2002

CHROMOSOMAL VARIATION IN NUCELLA LAPILLUS (L.) AND OTHER MURICID GASTROPODS

PASCOE, PHILIP LIONEL

http://hdl.handle.net/10026.1/2654

http://dx.doi.org/10.24382/4238

University of Plymouth

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.
CHROMOSOMAL VARIATION IN *NUCELLA LAPILLUS* (L.)

AND OTHER MURICID GASTROPODS

by

PHILIP LIONEL PASCOE

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

Department of Biological Sciences
Faculty of Science

In collaboration with
Plymouth Marine Laboratory

March 2002
Abstract

Candidate: Philip Lionel Pascoe

Title: Chromosomal variation in *Nucella lapillus* (L.) and other muricid gastropods

The Robertsonian polymorphism (numerical change in the chromosome complement by centric fusion or fission) in the dog-whelk (*Nucella lapillus*, Mollusca: Gastropoda) has been known and studied sporadically for almost 50 years. However, the possible causes, consequences and proposed correlations of this phenomenon remain enigmatic. *Nucella lapillus* (*2n = 26 to 36*), has undergone a marked reduction in chromosome number from its ancestral form; most other muricid species have a diploid chromosome number in the range *2n = 60 – 70*. Correlations have been proposed between chromosome number (or karyotype) and habitat (wave exposure), and also directly or indirectly with allozymes, shell shape and physiology. However, firm conclusions are lacking due to either, insufficient knowledge of the karyotypes in the populations studied, or the fact that any correlations are not consistent throughout the broad geographic range. This study reviews all the past research on the chromosomal polymorphism in *N. lapillus*, advances the karyology of this species by successfully labelling individual chromosomes through silver-staining of nucleolar organizer regions (NORs) and fluorescence *in situ* hybridisation (FISH) with rDNA and telomeric probes, and proposes a new system of nomenclature for this polymorphism. Knowledge of the geographic variation in chromosome number and karyotype is advanced by studying around 70 populations throughout the range of *N. lapillus* (mainly in the UK, but also the USA and northern and southern Europe). Evidence is shown that, (1) the polymorphism is far more widespread geographically than was previously thought; (2) more than five pairs of metacentric chromosomes are involved in the Robertsonian polymorphism; and (3) inversions also occur in some populations. Putative links between chromosomal polymorphism in *N. lapillus* and Darwinian fitness are reviewed in the light of the new findings and more recent work on other species; data are presented on fecundity, fertility, inter- and intra-individual variation in karyotype, selection within populations (adults v. embryos, homo- v. heterozygotes) and a possible genetic/karyotypic link with phenotype (Dumpton syndrome, a reproductive abnormality). Cytogenetic records for other muricids are also reviewed and karyotypes and chromosome lengths of four other species (*Ocenebra erinacea, Thais haemastoma, Murex trunculus, Ocinebrina aciculata*) are recorded for the first time. Genome size (DNA content) of 8 species of muricid, including the *2n=26* and *2n=36* forms of *N. lapillus*, are determined by Feulgen densitometry and compared in the context of evolutionary studies on the family. Although chromosome number and haploid length in *N. lapillus* has been reduced, its genome size is actually greater than all but one of the other muricids studied, and consequently has a much higher DNA packing ratio (>13000). Whether the chromosomal variation in *N. lapillus* represents polymorphism, polytypy, or speciation in progress remains unresolved, but substantial advances have been made in this dissertation and the required direction and focus for future work are clarified.
## Contents

*Abstract*.................................................................................................. 2  

*List of Contents* ...................................................................................... 3  

*List of Tables* ....................................................................................... 7  

*List of Figures* ..................................................................................... 8  

*Acknowledgements* ............................................................................. 13  

*Author's Declaration ( & Publications)* ............................................... 14  

**Chapter 1.** General Introduction .......................................................... 17  

  1.1 Introduction ........................................................................................... 18  

  1.2 The Nature of Robertsonian chromosomal polymorphism in  

  *Nucella lapillus*: a re-examination .......................................................... 22  

    1.2.1 Abstract ............................................................................................ 22  

    1.2.2 Introduction ....................................................................................... 22  

    1.2.3 Materials and methods ...................................................................... 25  

    1.2.4 Results ............................................................................................... 27  

    1.2.5 Discussion .......................................................................................... 33  

**Chapter 2.** Structural chromosomal polymorphism in the dog-whelk *Nucella lapillus*  

  (Mollusca: Neogastropoda) ...................................................................... 39  

  2.1 Abstract ............................................................................................... 39  

  2.2 Introduction .......................................................................................... 40  

  2.3 Materials and Methods ......................................................................... 42  

    2.3.1 Preparation of metaphase spreads ..................................................... 42  

    2.3.2 NOR staining and karyotyping ........................................................ 43  

  2.4 Results .................................................................................................. 44
2.4.1 $2n = 26$ karyotype ........................................................................................................ 44
2.4.2 Variations in NOR activity .......................................................................................... 47
2.4.3 Polymorphic populations ......................................................................................... 48

2.5 Discussion ...................................................................................................................... 53
2.5.1 NOR expression and heteromorphism ...................................................................... 53
2.5.2 Structural polymorphism .......................................................................................... 53
2.5.3 Causes and consequences of chromosomal polymorphism ..................................... 54

advances in karyology using rDNA loci and NORs ......................................................... 57

3.1 Abstract ......................................................................................................................... 57
3.2 Introduction ................................................................................................................... 57

3.3 Material and methods .................................................................................................. 59
3.3.1 Fluorescence in situ hybridization (FISH) ................................................................. 60
3.3.2 Probe production and labelling .................................................................................. 60
3.3.3 Denaturation / hybridization ..................................................................................... 60
3.3.4 Detection .................................................................................................................... 61

3.4 Results ......................................................................................................................... 62
3.4.1 The $2n=36$ karyotype ............................................................................................. 62
3.4.2 Ag-NOR staining ....................................................................................................... 64
3.4.3 Results of FISH ......................................................................................................... 66

3.5 Discussion ..................................................................................................................... 69

3.6 Application of the rDNA probe to *Ocenebra erinacea* ............................................. 72

3.7 Fluorescence in situ hybridisation (FISH) with a telomeric probe ............................... 73
3.7.1 Introduction ............................................................................................................... 73
3.7.2 Material and methods ............................................................................................... 74
3.7.3 Results and Discussion ............................................................................................ 75
3.7 Proposal for Nomenclature ................................................................. 76

Chapter 4. Geographical variation in the karyotype of *Nucella lapillus* .................. 78

4.1 Introduction ......................................................................................... 78

4.2 Material and Methods ......................................................................... 82

4.3 Results ................................................................................................. 83

4.3.1 Numerical variation ............................................................................... 83

4.3.2 Karyotypic variations and inversions ......................................................... 87

4.4 Discussion .............................................................................................. 109

Chapter 5. Karyotypes and Genome Size in Muricid gastropods ....................... 114

5.1 Introduction .......................................................................................... 114

5.2 Material and methods ........................................................................... 117

5.2.1 Karyotyping .......................................................................................... 117

5.2.2 Genome Size ........................................................................................ 118

5.3 Results .................................................................................................... 119

5.3.1 Karyotypes .......................................................................................... 120

5.3.2 Genome size ........................................................................................ 134

5.4 Discussion .............................................................................................. 138

Chapter 6. The possible causes, consequences and correlations of the chromosomal polymorphism in *Nucella lapillus* ........................................ 142

6.1 Introduction .......................................................................................... 142

6.2 Fecundity and Fertility ......................................................................... 146

6.2.1 Number of embryos ............................................................................. 146

6.2.2 Breeding experiments ......................................................................... 148

6.3 Is the polymorphism neutral? ................................................................. 149

6.3.1 Numerical variation within and between individual embryos ................. 150
6.3.2 Variation between embryos and adults ................................................................. 157
6.3.3 Variations within and between nearby populations ................................................. 157
6.3.4 Statistical analysis ................................................................................................ 159

6.4 Correlations: Environment/Wave action, Phenotype and Physiology ................ 161

6.5 Dumpton Syndrome ............................................................................................... 166

6.6 Discussion .............................................................................................................. 167

Summary by quotations ............................................................................................... 171

Appendix 1: Banding Methods - trials and notes ......................................................... 172

Appendix 2. The metaphase spreads of Nucella lapillus - karyotyped in Chapter 4 ........ 184

References .................................................................................................................. 202


Tables

Table 1.1 Chromosome number counts for adult *N. lapillus* from five sites around La Coruña, northwestern Spain (Galician coast)................................................................................................... 25

Table 1.2 Total chromosome lengths, relative lengths and arm ratios (where $x$ represents the mean) for *N. lapillus* (testes, $n = 13$) from Whitsand Bay, southeastern Cornwall. .................................................. 28

Table 2.1 Total chromosome lengths, relative lengths and arm ratios for *N. lapillus* from three $2n = 26$ populations: Whitsand Bay, Polzeath and West Runton ........................................................................ 45

Table 2.2 Frequency of structural chromosome rearrangements (Q types) in four numerically polymorphic populations of *N. lapillus*, including results for 2 individuals from the same egg capsule (Renney A & B). ........................................................................................................ 52

Table 4.1 Summary data for *Nucella lapillus* - Geographical variation in chromosome numbers and karyotype (continued overleaf).................................................................................................. 84

Table 5.1 Published data on chromosome number in the muricidae including confirmation or new records from this study. ...................................................................................................................... 119

Table 5.2 Total chromosome lengths, relative lengths and arm ratios for *N. lapillus* from three $2n = 26$ populations: Whitsand Bay, Polzeath and West Runton ................................................................ 122

Table 5.3 Total chromosome lengths, relative lengths and arm ratios for *Nucella lapillus* ($2n = 36$, embryos, $n = 7$) from Mount's Bay and Roscoff ............................................................................. 122

Table 5.4 Total chromosome lengths, relative lengths and arm ratios for *Ocenebra erinacea* (embryos, $n = 6$) from Looe (3) and Brest (3) ..................................................................................................... 125

Table 5.5 Total chromosome lengths, relative lengths and arm ratios for *Thais haemastoma* (embryos, $n = 3$) from the Algarve, Portugal ................................................................. 128

Table 5.6 Total chromosome lengths, relative lengths and arm ratios for *Murex (Hexaplex) trunculus*, $2n = 70$, (embryo, $n = 1$) from Portugal ............................................................................................ 131

Table 5.7 Results of Feulgen densitometry on gill nuclei of three molluscan species of known genome size to determine regression .......................................................................................... 135

Table 5.8 Chromosome number, haploid chromosome length (where determined) and genome size ($C$ value = pg DNA in haploid) derived from the Feulgen reaction, in 9 muricid species. ................. 135
Figures

Figure 1.1 The dog-whelk, *Nucella lapillus*: A. Adults and juveniles. B. Chromosomes labelled using Fluorescence in situ hybridisation to show the nucleolar organiser regions. C. $2n = 26$ karyotype. D. $2n = 36$ karyotype. Scale bar = 10 μm.

