOVA, four year classes at two locations					
Source of variation	df	Sum of squares	Variance components	Percentage of variation	
Among samples	7	19.64	-0.00198	-0.11	**F_{ST} = -0.0011 (n.s.)**
Among individuals within samples	136	391.14	1.07169	59.46	F_{IS} = 0.594 (p < 0.05)
Within individuals	144	105.5	0.73264	40.65	
Total	*287*	*516.27*	*1.80234*		
C: Two-level AMOVA, Kapellenbank versus Suikerplaat with four year classes					
Source of variation	df	Sum of squares	Variance components	Percentage of variation	
Among groups	1	3.423	0.00504	0.28	**F_{CT} = 0.00278 (n.s.)**
Among samples within groups	6	16.21	-0.00486	-0.27	F_{SC} = -0.0027 (n.s.)
Among individuals within samples	136	391.14	1.072	59.39	F_{IS} = 0.594 (p < 0.05)
Within individuals	144	105.5	0.733	40.6	
Total	*287*	*516.27*	*1.805*		

Table 5 - Posterior probabilities (ln Pr(data|K)) of number of groups K according to simulations run with Structure version 2.3.4.

K	ln Pr(data|K)
1	-1900.0
2	-1962.6
3	-1989.3
4	-1951.2
5	-1985.5
6	-1975.4
7	-1956.3
8	-2063.1

Table 6 – Linear model of shell shape of *Limecola balthica* on Suikerplaat versus Kapellenbank as estimated using statsmodels version 0.9.0 in Python version 3. Dependent variable: lnwid (natural log of shell width), $r^2 = 0.769$; N = 158, $df_{model} = 3$, $df_{residuals} = 154$.

	Coefficient	Standard error	*P*
Intercept	-1.23	0.195	0.000
Origin	0.59	0.296	0.048
Lnlen	1.14	0.067	0.000
Lnlen * Origin	-0.20	0.101	0.045