Figure 1.2 The $2n = 26$ karyotype of *N. lapillus* (here collected from West Runton, Norfolk) comprises five groups based on chromosome size and arm length parameters.

Figure 1.3 Contrasting karyotypes from a single *N. lapillus* (the same individual featured in Figure 1.4 (b)) (A) $2n = 30$ cell, putatively polymorphic for chromosome pairs 2, 3 and 4; (B) $2n = 33$ cell, putatively polymorphic for pairs 1, 2, 4, 8 and 9.

Figure 1.4 Chromosome number—frequency histograms for *N. lapillus* from Thurlestone, southwestern Devon, a $2n > 26$ population.

Figure 1.5 Chromosome number—frequency histogram for adult *N. lapillus* ($n = 11$ animals: 217 cells) from Whitsand Bay, southeastern Cornwall, a typical $2n = 26$ population.

Figure 2.1 *Nucella lapillus*: The karyotype of a $2n = 26$ embryo from Whitsand Bay, showing the NORs (arrowed) on chromosome pairs 2, 7 and 10. (Scale bar = 10 μm).

Figure 2.2 *Nucella lapillus*: An idiogram of the $2n = 26$ chromosome form.

Figure 2.3 *Nucella lapillus*: Variation in NOR expression for different embryos from the same egg capsule from each of three populations.

Figure 2.4 *Nucella lapillus*: The karyotype of a $2n = 32$ spread from Renney Rocks showing the NORs (arrowed) on the product of one chromosome of pair No. 2, and pairs 7 and 10.

Figure 2.5 *Nucella lapillus*: (A) Examples of NOR-bearing chromosomes involved in the polymorphism; the 'normal' No. 2 chromosome, and Q types A, B and C.

Figure 3.1 *Nucella lapillus*: A. $2n = 36$ metaphase spread from a Mount's Bay embryo. B. The same spread karyotyped in the 13 'pairs' format. Scale bar = 10 μm.

Figure 3.2 *Nucella lapillus*: Karyotyped metaphase spreads with silver-stained NORs (arrowed). A: $2n = 26$ karyotype (Whitsand Bay). B: $2n = 36$ karyotype (Mount's Bay).

Figure 3.3 Histograms showing variation in silver-stained NOR expression (A-C) compared with FISH signals using the rDNA probe (D), within and between different embryos from Mount's Bay ($2n = 36$).

Figure 3.4 *Nucella lapillus*: A-C. Metaphase spreads with rDNA loci marked by fluorescent signals using FISH with a rDNA probe. D. Same spread as C, silver-stained for NORs.

Figure 3.5 The application of FISH with the rDNA probe to metaphase chromosomes of *Ocenebra erinacea*.

Figure 3.6 The application of FISH with a telomeric probe (TTAGGG)$_n$ to metaphase chromosomes of *Nucella lapillus*.

Figure 3.7 Nomenclature shown in idiogram form for chromosome pair 2.
Figure 3.8  Idiogram showing form of the polymorphic chromosomes in a karyotype designated H 1,8,9; A 2,3 .............................................................. 77

Figure 4.1  *Nucella lapillus*: 2n = 36 karyotype from Lulworth Cove, Dorset ........................................... 80

Figure 4.2  2n = 36 karyotype from Mount's Bay, Cornwall................................................................. 81

Figure 4.3  A summary of the distribution of chromosome number variation in *N. lapillus* at a selection of the sites examined in the N. Atlantic. Red sectors in the pie diagrams relate to the mean number of chromosomes exhibiting the Robertsonian polymorphism, each chromosome is represented by 36° .................................. 86

Figure 4.4  2n = 26, 881 karyotype from Slaattholmen, Norway .............................................................. 89

Figure 4.5  2n = 27, H9 karyotype from Dumpton Gap, Kent .............................................................. 90

Figure 4.6  2n = 27, H9, 5I* karyotype from Dumpton Gap, Kent .......................................................... 90

Figure 4.7  2n = 27, H4 karyotype from Maine, USA ................................................................. 91

Figure 4.8  2n = 27, H4 karyotype from Slaattholmen, Norway .......................................................... 91

Figure 4.9  2n = 27, H4 karyotype from the Isle of Man ................................................................. 92

Figure 4.10  2n = 27, H3 (55I*) karyotype from the Isle of Wight ............................................................. 92

Figure 4.11  2n = 27, H5 karyotype from Cuckmere Haven, Sussex ..................................................... 93

Figure 4.12  2n = 27, aneuploid* spread (type X) from Old Stairs Bay, Kent ............................................ 94

Figure 4.13  2n = 28, A3 karyotype from Slaattholmen, Norway ............................................................ 97

Figure 4.14  2n = 28, A4 karyotype from Dover, Kent ............................................................. 97

Figure 4.15  2n = 28, A9, 8I* karyotype from North Foreland, Kent ................................................ 98

Figure 4.16  2n = 28, H4,9 karyotype from Folkestone, Kent ............................................................. 98

Figure 4.17  2n = 29, H9; A3 karyotype from Jennycliff, Plymouth Sound ............................................. 99

Figure 4.18  2n = 29, H1,8,9 karyotype from Newlyn, S. Cornwall ...................................................... 99

Figure 4.19  2n = 29, H3,8,9; 44I* karyotype from Westcombe Cove, S. Devon ................................. 100

Figure 4.20  2n = 29, H5,8,9 karyotype from Rade de Brest, N.W. France ........................................... 100

Figure 4.21  2n = 30, A8,9 karyotype from Horse Ledge, Isle of Wight ............................................... 101

Figure 4.22  2n = 30, H1,8; A9 karyotype from Thurlestone, S. Devon ............................................. 101

Figure 4.23  2n = 31, H9; A3,8 karyotype from Westcombe Cove, S. Devon ..................................... 102

Figure 4.24  2n = 31, H9; A3,8 karyotype from Rade de Brest, N.W. France ..................................... 102

Figure 4.25  2n = 31, H1,3,4,8,9 karyotype from Hanover Point, Isle of Wight ................................. 103
Figure 4.26 2n = 31, H1,2,4; A5 karyotype from St. Peter Port, Guernsey......................... 103
Figure 4.27 2n = 32, A1,3,8 karyotype from Renney Rocks, S. Devon.......................... 104
Figure 4.28 2n = 32, H1,5; A8,9 karyotype from Thurlestone, S. Devon...................... 104
Figure 4.29 2n = 32, H1,2,3,4,8,9 karyotype from Penlee Point, Cornwall.................... 105
Figure 4.30 2n = 33, H1,8,9; A2,3 karyotype from Renney Rocks, S. Devon................. 105
Figure 4.31 2n = 34, H1,3,9; A2,8 karyotype from Renney Rocks, S. Devon................. 106
Figure 4.32 2n = 34, H1,3; A2,8,9 karyotype from Renney Rocks, S. Devon................. 106
Figure 4.33 2n = 35, H1; A2,3,8,9 karyotype from Mount’s Bay, Cornwall.................. 107
Figure 4.34 2n = 35, H1; A2,3,8,9 karyotype from Mount’s Bay, Cornwall.................. 107
Figure 4.35 2n = 35, H1; A2,3,8,9 karyotype from Roscoff, N. Brittany....................... 108
Figure 4.36 2n = 36, A1,2,3,8,9 karyotype from Roscoff, N. Brittany......................... 108
Figure 5.1 Shells of the muricid species in this study..................................................... 116
Figure 5.2 Karyotype of Nucella lapillus 2n = 26 form, grouped by centromere position or arm ratio and in descending size................................................................. 121
Figure 5.3 Nucella lapillus: 2n = 36 spread grouped by chromosome type and size........... 121
Figure 5.4 Chromosomes of Ocenebra erinacea (2n = 70) from Looe, S. Cornwall............. 123
Figure 5.5 Karyotype of Ocenebra erinacea (2n = 70) from Looe, S. Cornwall.................. 124
Figure 5.6 Chromosomes of Thais haemastoma (2n = 70) from Portugal.......................... 126
Figure 5.7 Karyotype of Thais haemastoma (2n = 70) from Portugal............................... 127
Figure 5.8 Chromosomes of Murex (Hexaplex) trunculus (2n = 70) from Portugal............. 129
Figure 5.9 Karyotype of Murex (Hexaplex) trunculus (2n = 70) from Portugal................. 130
Figure 5.10 Metaphase spread of Ocinebrina aciculata (2n = 86) from Ria Formosa, Portugal................. 132
Figure 5.11 Karyotype of Ocinebrina aciculata (2n = 86) from Ria Formosa, Portugal........ 133
Figure 5.12 Correlation between relative density of interphase nuclei in the Feulgen reaction and Genome Size (pg DNA) derived from three reference species (red). The other species (green) are placed on the regression line based on mean relative density measurements............................ 136
Figure 5.13 Relationships between chromosome length and genome size.......................... 136
Figure 6.1 Mean number of developing embryos per egg capsule versus the mean diploid chromosome number (approximate in some cases) of the population...................................... 147
Figure 6.2 Whitsand Bay, SE Cornwall, an example of a high energy shore (shown here in relatively calm conditions)................................................................. 148