8. Figures

Figure 1 - Results of sampling on the 7th and 8th of February 2018 by Rijkswaterstaat on the map of water surrounding 'de Suikerplaat' (dark blue). A total of 29 boxcore samples were taken. Size of the green circle indicates the number of individuals found.
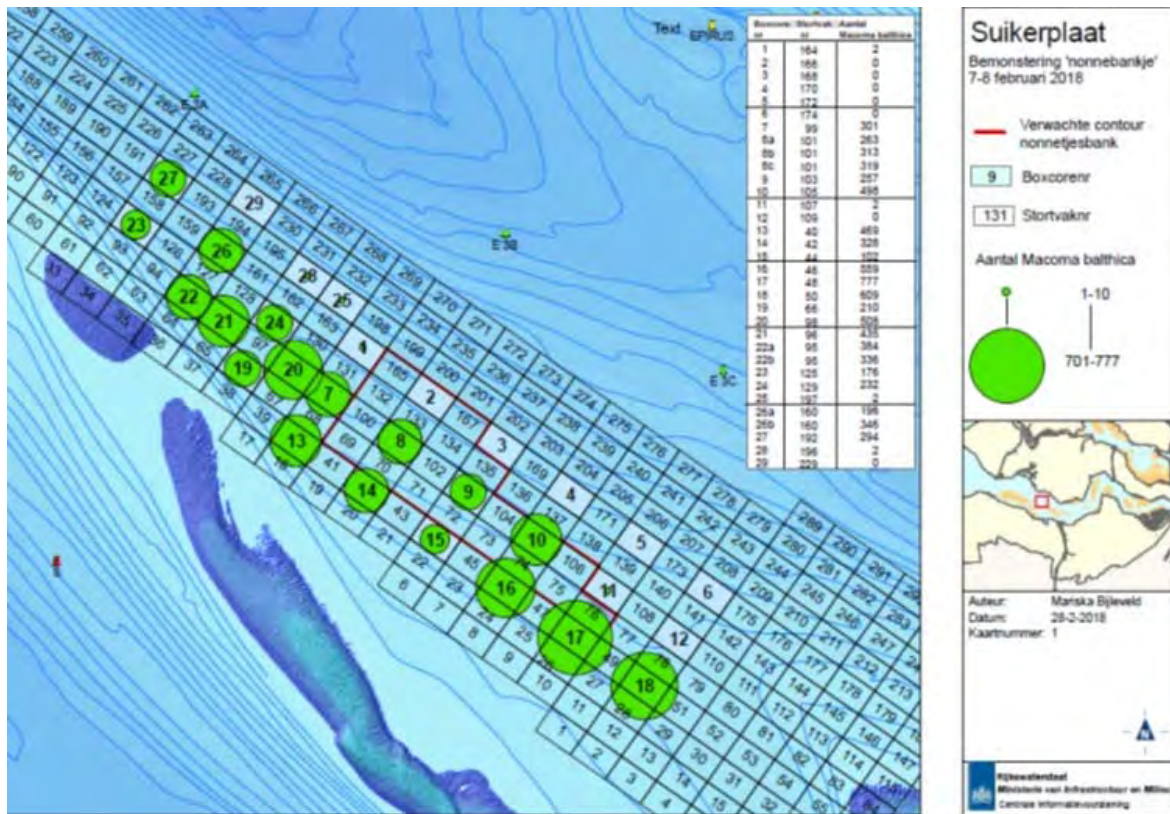
Figure 2 – Allelic variation for five microsatellite loci in *Limecola balthica* from two locations in the Western Scheldt.
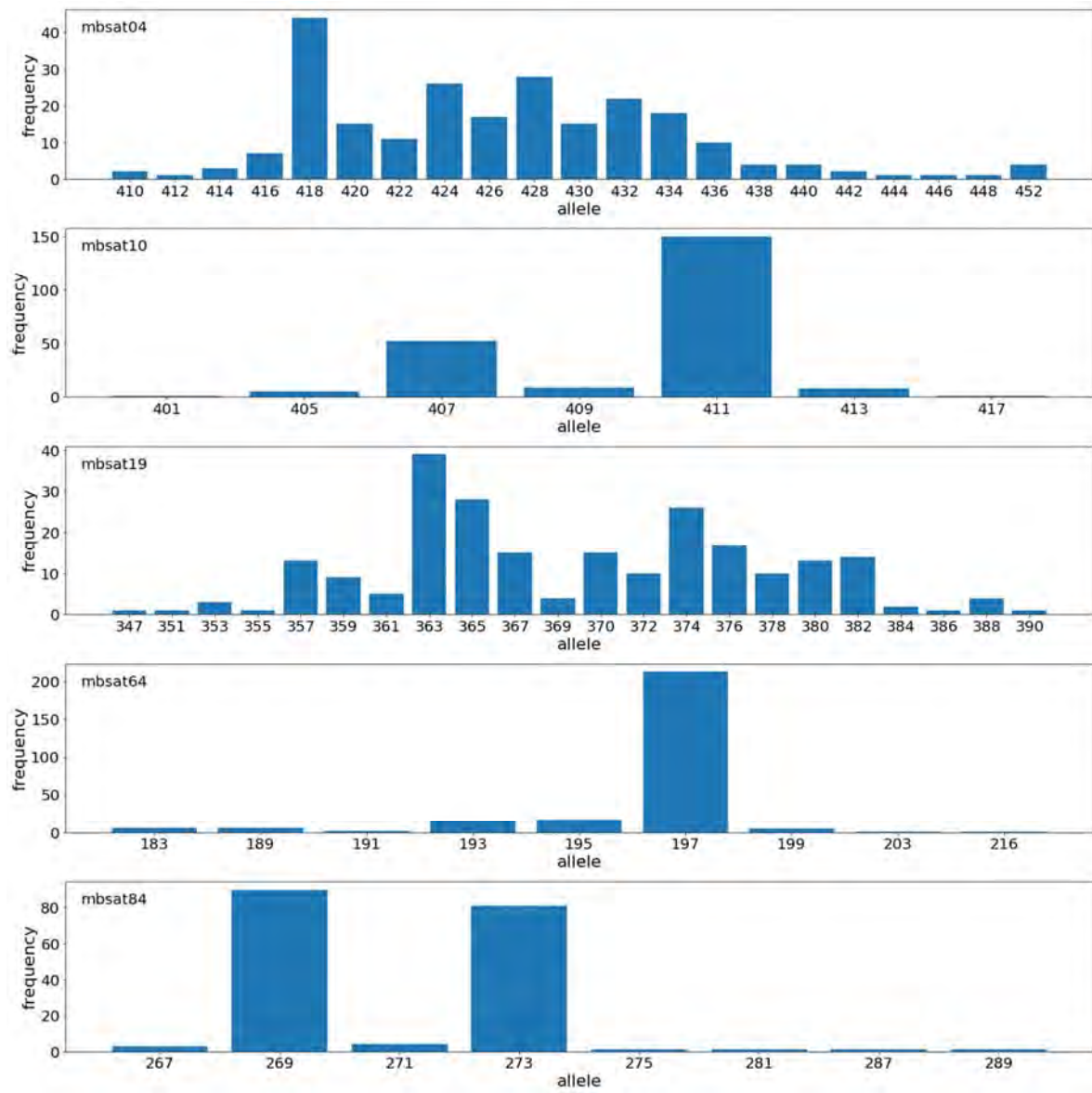