Figure 6.3 Breeding aggregation of *Nucella lapillus* at Whitsand Bay with numerous recently laid egg capsules and others at various stages of development........................................................................................................ 148

Figure 6.4 Frequency of diploid chromosome numbers in embryos from Port St. Mary, Isle of Man........................................................................................................ 151

Figure 6.5 Frequency of diploid chromosome numbers in embryos from Slaattholmen, Norway ............................................................. 152

Figure 6.6 Frequency of diploid chromosome numbers in embryos from Mount's Bay, Cornwall.................................................................................. 153

Figure 6.7 Frequency of diploid chromosome numbers in embryos from Renney Rocks, S. Devon............................................................. 154

Figure 6.8 Frequency of diploid chromosome numbers in embryos from Cawsand, SE Cornwall............................................................. 155

Figure 6.9 Frequency of diploid chromosome numbers in embryos and adults from Thurlestone, S. Devon........................................................................................................ 156

Figure 6.10 Frequency of diploid chromosome numbers in embryos and adults from sites in Kent........................................................................................... 158

Figure 6.11 Frequency of diploid chromosome numbers in embryos from sites in Maine, USA.............................................................................. 159

Figure 6.12 Frequency of diploid chromosome numbers in embryos from sites on the Isle of Wight................................................................. 160

Figure 6.13 Views of Renney Rocks, SW Devon. A. The reef faces south-west and is often exposed to severe swells and wave action. B. Behind the main outcrop an extensive area of small boulders provides a more sheltered micro-habitat........................................................................................................ 162

Figure 6.14 A & B. Embryos of *N. lapillus* just before and at hatching respectively, showing reduction in size of the velum. C & D. Hatchlings of *O. erinacea* with the large velum extended and retracted respectively. ................................................................. 164

Appendices

Figure A 1.1 Scanning electron micrograph of *Nucella lapillus* chromosomes........................................................................................................ 183

Figure A 2.1 2n=26 from West Runton, Norfolk........................................................................................................ 184

Figure A 2.2 2n = 36 from Mount's Bay, Cornwall........................................................................................................ 184

Figure A 2.3 Full legend for chromosome number variation in *N. lapillus* at sites examined in the N. Atlantic, as shown in Figure 4.3........................................................................................................ 185

Figure A 2.4 2n = 26, (88I) from Slaattholmen, Norway .............................................................................. 185

Figure A 2.5 2n = 27, (H9) from Dumpton Gap, Kent........................................................................................................ 186

Figure A 2.6 2n = 27, (H9, 5I) from Dumpton Gap, Kent........................................................................................................ 186

Figure A 2.7 2n = 27, (H4) from Maine, USA...................................................................................... 187

Figure A 2.8 2n = 27, (H4) from Slaattholmen, Norway...................................................................................... 187
Figure A 2.9 2n = 27, (H4) from the Isle of Man ................................................................. 188
Figure A 2.10 2n = 27, (H3, 55) from the Isle of Wight .......................................................... 188
Figure A 2.11 2n = 27, (H5) from Cuckmere Haven, Sussex ................................................... 189
Figure A 2.12 2n = 27, aneuploid (type X) from Old Stairs Bay, Kent ................................. 189
Figure A 2.13 2n = 28, (A3) from Slaaatholmen, Norway ...................................................... 190
Figure A 2.14 2n = 28, (A4) from Dover, Kent ..................................................................... 190
Figure A 2.15 2n = 28, (A9) from North Foreland, Kent ...................................................... 191
Figure A 2.16 2n = 28, (H4,9) from Folkestone, Kent ......................................................... 191
Figure A 2.17 2n = 29, (H9; A3) from Jernycliff, Plymouth Sound ........................................ 192
Figure A 2.18 2n = 29, (H4,8,9) from Pudcombe Cove, S. Devon ........................................ 192
Figure A 2.19 2n = 29, (H3,8,9; 441) from Westcombe Cove, S. Devon ......................... 193
Figure A 2.20 2n = 29, (H5,8,9) from Rade de Brest, N.W. France ................................. 193
Figure A 2.21 2n = 30, (A8,9) from Horse Ledge, Isle of Wight ......................................... 194
Figure A 2.22 2n = 30, (H1,8; A9) from Thurlestone, S. Devon ........................................... 194
Figure A 2.23 2n = 31, (H9; A3,8) from Westcombe Cove, S. Devon ................................. 195
Figure A 2.24 2n = 31, (H9; A3,8) from Rade de Brest, N.W. France ............................. 195
Figure A 2.25 2n = 31, (H1,3,4,8,9) from Hanover Point, Isle of Wight ......................... 196
Figure A 2.26 2n = 31, (H1,2,4; A5) from St. Peter Port, Guernsey ................................. 196
Figure A 2.27 2n = 32, (A1,3,8) from Renney Rocks, S. Devon ......................................... 197
Figure A 2.28 2n = 32, (H1,5; A8,9) from Thurlestone, S. Devon ........................................ 197
Figure A 2.29 2n = 32, (H1,2,3,4,8,9) from Penlee Point, Cornwall ............................... 198
Figure A 2.30 2n = 33, (H1,8,9; A2,3) from Renney Rocks, S. Devon .............................. 198
Figure A 2.31 2n = 34, (H1,3,9; A2,8) from Renney Rocks, S. Devon .............................. 199
Figure A 2.32 2n = 34, (H1,3; A2,8,9) from Renney Rocks, S. Devon .............................. 199
Figure A 2.33 2n = 35, (H1; A2,3,8,9) from Mount's Bay, Cornwall .............................. 200
Figure A 2.34 2n = 35, (H1; A2,3,8,9) from Mount's Bay, Cornwall .............................. 200
Figure A 2.35 2n = 35, (H1; A2,3,8,9) from Roscoff, N. Brittany ..................................... 201
Figure A 2.36 2n = 36, (A1,2,3,8,9) from Roscoff, N. Brittany ......................................... 201
I thank Peter Gibbs and David Dixon of Plymouth Marine Laboratory for introducing me to the fascinating subjects of the dog-whelk and cytogenetics respectively. I also thank David Dixon, my original supervisor, for 'getting me into this mess' i.e. encouraging me to embark on this study, and my current supervisors, Tony Hawkins (Plymouth Marine Laboratory) and Awadhesh Jha (University of Plymouth), for 'getting me out of it!', i.e. supplying encouragement and patience to enable me to finish it.

I am also indebted to several of the staff at Plymouth Marine Laboratory for collecting some of the material from distant sites, the Library staff for their advice and patience and in particular to Nick Pope, Gary Burt and Martyn Atkins for keeping me in touch with the basics of advancing computer technology.

Finally, I thank my thesis examiners, Drs. John Bishop, David Lloyd and John Crothers, for the stimulating discussions and helpful suggestions towards the final polishing.
AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was undertaken on a part-time basis with no direct funding made available; the author is a member of staff at the partner institution, Plymouth Marine Laboratory (Natural Environmental Research Council).

Relevant courses, scientific seminars and conferences were attended throughout the registration period. Publications and presentations made by the author are given below:

**Published papers (Cytogenetics):**


**Published papers (Chromosomal Genetic Toxicology):**


**Posters**

Pascoe, P.L.; Dixon, D.R., 1993
Structural chromosomal rearrangements associated with Robertsonian variation in the dog-whelk Nucella lapillus (Mollusca: Neogastropda) [abstract]. Workshop on chromosomal variation in nature - focus on whole-arm rearrangements. 17th International Congress of Genetics, Birmingham, 15-21 August 1993.


Fluorescence in situ hybridization applied to a chromosomal polymorphism in the dog-whelk, Nucella lapillus. Marine Biology: Molecular and Genetic advances. NERC/PML/MBA meeting, University of Plymouth, April 1995.

Karyotype evolution and genome size in muricid gastropods. Fifth Congress of the European Society for Evolutionary Biology, University of Edinburgh, Sept 1995.


[The above poster (or v. similar; same authors) was also presented at:
1. UKEMS annual meeting, York, April 6-8- 1998.
3. Oceanography 98, University of Southampton.
Oral Presentations (P.L. Pascoe)


Molecular markers in genotoxicology. Seminar: Group meeting, Molecular Marine Biology and Chemistry at PML. March 1996, Plymouth Marine Laboratory.


Signed

Date

22/3/02
Chromosomal variation in *Nucella lapillus* and other muricid gastropods

Chapter 1. General Introduction

Figure 1.1 The dog-whelk, *Nucella lapillus*: A. Adults and juveniles. B. Chromosomes labelled using fluorescence *in situ* hybridisation to show the nucleolar organiser regions (NORs). C. $2n = 26$ karyotype D. $2n = 36$ karyotype. Scale bar = 10 µm.
1.1 Introduction

The Muricidae are a widely-distributed family of carnivorous marine snails inhabiting the littoral or sub-littoral zones of rocky shores. Worldwide research on the muricids, particularly *Nucella lapillus* (L.), is extensive in several different fields, viz. general biology, cryptic speciation, physiology, reproduction and ecotoxicology (e.g. see Crothers, 1985; Butler, 1985; Etter, 1989; Palmer *et al.*, 1990; Gibbs *et al.*, 1991a; Gibbs & Bryan, 1994). Although the family does not include commercially important species, they are ecologically important as major predators of barnacles and mussels and serve as extremely useful bio-indicators in various ecotoxicological programmes, many of which were initiated through work at Plymouth (Bryan, *et al.*, 1986; Gibbs & Bryan, 1986; Gibbs *et al.*, 1987; Bailey & Davies, 1989; Bright & Ellis, 1990; Gibbs *et al.*, 1990, 1991b; Stewart *et al.*, 1992). Evolution of the group has also received considerable attention, particularly the species in the northern hemisphere (Kool, 1993; Collins *et al.*, 1996; Marko, 1998). Cytogenetic studies to date have revealed that in all but one of the muricid species the diploid chromosome number is in the range $2n = 60 - 70$. However, the Atlantic dogwhelk, *Nucella lapillus*, has undergone a marked reduction in chromosome number since its ancestral form dispersed into the North Atlantic from the NE Pacific, via an Arctic route, during a period of global warming, $< 5$ MYA (Briggs, 1970). *N. lapillus* is also unusual in having a numerical chromosomal polymorphism, the diploid number varying from 26 to 36 (Fig. 1.1). This may relate to theories that, due to post-Pliocene glaciations (e.g. in the North Sea), modern *Nucella lapillus* may be a combination of several genetically distinct populations, (Cambridge & Kitching, 1982; Crothers, 1983).