Figure 3 - Principal Coordinates Analysis (PCoA) showing variability among eight samples of *L. balthica* in the Western Scheldt genotyped for five microsatellite loci. K3 - K6: four year classes from Kapellenbank; S3 - S6: four year classes from Suikerplaat.
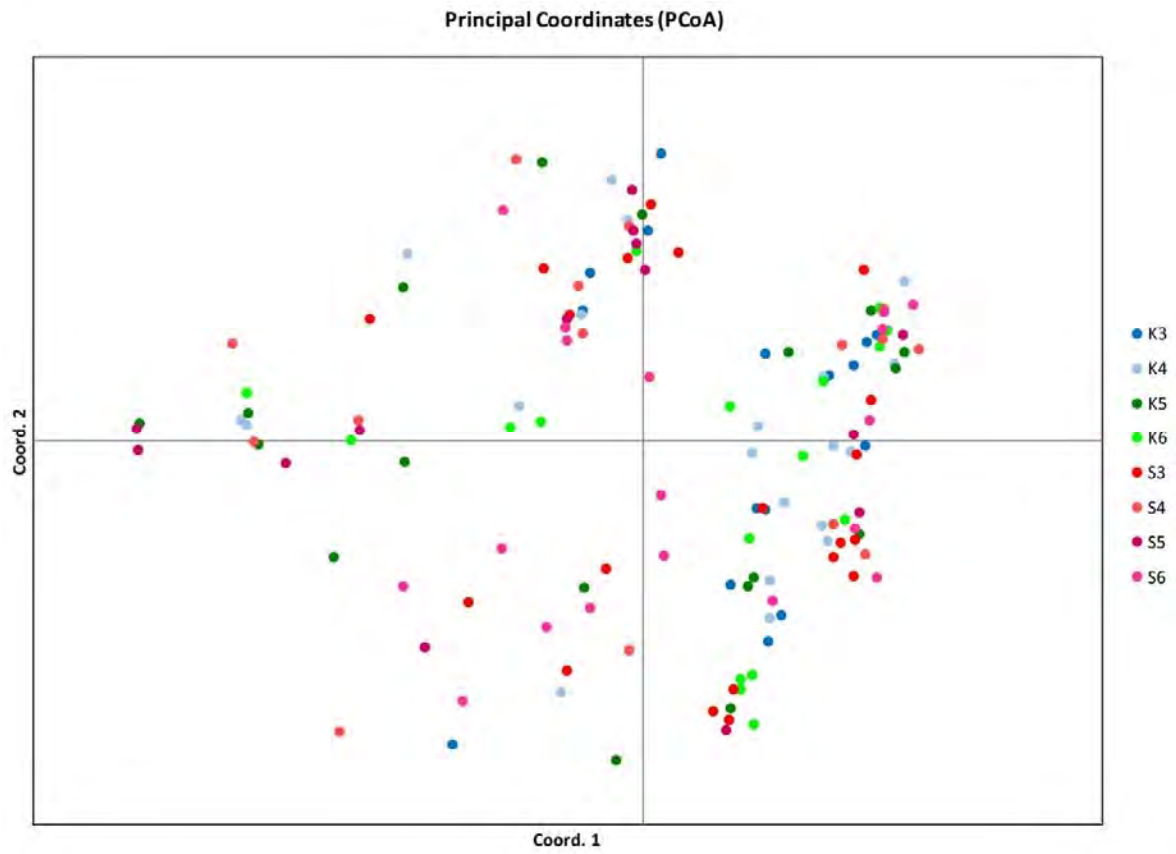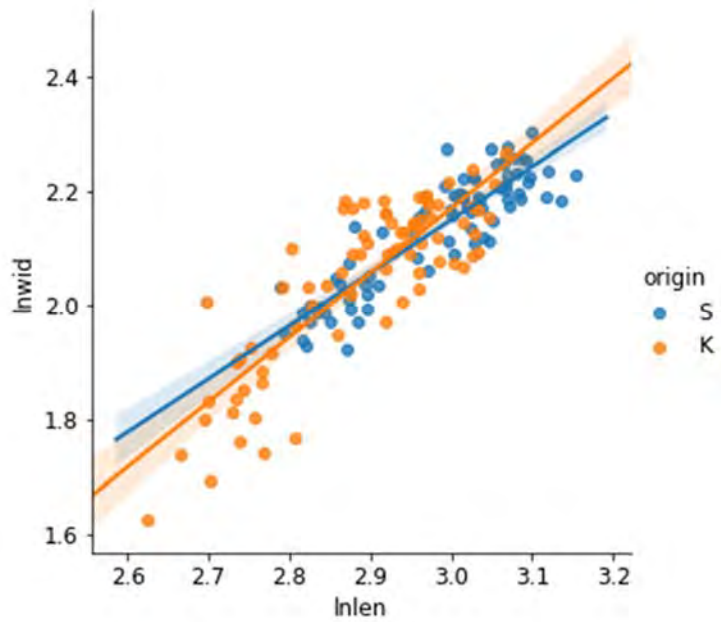
Figure 4 – Plot of *Limecola balthica* shell shape at Suikerplaat (S) and Kapellenbank (K) with fitted linear regression lines per sample. 'Lnwid' = natural log of shell width in mm; 'lnlen' = natural log of maximum shell length in mm.