The muricidae therefore, are a well studied group which provide an interesting subject for genetic and cytogenetic research as they exhibit both interspecific and intraspecific
variation in chromosome number which may relate to physiological/behavioural, geographical and evolutionary factors.

Chromosomal variation in the dog-whelk *Nucella lapillus* (Fig. 1.1, C, D) has been known and studied sporadically for almost 50 years. It takes the form of a Robertsonian polymorphism, i.e. the numerical change in the chromosome complement is brought about by centric (centromere) fusion or fission, and is thought to involve up to five pairs of metacentric (median) chromosomes in the more common 2n=26 form (Fig. 1.1, C). It has proven quite an enigmatic phenomenon in this species as several individual, or groups of, scientists (see below) have attempted to elucidate the causes and consequences of the chromosomal variation within this species, but have achieved only limited success. For example, evidence suggests that in *N. lapillus* there is a link between chromosome number and the degree of wave exposure to which it is subjected (shore type). The more common, 2n=26 form tends to be found on exposed shores, whereas higher chromosome number populations, up to the recorded maximum of 2n=36 (Fig. 1.1, D), are found on more sheltered shores. This numerical polymorphism, brought about by fission or fusion events, was thought to be restricted to a relatively small part of the large geographic range of this species, i.e. shores of the English Channel (Bantock & Cockayne, 1975). Several theories on the significance of these findings have been proposed, e.g. correlations have been found between karyotype (&/or habitat) and (1) allozymes (Day *et al.*, 1993), (2) shell shape and (3) physiology (Kirby *et al.*, 1994a, b). However, firm conclusions are lacking due to either, insufficient knowledge of the karyotypes in the populations studied, or the fact that any correlations are not consistent throughout the broad geographic range. Most previous authors suggest/assume that for these higher chromosome number types to become fixed in populations there must be a fitness advantage in different habitats or
niches. If this was true, one might expect to find the polymorphism (i.e. \(2n>26\) types) to be more widespread than recorded to date.

This study was originally initiated for two main reasons:

1. To identify individuals/populations by the karyotype and hence possibly the (geographical) origins of animals re-colonising habitats where this species had become extinct through the sterilising effects of tributyltin (TBT) pollution. Several studies on populations of *N. lapillus* showed conclusively that the leachate from TBT-containing anti-fouling paints was causing the phenomenon of 'imposex' in female dog-whelks and, in some highly polluted areas, leading to sterility and eventual extinction of the population (Bryan *et al*., 1986; Gibbs & Bryan, 1986; Gibbs, *et al*., 1987, 1988). Partly as a consequence of these studies, restrictions on the use of this biocide were implemented in many countries (e.g. France in 1982, UK in 1987, Europe in 1991; see Waite *et al*., 1991) and after environmental concentrations diminished, re-colonisation of some habitats was recorded (Crothers, 1998; and personal observations). However, the dog-whelk has very low powers of dispersal; the young are direct-developing, non-planktonic and adults have very low vagility, their range extending only a few metres over their life span of around 10 years (Hughes, 1972). Therefore, re-colonisation or dispersal would normally occur by individuals 'yomping', albeit rather slowly, along the shore or by animals rafting on flotsam. The ability to identify animals cytogenetically was also desirable for related studies where animals from different shore types (habitats) were transplanted to areas where *N. lapillus* had become extinct (e.g. Gibbs, 1993a).

To achieve the above, the second reason was necessary and became the dominant goal:

2. To advance the karyology of this species by overcoming the previous lack of success in identifying, unequivocally, individual chromosomes through labelling or marking techniques. *N. lapillus*, as with many other marine invertebrates, has proven
largely refractory to the standard (mammalian) chromosome banding techniques. The aims were to develop and apply different techniques, possibly utilising molecular methods, to identify the individual chromosomes involved in the polymorphism, and then with increased knowledge of the variation, address the following questions:

a. How widespread is the Robertsonian polymorphism?

b. How strong is the link between chromosome number and environment/shore type?

c. Is a given chromosome number represented by only one karyotype arrangement?

d. Does the polymorphism involve only fission / fusion events or are there also related structural rearrangements (e.g. inversions) as indicated by Page (1988)?

e. Is there selection within a population for certain karyotypes or chromosome number, e.g. comparing embryos with adults?

f. Is the polymorphism neutral within populations, i.e. are karyotypes biased towards homozygous or heterozygous forms of the polymorphism?

g. What are the relationships between chromosome number, total haploid length and genome size (DNA content) within the Muricidae?

h. Is there an evolutionary pattern within the species, genus or family?

i. Can the above be related to geography or geological events?

The above questions relate to structural, geographical and evolutionary aspects of chromosomal variation, particularly the Robertsonian polymorphism, and most have been at least partially answered in this study.

N.B. Throughout this study the term 'polymorphism' is in accordance with common usage in the context of Robertsonian rearrangements or translocations, but might more accurately be characterised in this case as a dimorphism with intermediates. Also, the terms homozygote and heterozygote, which are conventionally applied to genes, are often used
here to describe the form of pairs of whole chromosomes that exhibit Robertsonian translocations in *N. lapillus*, as suggested by Searle (1988b) for the common shrew.

Firstly, a co-authored paper (Dixon *et al.*, 1994), is reproduced here; this serves as a review of the historical data relating to the polymorphism and presents some modified methods and initial new findings on the numerical and structural chromosomal variation in some populations throughout the geographical range of the species.

### 1.2 THE NATURE OF ROBERTSONIAN CHROMOSOMAL POLYMORPHISM IN *NUCELLA LAPILLUS*: A RE-EXAMINATION

David R. Dixon, Philip L. Pascoe, Peter E. Gibbs and Juan J. Pasantes (1994)


#### 1.2.1 Abstract

Over the past half century several different classes of large-scale chromosomal rearrangements have been reported for many different animal groups. In the dog-whelk *Nucella lapillus* (Mollusca: Gastropoda) this variation takes the form of Robertsonian polymorphism (centric fusion or fission), the diploid chromosome number varying between 26 and 36 depending on the number of median/subterminal chromosomes in the karyotype. Historical data relating to the polymorphism exhibited by *N. lapillus* are reviewed, and new data regarding numerical and structural chromosomal variation are also presented. A high level of intra-individual variation in chromosome number has been discovered in some populations: the link between chromosome number and adaptation to specific environmental conditions, proposed by early investigators, thus seems in doubt at least for some populations.
1.2.2 Introduction

*Nucella (= Thais) lapillus* (L.), the common dog-whelk, is widely distributed on rocky shores on both sides of the North Atlantic, in the east from northern Russia to Portugal, and in the west from Southern Newfoundland to New York. Its ancestors are likely to have been Pacific in origin, these migrating into the North Atlantic during the Miocene or Pliocene periods when milder climatic conditions prevailed (Briggs, 1970). The species has a direct larval development; this takes place over 3—4 months within a durable capsule attached to rock, the juveniles emerging as miniature adults. Dispersal of the species is thus limited by its lack of a planktonic larval phase and also by the fact that the movement of individual adults during their lifetime (> 6 years) is restricted to a few metres (Hughes, 1972). Several lines of evidence suggest that genetic interchange between *N. lapillus* populations is restricted. These include high levels of phenotypic, viz. shell morphology, (e.g. Crothers, 1985), genotypic (allozymic and ribosomal DNA polymorphism) (e.g. Day, 1990; Patton and Dixon, unpublished data) and karyotypic (e.g. Staiger, 1950a, 1954) variation.

Staiger (1950a, 1954) was the first to show that *N. lapillus* populations in the region around Roscoff on the Channel coast of France contained a numerical (Robertsonian) chromosomal polymorphism (he used the term ‘dimorphism’) affecting five pairs of metacentric chromosomes in the $2n = 26$ form, which were represented by 10 pairs of acrocentrics in the $2n = 36$ form. Staiger (1950a, 1954) examined individuals from 48 sites in the Roscoff region of Brittany, representing a range of exposure conditions, and concluded that the degree of chromosomal heterogeneity correlated well with environmental factors. He found that high chromosome numbers were characteristic of, although not exclusively found on, shores sheltered from wave action, whereas a low chromosome number was commonly found at exposed sites typified by strong wave action,
and animals with intermediate chromosome numbers occupied shores of intermediate exposure. What adaptive significance there is in such chromosome number variation and how this translates into differences in fitness between the different chromosomal genotypes under differing conditions of exposure remains a matter of conjecture. Recently, it has been suggested that crossover suppression around the centromeres in heterozygotes will assist these regions of the chromosomes to evolve independently in the two types of homozygotes \(2n = 26\) and \(2n = 36\) to become adapted to different ecological niches within the same environment (racial separation), with the heterozygote possibly becoming better adapted to intermediate habitats (heterozygote advantage) (Searle, 1988a, b).