9. Supplement A: raw data

Raw data for the project are listed here; 'individual' is the code for the individual *Limecola balthica* bivalve, where 'K' stands for Kapellenbank and 'S' for Suikerplaat, the first digit following indicates the number of years since fertilization, and the last two digits consecutively number the individuals within samples; length, height and width (mm) of the shells were measured as in (Luttikhuizen et al. 2003); columns 5-14 give (PCR product) sizes for both alleles of each of five microsatellite loci.

individual	length	height	width	mbsat04		mbsat10		mbsat19		mbsat64		mbsat84	
K301	13.79	10.51	5.08	434	448	407	411	363	370	197	197	269	269
K302	14.36	11.5	5.68	418	418	411	411	359	359	195	195	0	0
K303	15.41	11.59	6.27	#N/A	#N/A	#N/A	#N/A	0	0	0	0	#N/A	#N/A
K304	16.29	12.04	7.64	0	0	0	0	372	372	197	197	269	269
K305	15.88	12.88	6.58	418	418	409	411	365	376	197	199	273	273
K306	16.07	12.39	6.8	424	430	411	411	363	378	197	197	269	273
K307	16.57	12.15	7.14	#N/A	#N/A	411	411	374	374	197	197	269	269
K308	15.89	12.21	6.45	424	440	411	411	#N/A	#N/A	197	197	273	273
K309	15.68	12.18	6.87	430	430	407	411	355	374	195	195	273	273
K310	15.93	12.32	5.71	418	424	411	411	363	372	191	197	#N/A	#N/A
K311	15.76	11.95	6.07	418	428	407	411	367	378	193	197	#N/A	#N/A
K312	15.53	11.95	6.37	418	440	407	407	372	376	197	197	273	273
K313	15.48	11.69	5.81	426	426	411	411	359	367	197	197	273	273
K314	17.47	12.69	7.01	436	440	405	405	365	380	195	195	269	273
K315	16.89	13.09	7.39	434	434	407	411	367	372	195	195	#N/A	#N/A
K316	17.22	13.05	7.65	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	197	197	#N/A	#N/A
K317	16.82	12.78	7.64	422	436	411	411	361	376	197	197	273	273
K318	16.56	12.98	5.86	428	428	407	411	353	370	197	197	269	269