Bantock and Cockayne (1975) and Bantock and Page (1976) carried out a chromosome survey of dog-whelk populations on the Channel coast of southern England and reported finding a similar relationship to that found by Staiger (1954) between chromosome numbers and ecological conditions. Subsequently, Hoxmark (1970) examined Norwegian populations in an attempt to repeat Staiger’s work but was only able to find \(2n = 26\) populations on all shore types. Mayr (1969) stated that only the \(2n = 26\) type occurs on the Atlantic coast of North America, an observation not supported by any data. Ahmed (1974) reported finding no evidence of any similar polymorphism in other species of *Nucella* on the Pacific coast of North America. One of us (J.J.P.) has examined *N. lapillus* from five localities (three exposed; two sheltered) around La Coruña, northwestern Spain, and could find only the \(2n = 26\) form (Table 1.1). It would appear, therefore, that the \(2n = > 26\) form is restricted to one relatively small, central part of a large geographic range, within which *N. lapillus* occupies a broader niche compared with the other (Pacific) species, presumably due to the lack of competing gastropod species on Atlantic shores (Kitching, 1985). If one accepts the argument put forward by Staiger and others that

\[\text{Erratum: Hoxmark (1970) did record chromosome numbers in meiotic metaphases of } n > 13, \text{ but was unable to show any correlation with exposure (wave action).}\]
chromosomal polymorphism confers some adaptive advantage to dog-whelk populations along the English and French Channel coasts, then there remains a need to explain how *N. lapillus* succeeds throughout the greater part of its geographic range, and under a full range of exposure conditions, with a karyotype consisting of only 2n = 26 chromosomes.

This section describes results of a study of karyotype variation in dog-whelk populations of intermediate and low chromosome number in southwestern England and elsewhere which cast new light on the nature of Robertsonian polymorphism in *N. lapillus* and its possible evolutionary significance.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Chromosome number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Reira (exposed)</td>
<td>1</td>
</tr>
<tr>
<td>Calon (exposed?)</td>
<td>1</td>
</tr>
<tr>
<td>Sahon (exposed)</td>
<td>1</td>
</tr>
<tr>
<td>Muinos (sheltered)</td>
<td>3</td>
</tr>
<tr>
<td>Santa Christina (sheltered)</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1.1 Chromosome number counts for adult *N. lapillus* from five sites around La Coruña, northwestern Spain (Galician coast), based on testis preparations. Numbers refer to the total number of metaphases counted from *n* number of individuals.

1.2.3 Materials and methods

Samples of adult *N. lapillus* and/or egg capsules were collected from sites representing a range of exposure conditions in southern England, northwestern Spain and western Norway (Svartholmane, off the Biological Station, Bergen, i.e. a site of intermediate exposure which featured previously in Hoxmark (1970)). The animals were housed
temporarily in a recirculating seawater aquarium where they were fed small *Mytilus edulis*. Egg capsules laid in the aquarium were kept for 4—6 weeks prior to analysis.

1.2.3.1 Preparation of mitotic metaphase spreads

Chromosome spreads were prepared from adult testes or early-veliger-stage embryos (4—6 weeks old) taken from intact egg capsules. Testes samples were dissected away from the underlying digestive gland and cut into small (1 mm square) pieces; embryos were teased from the early shell and remaining yolk. Both tissues were given two combined colchicine and hypotonic treatments: 0.08% colchicine (Sigma) in 50% seawater, 30—45 min followed by 0.04% colchicine in 25% seawater, 30—45 min. After a further hypotonic treatment, 0.075 M KCl, 2 x 5 min, the tissues were fixed in fresh, cold, Carnoy's solution (3:1, ethanol/glacial acetic acid), three or four changes for a minimum of 1 h.

Slide making was based on the procedure for solid tissues described by Kligerman and Bloom (1977). Following dissociation of the Carnoy's fixed tissue in 60% acetic acid, concentric rings of spread nuclei were produced by pipetting drops of the cell suspension onto the surface of a warm slide at 40 °C. Slides were stained for 10 min in freshly prepared and filtered 10% Giemsa (Gurr’s Improved R66) in phosphate buffer (Gurr’s pH 6.8 buffer tablets). After rinsing and blueing in tapwater containing a few drops of ammonium hydroxide, the slides were blotted dry. Photographs were taken using a Zeiss photomicroscope (63 x or 100 x oil immersion lens) on Kodak Panatomic-X Technical Pan film, employing a green filter to enhance contrast. In excess of 1000 cells were counted during the course of this study.
1.2.3.2 Karyotyping

Karyotyping followed the standard procedure based on arm length parameters. Chromosomes were first grouped by arm ratio, then arranged according to length, and finally matched by eye, rearranging to improve pairing where necessary. Measurements of chromosome arm length were made with the aid of a Kontron IBAS Image Analyser. Total length (nearest 0.01 μm), relative length (100 x chromosome length/total haploid length) and arm ratio (length of short arm, p/length of long arm, q) were computed based on the average length values for each pair of chromosomes, excluding centromeric distances. Centromere position was described according to the nomenclature of Levan, Fredga and Sandberg (1964). Chromosomes were classified according to centromere position as follows:

'median' (m), AR 1—0.59; 'submedian' (sm), AR 0.59—0.33; 'subterminal' (st), AR 0.33—0.14; and 'terminal' (t), AR 0.14—0. In the text, use is made also of the more widely employed, though less precise, terms metacentric (m), submetacentric (sm) and acrocentric (st-t), to aid comparison with previous work.

1.2.4 Results

1.2.4.1 The 2n = 26 karyotype

The 2n = 26 karyotype of *N. lapillus* consists of five groups of chromosomes (Figure 1.2). based on relative chromosome size and centromere position: group A (m, n = 4 pairs); group B (sm, n = 2 pairs); group C (m, n = 4 pairs); group D (st, n = 1 pair); and group E (m and sm, n = 2 pairs). Specific chromosome pairs, with the exception of subterminal pair 11, could not be unequivocally identified due to only small gradations in size within and between groups, and an absence of any consistent secondary features (e.g. secondary constrictions). This confirms the basic karyotype proposed for *N. lapillus* by Page (1988).
Table 1.2 shows the results of measurements of total length, relative length and arm ratio for each of the 13 pairs of chromosomes in the karyotype of *N. lapillus* from Whitsand Bay, southeastern Cornwall, UK. There was no difference between this population with respect to the composition of the 2n = 26 karyotype and other 2n = 26 populations from eastern and southern England (Polzeath, Bude and Norfolk), southwestern Brittany (Pointe de la Torche), northwestern Spain (Galicia) and Norway (Svartholmane, near Bergen). These additional data are available from the authors.

<table>
<thead>
<tr>
<th>Chromosome Pair No.</th>
<th>Total Length (µm) (x ± 2SE)</th>
<th>Relative Length (%) (x ± 2SE)</th>
<th>Arm Ratio (x ± 2SE)</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.23 ± 1.37</td>
<td>13.9 ± 0.4</td>
<td>0.81 ± 0.03</td>
<td>m</td>
</tr>
<tr>
<td>2</td>
<td>9.28 ± 1.23</td>
<td>12.5 ± 0.2</td>
<td>0.88 ± 0.02</td>
<td>m</td>
</tr>
<tr>
<td>3</td>
<td>8.30 ± 1.05</td>
<td>11.2 ± 0.4</td>
<td>0.64 ± 0.02</td>
<td>m</td>
</tr>
<tr>
<td>4</td>
<td>7.79 ± 1.02</td>
<td>10.5 ± 0.2</td>
<td>0.71 ± 0.04</td>
<td>m</td>
</tr>
<tr>
<td>5</td>
<td>6.70 ± 0.81</td>
<td>9.1 ± 0.4</td>
<td>0.37 ± 0.02</td>
<td>m</td>
</tr>
<tr>
<td>6</td>
<td>5.20 ± 0.64</td>
<td>7.0 ± 0.2</td>
<td>0.35 ± 0.04</td>
<td>sm</td>
</tr>
<tr>
<td>7</td>
<td>5.37 ± 0.63</td>
<td>7.3 ± 0.2</td>
<td>0.79 ± 0.06</td>
<td>m</td>
</tr>
<tr>
<td>8</td>
<td>4.57 ± 0.54</td>
<td>6.2 ± 0.2</td>
<td>0.60 ± 0.06</td>
<td>m</td>
</tr>
<tr>
<td>9</td>
<td>4.51 ± 0.51</td>
<td>6.1 ± 0.1</td>
<td>0.63 ± 0.04</td>
<td>m</td>
</tr>
<tr>
<td>10</td>
<td>3.99 ± 0.40</td>
<td>5.4 ± 0.2</td>
<td>0.75 ± 0.04</td>
<td>m</td>
</tr>
<tr>
<td>11</td>
<td>3.81 ± 0.34</td>
<td>5.2 ± 0.2</td>
<td>0.24 ± 0.02</td>
<td>st</td>
</tr>
<tr>
<td>12</td>
<td>2.39 ± 0.22</td>
<td>3.3 ± 0.2</td>
<td>0.75 ± 0.05</td>
<td>m</td>
</tr>
<tr>
<td>13</td>
<td>1.62 ± 0.15</td>
<td>2.2 ± 0.2</td>
<td>0.61 ± 0.06</td>
<td>m/sm</td>
</tr>
</tbody>
</table>

Table 1.2  Total chromosome lengths, relative lengths and arm ratios (where x represents the mean) for *N. lapillus* (testes, n = 13) from Whitsand Bay, southeastern Cornwall.
Figure 1.2 The 2n=26 karyotype of *N. lapillus* (here collected from West Runton, Norfolk) comprises five groups based on chromosome size and arm length parameters. In order of decreasing size, group A, median (AR = 1 - 0.59, 4 pairs; group B, submedian (AR = 0.59 - 0.33), 2 pairs; group C, median, 4 pairs; group D, subterminal (AR = 0.33 - 0.14), 1 pair; and group E, median/submedian, 2 pairs. Individual identification is possible only for pair 11. Note prominent secondary constrictions on single chromosomes in pairs 2 and 7 (arrowed). Scale bar = 10μm.
Figure 1.3  Contrasting karyotypes from a single *N. lapillus* (the same individual featured in Figure 1.4 (b)) (A) 2n = 30 cell, putatively polymorphic for chromosome pairs 2, 3 and 4; (B) 2n = 33 cell, putatively polymorphic for pairs 1, 2, 4, 8 and 9. Scale bars = 10 μm
1.2.4.2  The 2n > 26 karyotype

Figure 1.3 shows 2n = 30 and 2n = 33 karyotypes from Thurlestone, southern Devon, a site of intermediate chromosomal heterozygosity. (Note: both spreads are from the same animal.) The fundamental number (nombre fondamental, NF; Matthey, 1945), the number of major chromosome arms in the 2n karyotype, was the same as that recorded for the 2n = 26 form, i.e. 48. This supports the view that the observed numerical variation was a result of a Robertsonian rearrangement (i.e. centric fusion or fission). (Note: in this study, short arms such as those of pairs 11 and 13 (Figure 1.2) were omitted from the NF calculation; this phenomenon will be addressed in more detail in a later paper (Pascoe and Dixon, 1994)). A significant proportion of 2n = > 26 karyotypes analysed during this study were checked to ensure that the NF did not deviate from 48 to avoid including any that contained 'extra' chromosomes (i.e. hyperdiploid spreads) resulting from cell mixing during slide manufacture. Our investigation revealed that of the five pairs of metacentric chromosomes contributing to the polymorphism, three (or possibly four) were from group A and the remaining two pairs were from group C. This contrasts with the findings of Page (1988), who reported that two pairs from each of these groups and one pair from group B were involved in the polymorphism: this undoubtedly reflects population differences. The precise identification of chromosomes contributing to the Robertsonian polymorphism awaits the successful application of chromosome banding or related techniques. Work of this nature is currently in progress in our laboratory.

A most interesting feature of the 2n = > 26 karyotype of N. lapillus was the presence of 'rabbit-ear' acrocentrics (terminology after Levan, Hsu and Stich, 1962), i.e. products of centric fission which display a short arm in addition to the predicted long arm. In fact, the majority of acrocentric chromosomes examined during this study were of the 'rabbit-ear' type (e.g. Figure 1.3, chromosomes 3 and 4).
Figure 1.4 Chromosome number—frequency histograms for *N. lapillus* from Thurlestone, southwestern Devon, a 2n = > 26 population. (a) Pooled counts based on 20 adults; (b) counts from a single adult showing a high level of intra-individual variation; (c) pooled embryo counts for the same population; and (d) counts from a single embryo showing a reduced level of numerical variation.
Figure 1.4 (a) shows pooled chromosome counts for 20 adult *N. lapillus* (257 cells; 1—37 counts performed per individual) from Thurlestone, South Devon. This pattern was typical of all those ‘high’ chromosome number populations we examined. No pure 2n = 36 populations or, for that matter, animals, were discovered during our survey of sites on the English Channel coast (Pascoe, Gibbs and Dixon, unpublished data). This absence can be attributed to the effects of tributyltin antifouling paint which has seriously decimated dogwhelk populations around the UK and elsewhere during the past decade (Gibbs and Bryan, 1986), particularly those at sheltered sites (boat harbours) where the high chromosome number form appears to have been dominant, e.g. Salcombe (Bantock and Cockayne, 1975). Figure 1.4 (b) shows the range of chromosome number counts from one adult animal from Thurlestone. When checked for fundamental number, about 25% of spreads were found to be the result of cell mixing and were consequently omitted from the analysis.

Figures 1.4 (c) and 1.4 (d) show chromosome counts for dog-whelk embryos from egg capsules collected at Thurlestone. These showed the same overall pattern of between-cell variation as was recorded for adults, although the range of numerical variation per individual embryo was somewhat reduced. A Mann-Whitney U-test revealed a significant difference (P < 0.05) between the range of intra-individual chromosome number variation in dog-whelk embryos and adults from Thurlestone.

1.2.5 Discussion

This investigation has revealed significant levels of intra and inter-individual variation in chromosome number in dog-whelks collected from several geographically separated sites on the south coast of England. Robertsonian transformations refer to chromosome structural changes due to centric fusion or centric fission of non-homologous acrocentric or
telocentric chromosomes (Robertson, 1916). Such a mechanism involves changes in the number of centromeres without a major change in NF, i.e. the total number of chromosome arms. Centric fusions are considered to be one of the main ways by which chromosome numbers have changed in animal karyotype evolution (White, 1973; John, 1976), and are generally considered to be the more frequent (White, 1965) since there are fewer morphological obstacles to their formation, i.e. no need for new centromeres and telomeres.

The conservation of fundamental number ignores the formation of so-called 'rabbit-ear' (i.e. short-armed) acrocentrics which we report here for _N. lapillus_ and which have already been described for several other species exhibiting Robertsonian polymorphism, e.g. the coccinellid beetle _Chilocorus stigma_ (White, 1973) and the pocket mouse _Perognathus goldmani_ (Patton, 1969).

Interestingly, Staiger (1954) described the chromosomes of the 2n = 36 form of _N. lapillus_ on the Brittany coast as consisting of a mixture of metacentric and 'rodshaped' (not strictly acrocentric, for the most part) chromosomes, which he depicted as having short arms (e.g. Staiger, 1954, Figure 18). This fact appears to have been overlooked by more recent investigators. White (1973) could see no reason why there should be any difference between centric fusions involving chromosomes with terminal centromeres (acrocentrics) and those, such as in the cases described above, including _N. lapillus_, where a short length of chromatin is lost. In other organisms (Patton, 1969), the short arms of 'rabbit-ear' acrocentrics have been shown to consist of heterochromatin, i.e. genetically inert (or dispensable) portions of chromosome arms which seemingly disappear upon fusion. This heterochromatin may serve to stabilize naked centromeres (Cooper, 1959). The presence of short arms on a large proportion of those acrocentrics involved in the polymorphism provides unequivocal evidence that the significant level of intra-individual variation in
chromosome number reported here cannot be simply accounted for by chromosome breakage during slide making.

It remains to be shown whether there is any structural difference between the centromeres of monomorphic (i.e. $2n = 26$) and polymorphic (i.e. $2n \geq 26$) populations of *N. lapillus*. The centromere is the primary constriction at which metaphase chromatids (chromosome arms) are held together. Using special staining techniques it is possible to visualize four dense structures, two in each (metacentric) chromatid (Bostock and Sumner, 1978), the precise nature of which is unknown. The presence of four in metacentric and two in telocentric chromosomes suggests the ready conversion of one to the other by splitting or fusion (Bostock and Sumner, 1978). The very low level of numerical variation recorded for the $2n = 26$ form (Figure 1.5) suggests that it may have reached a level of centromeric fixation not found in $2n \geq 26$ populations. Whether this reflects the presence of a gene responsible for centromeric fragility/stability remains to be discovered. An inherent stability of the $2n = 26$ karyotype is strongly indicated by the results of a dog-whelk transplant experiment in which adults from the exposed site at Bude were moved to a sheltered site in Plymouth Sound (Batten Bay to the north of Renney Rocks, a high chromosome number site) where the native population had ceased breeding because of TBT-induced sterilization (Gibbs and Bryan, 1986). Significantly, examples of the F1 generation (4-year-old males) produced by the Bude transplants resembled their parents in being $2n = 26$ ($n = 7$ individuals, 54 spreads, Gibbs, unpublished data). This indicates that the parental karyotype was conserved despite the altered environmental conditions, and supports the idea of centromeric fixation in the $2n = 26$ form.
The high level of intra-individual variation in *N. lapillus* casts considerable doubt on any supposed link between chromosome number and environmental conditions, *viz.* degree of exposure and/or shelter from wave action, as originally suggested by Staiger (1954) and Bantock and Cockayne (1975). White (1973) found it surprising that the adaptive properties of five different, non-homologous chromosomes were all similar in that they helped adapt the individual to 'exposed' environments. Hoxmark (1970) proposed the idea of two chromosomal races — form 13 (haploid, \( n \)), with a northern distribution, and form 18, with a southern distribution. He thought that the coast of Brittany and the Channel form an overlapping zone between the two forms with free mating between them. This racial zonation hypothesis can be discounted because the more southerly Spanish material described here (Table 1.1) proved to be \( 2n = 26 \) on all shore types. We have been unable to locate any data addressing the phenomenon of intra-individual variation in Robertsonian populations.
White (1973) distinguished between cytologically **polymorphic** species in which many or all demes (breeding groups) contain several different karyotypes, and **polytypic** species which consist of two or more chromosomal races differing in karyotype. Two models have been suggested by Patton (1969) to explain the types of Robertsonian variation observed in a wide variety of animal species: (1) geographically separated chromosome races showing limited degrees of hybridization where the racial zones overlap at the margins (e.g. Patton, 1969; Ford and Hamerton, 1970; Nevo and Shaw, 1972; Baker, 1981; Greenbaum, 1981; Porter and Sites, 1985; Searle, 1988b); and (2) dominance of one race over another with limited expression of one pure form and a greater amount of hybridization through introgression. Physiographic and ecological barriers dominate in the type 1 model, but have broken down in the type 2 situation, resulting in evolutionary ‘footprints’ (Patton, 1969). The pattern of chromosomal variation described here for *N. lapillus* appears to fit more closely with the type 2 model and not with the type 1 model favoured by some previous investigators (e.g. Hoxmark, 1970).

The hypothesis we propose to explain the chromosome number variation in *N. lapillus*, in the light of the new facts presented here, is that this represents an example of chromosomal evolution in action brought about by centric fusions. Related species in the Pacific, where the ancestor of *N. lapillus* has its origins (Kitching, 1985), have diploid chromosome numbers of 60 or 70 (Nishikawa, 1962: Ahmed, 1974), which suggests that the reduction in chromosome number in *N. lapillus* commenced after its ancestor invaded the North Atlantic from the Pacific, via the Bering Strait and Arctic Ocean, in the late Miocene/Pliocene (Briggs, 1970). It is interesting to record that Staiger (1950b) drew attention to unusually low chromosome number in *N. lapillus* compared to other stenoglossan prosobranchs.
Centric fusions are recognized to be the main way by which chromosome numbers have been changed during animal karyotype evolution (White, 1965, 1973; John and Lewis, 1968; Mayr, 1969). The fact that the $2n = 26$ form thrives on all shore types over the large part of its range, which extends on the eastern side of the Atlantic from northern Norway (73°N) to Portugal (36°N) and on the western side from 50° to 41°N (Hoxmark, 1970; Mayr, 1969), indicates that variation from this is not a necessary prerequisite for survival under any specific set of environmental conditions as determined by the level of wave action or other physical factors. The functional significance of chromosome number variation in *N. lapillus* is currently being investigated at the structural and molecular levels of chromosomal organization.