K401	14.9	11.21	5.44	418	418	407	407	367	374	197	197	273	273
K402	14.85	11.62	7.43	440	442	411	411	363	376	#N/A	#N/A	#N/A	#N/A
K403	14.8	11.28	6.05	452	452	409	409	351	353	189	197	273	273
K404	15.46	12.42	6.72	0	0	411	411	#N/A	#N/A	195	197	#N/A	#N/A
K405	15.32	11.69	6.12	424	424	411	411	#N/A	#N/A	195	197	269	273
K406	15.4	12.01	6.69	420	424	411	413	363	363	197	197	0	0
K407	18.67	14.02	8.12	414	414	411	411	357	378	197	197	269	269
K408	18.53	14.09	7.19	420	420	407	411	361	388	197	197	269	269
K409	17.53	13.39	7.83	418	436	407	411	365	380	197	197	269	273
K410	17.74	13.33	7.53	416	416	411	417	372	376	193	197	269	269
K411	18	13.54	8.36	428	428	409	411	363	374	183	195	273	273
K412	18.51	13.77	7.88	428	432	#N/A	#N/A	#N/A	#N/A	197	197	#N/A	#N/A
K413	19.29	14.12	7.84	428	428	#N/A	#N/A	370	373	197	199	269	269
K414	19.28	13.85	8.46	424	424	411	411	367	367	197	197	273	273
K415	18.57	14.22	8.1	428	428	411	411	363	363	197	197	269	273
K416	19.33	14.49	8.25	418	428	407	407	361	380	189	197	0	0
K417	18.91	14.06	7.43	416	430	407	411	365	380	197	197	273	273
K418	19.31	14.56	7.62	416	422	#N/A	#N/A	#N/A	#N/A	197	197	0	0
K419	20.6	14.93	8.06	420	452	411	411	357	376	197	203	#N/A	#N/A
K420	20.75	15.49	8.12	418	418	411	411	363	363	195	197	273	273
K501	14.87	11.33	6.25	426	428	#N/A	#N/A	372	382	193	197	269	273
K502	20.14	14.69	7.96	418	418	411	411	0	0	195	195	#N/A	#N/A
K503	18.03	14.19	8.87	#N/A	#N/A	#N/A	#N/A	0	0	0	0	#N/A	#N/A
K504	16.49	12.85	8.16	#N/A	#N/A	#N/A	#N/A	363	363	197	197	#N/A	#N/A
K505	17.58	13.93	8.77	422	422	411	411	370	370	193	197	273	273
K506	18.1	14.1	8.24	424	424	411	411	374	376	197	197	273	273
K507	18.63	14.35	8.54	420	434	411	411	359	370	183	197	0	0

K508	17.92	13.36	8.09	420	436	#N/A	#N/A	0	0	197	197	#N/A	#N/A
K509	17.77	13.53	8.08	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	193	197	#N/A	#N/A
K510	18.5	13.83	8.68	426	434	407	407	374	376	193	197	269	273
K511	19.74	14.84	8.33	426	434	409	411	363	363	193	197	267	271
K512	17.62	13.49	8.89	426	426	411	411	370	374	193	197	275	287
K513	19.73	14.16	8.82	#N/A	#N/A	407	407	365	390	197	197	269	269
K514	19.06	14.37	8.08	#N/A	#N/A	407	407	364	370	197	197	#N/A	#N/A
K515	18.53	13.94	8.69	418	430	407	407	367	374	197	197	269	269
K516	20.36	14.54	7.91	434	434	411	411	363	365	197	197	269	269
K517	20.66	14.82	8.38	434	434	411	411	365	374	197	197	273	273
K518	19.17	14.45	8.52	#N/A	#N/A	411	411	#N/A	#N/A	197	197	#N/A	#N/A
K519	19.18	13.9	8.55	418	432	407	407	363	382	183	197	269	273
K520	19.33	14.47	8.59	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	197	197	267	273
K601	18.96	14.21	8.41	418	430	411	411	367	382	197	197	273	273
K602	19.45	14.99	8.97	#N/A	#N/A	#N/A	#N/A	0	0	197	197	#N/A	#N/A
K603	18.46	14.03	8.88	430	430	#N/A	#N/A	#N/A	#N/A	191	197	#N/A	#N/A
K604	19.47	15.01	8.91	424	442	409	409	365	365	183	197	269	269
K605	19.31	15.13	8.93	428	428	#N/A	#N/A	388	388	197	197	#N/A	#N/A
K606	20.63	15.62	9.4	430	430	411	411	369	380	197	197	273	273
K607	17.76	13.75	8.77	426	426	411	411	364	364	197	197	269	281
K608	18.87	13.88	8.4	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	197	197	#N/A	#N/A
K609	19.99	14.91	9.17	420	438	407	407	361	367	197	197	0	0
K610	21.17	15.88	9.16	424	424	405	405	365	378	197	197	269	269
K611	18.97	13.9	8.21	424	430	407	407	365	365	183	197	269	269
K612	21.5	15.88	9.65	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	197	197	#N/A	#N/A
K613	19.55	14.58	8.62	420	420	411	411	370	378	189	197	273	273
K614	20.75	15.22	8.75	418	451	411	411	363	363	197	197	269	269