**Acknowledgements**

We wish to express our grateful thanks to Prof. Annetrudi Kress (University of Basel) for her painstaking translations of Staiger’s papers. We would also like to thank Dr John Green (Plymouth Marine Laboratory) for providing the Norwegian material, and Dr Eve Southward (Marine Biological Association) who kindly commented on an earlier draft of the manuscript.
Declaration of Coauthored publication

A coauthored paper is included in Chapter 1 of this thesis as part of the general introduction to the subject and relevant literature:


The percentage input the candidate made to each aspect of the collaborative work is declared below.

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intellectual</td>
<td>26%</td>
</tr>
<tr>
<td>Practical</td>
<td>75%</td>
</tr>
<tr>
<td>Literary</td>
<td>10%</td>
</tr>
</tbody>
</table>

First Author

Candidate

Signed  

Date  

10/5/01  

10/5/01  
Chapter 2. Structural chromosomal polymorphism in the dog-whelk *Nucella lapillus* (Mollusca: Neogastropoda)


Published in: Marine Biology, 118, 247-253.

2.1 Abstract

On the English and French Channel coasts the dog-whelk, *Nucella lapillus*, exhibits variation in chromosome number which appears to correlate with the degree of wave action on the shore. The more common, 2n=26 morph is typically found on exposed shores subjected to a high degree of wave action, whereas those with higher chromosome numbers, up to the recorded maximum of 2n=36, are restricted to more sheltered environments. The polymorphism is thought to be Robertsonian in nature, involving centric (centromere) fission or fusion, but detailed analysis of the polymorphism has been restricted by lack of success in labelling individual chromosomes. Using a silver staining technique for the nucleolar organiser regions (NORs), three pairs of chromosomes, in the basic 2n=26 karyotype, have been positively identified. A series of structural chromosomal rearrangements (pericentric and paracentric inversions) affecting one pair of chromosomes involved in the numerical polymorphism is described. Significant differences exist between populations with respect to this character. These chromosomal rearrangements have the potential to reduce the level of interbreeding between the different types, and may act as isolating mechanisms between breeding groups. Structural chromosomal polymorphism is likely therefore, to have greater significance in relation to adaptation than simple numerical variation. This finding raises important questions concerning the (cyto)taxonomic status of *N. lapillus* in different parts of its range.
2.2 Introduction

Over much of its wide geographic range the common dog-whelk *Nucella lapillus* (L.) has a karyotype consisting of 2n = 26 but in a restricted part of its range chromosome counts of 2n >26 are common (Staiger 1954; Bantock & Cockayne 1975). This variation is mainly confined to populations on the English and French Channel coasts, although a few sites outside this region have also been identified (Bantock & Page 1976; Pascoe et al., unpublished). Primarily, the polymorphism in *N. lapillus* is thought to be Robertsonian in nature, involving centric fusions or fissions affecting up to five pairs of median or submedian chromosomes in the 2n = 26 form. Staiger (1950a, 1954) working in the region around Roscoff, NW France, was the first to identify the numerical polymorphism in *N. lapillus*, and to describe the relationship between chromosome number and the degree of wave action on the shore. These observations were subsequently extended by Bantock and co-workers to include dog-whelk populations on the English Channel coast (Bantock & Cockayne 1975; Bantock & Page 1976). In both these studies, the 2n = 26 morph was reported to occur on exposed shores typified by high levels of wave action, whereas dog-whelks with higher chromosome numbers were restricted to sea coasts where the level of wave action was attenuated to some extent; the highest chromosome number form (2n = 36) occurred almost exclusively in sheltered bays and harbours where the effects of wave action were reduced to a minimum. These authors speculated on the possible adaptive significance of this relationship in relation to certain, polymorphic phenotypic characters, viz. shell morphology (Bantock & Cockayne 1975) and body size (Staiger 1957), but the fact that the numerical polymorphism occurs only in a small part of the species' range, which extends between latitudes 73°N and 36°N on the eastern side of the Atlantic (and between 50°N and 41°N on the western side), tends to support a genetic explanation, i.e. a hybrid zone or racial differentiation. Additional evidence (viz. intra-individual variation in
chromosome number) which casts further doubt over any direct relationship between chromosome number per se and phenotypic adaptation has recently been reported by Dixon et al. (1994).

The relationship between Robertsonian polymorphism in *Nucella lapillus* and ecological conditions has attracted considerable attention in the genetics literature since the time it was first described in the mid 1950s (e.g. Mayr 1969; White 1973), but detailed analysis has been severely limited by lack of success in labelling individual chromosomes (see Bantock & Page 1976, for discussion). Some progress has been made, however, using standard karyotyping procedures based on chromosome length, centromeric position and the presence of any secondary constrictions (e.g. Page 1988), but the identification of specific chromosomes, beyond this basic grouping, has not been possible.

The silver staining technique for the nucleolar organiser regions (NORs), the chromosomal regions encoding for ribosomal RNA (rRNA), was developed separately by Howell et al. (1975) and Goodpasture & Bloom (1975). It has proved a useful tool in cytogenetics for visualising secondary features (Howell & Black 1979), identifying specific chromosomes (Long & Dawid 1980), and for investigating the activity of the NORs themselves (Howell 1977). Essentially it is thought that metallic silver is deposited at the NOR site through reduction of ionic silver by the acidic or non-histone proteins associated with the rRNA. These proteins are known to be present only at active NOR sites, i.e. where rRNA was transcribed during the preceding interphase (Howell 1977; Hubbell 1985). More recently the potential of this technique has begun to be realised in marine biology and cytotaxonomy of molluscs (Dixon et al., 1986; Dixon & McFadzen 1987; Vitturi & Catalano 1989, 1990; Thiriot-Quiévreux & Insua 1992).
This paper reports on the results of an investigation to assess the use of silver-stained NORs as markers for studying numerical and structural chromosomal variation in *N. lapillus* populations.

2.3 Materials and Methods

Animals used in this study were mid-veliger stage embryos taken from egg capsules collected from various sites to include 2n = 26 and polymorphic populations. The sites were: Widemouth Bay (NE Cornwall), Whitsand Bay and Cawsand (SE Cornwall), Renney Rocks (SW Devon), St. Peter Port (Guernsey, Channel Is.) and Rade de Brest (NW France). A few spreads, obtained by the same method, from the testes of adult males (Whitsand Bay) and embryos from Polzeath (NE Cornwall) and West Runton (Norfolk), were also used (see Table 2.1).

2.3.1 Preparation of metaphase spreads

Embryos were taken from intact egg capsules and teased free from the early shell and remaining yolk. The tissue was given two, combined colchicine and hypotonic treatments: 0.08% colchicine in 50% sea water and 0.04% colchicine in 25% sea water, 30 - 45 min each, followed by a further hypotonic treatment, 0.075 M KCl, 2 x 5 min. Fixation was in Carnoy's solution (ethanol: acetic acid, 3:1) at 4°C, 3 changes, 20 min each (Meredith 1969). Slide making involved placing one or two of the fixed embryos on a clean microscope slide in a few drops of 60% acetic acid and allowing the cells to dissociate for a few minutes, before transferring to a hot-plate at 40°C for partial drying.
2.3.2 **NOR staining and karyotyping**

Silver staining of the NORs was achieved using the method of Gold and Ellison (1982). Spreads were karyotyped from photographic prints in the arrangement proposed by Page (1988), with centromere positions described according to the nomenclature of Levan *et al.* (1964). Thus in the 2n = 26 form the karyotype consists of five groups: group A - 4 pairs of large median chromosomes; group B - 2 pairs of sub-median chromosomes; group C - 4 pairs of medium-sized, median and sub-median chromosomes; group D - 1 pair of medium-sized, sub-terminal chromosomes; and group E - 2 pairs of small median/sub-median chromosomes (Dixon *et al.*, 1994).

Chromosome arm-lengths (short arm, p; long arm, q) were measured to the nearest 0.1 μm from 4800x photographic enlargements, using a Kontron IBAS Image Analyser. Total length (p + q), arm ratio (p/q) and relative haploid length ([(p + q/ total haploid length) 100] measurements were determined for each chromosome. NOR positions were recorded as a percentage distance from the centromere, described hereafter as 'np' or 'nq' depending on whether the NOR was on the short (p) or long (q) arm. Data recorded for each complete silver-stained spread were the total number of chromosomes, the number of NORs and on which chromosomes they occurred, noting if any were single (i.e. on one chromatid only), or multiple (more than one NOR on a chromatid), rather than the usual pair or double arrangement of one NOR on each chromatid. Attempts were made to karyotype polymorphic spreads into the conventional groupings (Dixon *et al.* 1994) assuming that the NORs occurred on the same chromosomes as in the 2n = 26 form. Only clearly stained NORs were used in the analyses.
2.4 Results

Silver-stained NORs usually appeared as a pair of brown or black spots, positioned interstitially, on both sister chromatids of some chromosomes (Fig. 2.1). However, in a small percentage of chromosomes (<3%) only one of the two chromatids showed any silver-staining, and in a few cases a double structure was observed at the NOR site indicating a possible duplication event or separation of the rRNA gene clusters (Long & Dawid 1980; Cheung et al., 1989).

The size of NORs, i.e. diameter (apparent intensity) of the spot, did exhibit some variation between slides, spreads and chromosomes, but the NORs always appeared as a spot and not a band extending along the chromatid arm. It is thought that this size variation was probably attributable to minor differences in stain composition or temperature, and although inter-chromosome variation may reflect differences in NOR activity (Howell 1977), no effort was made to record or quantify the size of NORs in this study.