K615	18.76	13.74	8.16	418	418	407	407	374	374	197	197	0	0
K616	20.4	15.06	8.55	418	428	409	409	365	374	197	197	269	269
K617	19.52	15.53	8.82	424	436	411	413	359	376	193	197	273	273
K618	21.02	15.56	8.63	418	436	411	411	382	382	197	197	273	273
K619	19.79	14.34	7.99	428	428	407	407	357	357	197	197	273	273
K620	20.62	15.31	8.79	420	420	411	411	374	382	197	197	#N/A	#N/A
S301	16.27	12.81	7.63	436	436	407	407	376	382	197	197	269	269
S302	16.85	12.65	7.4	426	426	407	407	359	380	197	197	273	273
S303	16.32	12.86	7.04	430	430	405	411	360	363	197	197	#N/A	#N/A
S304	17.03	12.98	7.29	#N/A	#N/A	411	411	0	0	197	197	269	269
S305	16.77	13.15	6.88	416	416	411	411	372	374	197	197	269	269
S306	17.18	13.91	7.3	432	432	411	411	353	374	197	197	269	269
S307	17.42	14.15	7.75	436	436	411	411	357	357	197	197	0	0
S308	16.86	14.01	7.17	424	424	407	407	369	369	189	197	269	269
S309	16.72	13.25	6.94	418	424	407	413	374	382	197	197	269	269
S310	17.64	14.04	6.83	418	418	411	411	365	365	197	197	0	0
S311	16.73	13.37	7.3	422	422	407	411	363	382	#N/A	#N/A	#N/A	#N/A
S312	17.11	13.4	7.37	416	418	411	411	365	384	197	197	269	269
S313	18.06	13.75	7.65	414	426	#N/A	#N/A	#N/A	#N/A	197	197	269	273
S314	17.75	14.31	7.35	428	428	411	411	365	365	197	197	269	269
S315	18.09	14.45	7.54	418	418	#N/A	#N/A	363	378	#N/A	#N/A	#N/A	#N/A
S316	17.68	14	7.47	432	432	411	411	363	381	0	0	273	273
S317	17.68	13.36	7.58	418	434	411	411	370	376	193	197	#N/A	#N/A
S318	18.1	14.19	7.34	434	434	411	411	363	370	195	195	269	269
S319	18.33	13.94	7.65	418	432	411	411	357	380	197	199	269	273
S320	18.13	13.97	7.78	418	422	#N/A	#N/A	357	372	189	197	269	269
S401	17.31	13.12	7.19	432	432	411	413	367	367	193	197	#N/A	#N/A

S402	18.44	14.46	8.4	426	432	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
S403	17.5	14.03	7.68	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
S404	17.88	13.37	7.18	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	197	197	269	269
S405	17.8	14.04	8.48	412	418	411	411	370	380	197	197	273	289
S406	17.71	13.62	7.96	428	428	411	411	374	376	195	197	269	269
S407	19.18	14.41	8.37	432	432	411	411	365	365	197	197	269	269
S408	19.23	14.76	8.04	418	434	411	411	365	365	197	197	273	273
S409	19.3	14.45	8.64	432	432	411	411	363	377	197	197	273	273
S410	19.41	15.09	8.71	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
S411	19.44	14.67	8.95	#N/A	#N/A	407	407	367	367	197	197	269	273
S412	19.93	15.42	9.11	422	428	#N/A	#N/A	363	363	197	197	#N/A	#N/A
S413	20.13	15.46	8.97	410	410	411	411	380	384	197	199	#N/A	#N/A
S414	20.1	15.43	8.67	432	434	407	411	380	382	197	197	0	0
S415	20.16	15.63	8.1	432	434	411	411	347	376	197	199	273	273
S416	19.51	14.4	7.85	#N/A	#N/A	#N/A	#N/A	376	376	197	197	#N/A	#N/A
S417	20.4	16.38	9.23	418	418	411	411	0	0	0	0	#N/A	#N/A
S418	20.84	15.42	8.77	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
S419	21.25	16.3	9.48	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	0	0	#N/A	#N/A
S420	20.67	15.68	8.26	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	0	0	0	0
S501	22.56	17.13	8.94	418	446	411	411	365	370	183	197	269	269
S502	22.61	17.43	9.36	#N/A	#N/A	407	407	0	0	197	197	#N/A	#N/A
S503	22.99	17.3	8.89	428	428	#N/A	#N/A	0	0	0	0	269	269
S504	21.03	15.74	8.27	#N/A	#N/A	#N/A	#N/A	0	0	0	0	#N/A	#N/A
S505	20.27	14.28	9.01	#N/A	#N/A	#N/A	#N/A	0	0	0	0	#N/A	#N/A
S506	19.97	15.49	9.73	422	422	#N/A	#N/A	374	374	197	197	#N/A	#N/A
S507	19.99	14.97	8.27	#N/A	#N/A	#N/A	#N/A	0	0	0	0	#N/A	#N/A
S508	20.05	15.15	8.73	432	426	411	411	365	380	0	0	0	0

S509	20.63	15.66	9.26	#N/A	#N/A	#N/A	#N/A	0	0	189	197	#N/A	#N/A
S510	20.24	15.46	8.95	426	426	411	411	370	370	197	197	#N/A	#N/A
S511	20.39	14.9	8.94	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	197	197	#N/A	#N/A
S512	22.18	16.11	10.02	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	0	0	#N/A	#N/A
S513	21.84	16.34	9.01	424	444	407	407	363	374	197	197	269	269
S514	21.44	15.91	9.47	#N/A	#N/A	#N/A	#N/A	0	0	197	197	#N/A	#N/A
S515	22.11	16.52	9.27	418	418	411	411	374	374	197	197	273	273
S516	21.59	16.05	8.79	438	438	411	411	359	380	197	197	269	273
S517	21.53	16.38	9.3	428	434	411	411	357	378	193	197	0	0
S518	20.58	15.66	8.46	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
S519	21.31	16.34	9.08	424	424	411	411	357	359	197	197	#N/A	#N/A
S520	20.91	16.57	8.32	428	432	401	411	382	382	197	197	0	0
S601	23.43	17.5	9.31	0	0	#N/A	#N/A	357	374	197	197	271	271
S602	21.72	16.05	9.56	424	424	411	411	365	365	197	197	269	269
S603	22	16.51	9.55	418	418	407	407	#N/A	#N/A	197	197	273	273
S604	21.94	16.11	9.58	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
S605	21.55	17.13	8.9	420	420	411	411	363	363	197	197	273	273
S606	21.49	16.43	9.58	#N/A	#N/A	#N/A	#N/A	363	363	193	197	269	269
S607	21.79	15.72	9.32	418	424	411	411	363	380	197	197	269	269
S608	22.09	16.45	9.19	432	432	407	407	357	357	197	197	267	271
S609	21.47	16.74	9.15	432	432	411	413	369	388	197	197	269	269
S610	21.88	16.32	8.92	420	420	411	411	365	372	193	197	269	273
S611	20.56	15.65	8.72	418	418	411	411	365	367	193	197	273	273
S612	20.81	16.47	8.92	424	424	413	413	#N/A	#N/A	#N/A	#N/A	269	269
S613	20.65	15.57	9.23	#N/A	#N/A	0	0	361	374	197	197	273	273
S614	21.48	16.59	9.06	430	434	411	411	378	378	197	216	273	273
S615	20.75	15.9	8.95	428	428	411	413	363	363	197	197	#N/A	#N/A

S616	20.36	15.48	8.97	422	430	#N/A	#N/A	363	367	0	0	269	269
S617	21.15	15.91	8.58	0	0	411	411	0	0	197	197	273	273
S618	21.07	16.21	9.73	#N/A	#N/A	#N/A	#N/A	0	0	#N/A	#N/A	#N/A	#N/A
S619	21.51	16.32	9.75	426	438	407	411	382	386	197	197	#N/A	#N/A
S620	20.48	15.48	8.81	0	0	411	411	376	376	0	0	#N/A	#N/A

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