2.4.1 2n = 26 karyotype

Previous analysis of the 2n = 26 karyotype of Nucella lapillus, comparing the parameters of relative haploid length and arm ratio for each chromosome pair, has shown this to be consistent over a large part of its geographical range, from Norway to NW Spain (Dixon et al., 1994). The positions of the NORs were found to be highly conserved in all the 2n = 26 populations which have been examined. With a few exceptions, the NORs were found on the short (p) arm of chromosome pairs 2, 7 and 10 (Fig. 2.1). Their positions with respect to the centromere were also surprisingly consistent considering the variation in chromatin condensation between the different spreads analysed. The mean np values for the NORs on chromosomes 2, 7 and 10 were 73.5% (SD = 4.9%), 29.2% (SD = 4.9%) and 34% (SD
= 5.5%), respectively (all based on over 50 measurements). Fig. 2.2 is an idiogram for the 2n = 26 karyotype of *N. lapillus* showing the total length, relative length, centromere position and location of the NORs for the different chromosome pairs, based on the mean values shown in Table 2.1.

<table>
<thead>
<tr>
<th>Chromosome Pair No.</th>
<th>Total Length (µm) (mean ± 2SE)</th>
<th>Relative Length (%) (mean ± 2SE)</th>
<th>Arm Ratio (mean ± 2SE)</th>
<th>NOR np % (mean ± 2SE)</th>
<th>Chromosome Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.72 ± 0.60</td>
<td>13.93 ± 0.22</td>
<td>0.796 ± 0.021</td>
<td>73.5 ± 0.662</td>
<td>m</td>
</tr>
<tr>
<td>2</td>
<td>8.72 ± 0.52</td>
<td>12.51 ± 0.19</td>
<td>0.838 ± 0.020</td>
<td></td>
<td>m</td>
</tr>
<tr>
<td>3</td>
<td>7.90 ± 0.49</td>
<td>11.32 ± 0.21</td>
<td>0.663 ± 0.028</td>
<td></td>
<td>m</td>
</tr>
<tr>
<td>4</td>
<td>7.32 ± 0.46</td>
<td>10.48 ± 0.16</td>
<td>0.734 ± 0.028</td>
<td></td>
<td>m</td>
</tr>
<tr>
<td>5</td>
<td>6.38 ± 0.40</td>
<td>9.16 ± 0.24</td>
<td>0.384 ± 0.018</td>
<td>29.2 ± 0.661</td>
<td>m</td>
</tr>
<tr>
<td>6</td>
<td>4.80 ± 0.27</td>
<td>6.91 ± 0.13</td>
<td>0.382 ± 0.020</td>
<td>34.0 ± 0.774</td>
<td>m</td>
</tr>
<tr>
<td>7</td>
<td>5.14 ± 0.30</td>
<td>7.39 ± 0.17</td>
<td>0.775 ± 0.033</td>
<td></td>
<td>m</td>
</tr>
<tr>
<td>8</td>
<td>4.39 ± 0.26</td>
<td>6.30 ± 0.14</td>
<td>0.597 ± 0.031</td>
<td></td>
<td>m/sm</td>
</tr>
<tr>
<td>9</td>
<td>4.15 ± 0.25</td>
<td>5.95 ± 0.09</td>
<td>0.614 ± 0.025</td>
<td></td>
<td>m/sm</td>
</tr>
<tr>
<td>10</td>
<td>3.67 ± 0.20</td>
<td>5.29 ± 0.12</td>
<td>0.720 ± 0.025</td>
<td></td>
<td>m</td>
</tr>
<tr>
<td>11</td>
<td>3.57 ± 0.19</td>
<td>5.13 ± 0.12</td>
<td>0.256 ± 0.013</td>
<td></td>
<td>st</td>
</tr>
<tr>
<td>12</td>
<td>2.26 ± 0.11</td>
<td>3.27 ± 0.11</td>
<td>0.785 ± 0.027</td>
<td></td>
<td>m</td>
</tr>
<tr>
<td>13</td>
<td>1.61 ± 0.07</td>
<td>2.35 ± 0.09</td>
<td>0.622 ± 0.031</td>
<td></td>
<td>m/sm</td>
</tr>
</tbody>
</table>

Table 2.1 Total chromosome lengths, relative lengths and arm ratios for *N. lapillus* from three 2n=26 populations: Whitsand Bay (13 spreads from testes), Polzeath and West Runton (15 spreads each, from embryos).

The number of chromosomes expressing NORs was found to vary considerably between spreads, and was often less than the nominal maximum of six. It was observed that some patterns of NOR expression i.e. combinations of the three pairs affected, were more common than others, and these heteromorphisms relating to the 2n = 26 form are detailed below.
Figure 2.1 *Nucella lapillus*: The karyotype of a 2n=26 embryo from Whitsand Bay, showing the NORs (arrowed) on chromosome pairs 2, 7 and 10. (Scale bar = 10μm)
Figure 2.2  *Nucella lapillus*: An idiogram of the 2n=26 chromosome form based on the mean figures for lengths and arm ratios given in Table 1. The positions of the NORs shown (black spots) are all based on means of over 50 measurements. (Scale bar = 5μm).

2.4.2 Variations in NOR activity

Two geographically separated 2n = 26 populations on opposite sides of the SW peninsula, several hundred kilometres apart, were selected for detailed comparison: Whitsand Bay (SE Cornwall) and Widemouth Bay (NE Cornwall). A small number of spreads from other 2n = 26 populations were also examined: N. Cornwall (Bude, Polzeath), Norway (Svartholmane, nr. Bergen), NW France (Brest) and NW Spain (Galician coast; J. Pasantes, pers. comm.); all showed NORs in the same positions as those described here. High levels of intra- and inter-individual variation in NOR activity appeared to be a characteristic of all those 2n = 26 populations we examined (Figs. 2.3A - D). These results show clearly that there was almost as much NOR variation between individuals in the same egg capsule as there was between sites. Of the three chromosome pairs containing NORs, pair 7 was the most stable in that the NORs appeared consistently on both chromosomes, whereas in pairs 2 and 10 NOR expression was more variable.
Figure 2.3 *Nucella lapillus*: Variation in NOR expression for different embryos from the same egg capsule from each of three populations: A & B - Whitsand Bay, C & D - Widemouth and E & F - Cawsand. Light columns represent one chromosome affected and black columns both chromosomes affected. The *n* values relate to the number of chromosome spreads analysed.

2.4.3 *Polymorphic populations*

Four *N. lapillus* populations exhibiting chromosome number variation (26<2n<36) were analysed using the silver-NOR technique: Cawsand and Renney Rocks (on either side of Plymouth Sound, SW Devon), St. Peter Port (Guernsey) and Pointe du Bindy (Rade de Brest). In common with 2n = 26 populations, there was considerable variation in the expression of NORs, both within and between individuals (e.g. Fig. 2.3, E & F), but it was
possible to distinguish spreads where NOR-bearing chromosomes were involved in the polymorphism, and others where this was not the case. In those spreads where the NOR-bearing chromosomes remained median, the NOR positions were 'normal' in most cases (see below), i.e. were located in the usual positions on pairs 2, 7 and 10.

Figure 2.4 *Nucella lapillus*: The karyotype of a 2n=32 spread from Renney Rocks showing the NORs (arrowed) on the product of one chromosome of pair No. 2, and pairs 7 and 10. (Scale bar = 5μm).
In 2n > 26 spreads where NOR-bearing chromosomes were involved in the polymorphism (e.g. Fig. 2.4), three different NOR-bearing products were recorded. These were identified by their relative size, arm ratio and NOR position not conforming to the normal configuration of pairs 2, 7 and 10. Nucleolar organiser regions occurred on the long (q) arm in all three cases, and their position with respect to the centromere and the relative length of the chromosome had changed. These were recorded as Q-types A, B and C with \( nq \) values of 50%, 81% and 34%, arm ratios of 0.81, 0.18 and 0.40, and relative lengths of 10.6, 6.5 and 6.4 respectively (Fig. 2.5A). Karyotyping evidence suggests that all three Q types were derived from chromosome pair 2 (as pairs 7 and 10 remained intact in all spreads karyotyped; see Fig. 2.4), but not all involved centric fission e.g. type A. However, the altered configurations, with the possible exception of type B, indicate that structural rearrangements (inversions) had to be involved. Schematic representation of the possible derivation of the Q types is shown in Fig. 2.5B. Evidence of population differences regarding the frequency of these different chromosomal rearrangements is presented in Table 2.2.
Figure 2.5 *Nucella lapillus*: (A) Examples of NOR-bearing chromosomes involved in the polymorphism; the 'normal' No. 2 chromosome, and Q types A, B and C. (Magnification: x4000). (B) Schematic representation of the proposed derivation of Q types A, B and C from chromosome No. 2. Small arrows indicate chromosome breakpoints and large arrows show the possible rearrangements and their products. Refer to Discussion. (Scale bars = 5 μm).
There appeared to be no direct correlation between the number of chromosomes and the NOR expression pattern, or the number of Q types. For example, some individuals with high chromosome numbers (2n=31-35) exhibited a high proportion of spreads with two Q type chromosomes, but these were not always spreads with the higher 2n values. In three other individuals (2n values up to 34), all from the same egg capsule, the frequency of spreads with two Q type chromosomes varied from over 50% to less than 5%. Other complicating factors observed in some polymorphic spreads included spreads with 7 pairs of NORs expressed, a 'normal' No. 2 chromosome and two Q types, or three Q type chromosomes (see Table 2.2). In a small number of spreads a different P-type chromosome (i.e. NOR positioned on p arm) was observed, conforming in size to chromosome 2 but with a np value of 35%, indicating an apparent paracentric inversion even when the chromosome was not contributing to the polymorphism (Table 2.2).

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of Embryos</th>
<th>No. of spreads</th>
<th>2n range</th>
<th>Mean No. of NORs</th>
<th>Q types</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None 1A 1B 1C 2C B + C B + 2C C + 'P'</td>
</tr>
<tr>
<td>Cawsand</td>
<td>4</td>
<td>85</td>
<td>29-32</td>
<td>4.7</td>
<td>84 1</td>
</tr>
<tr>
<td>Brest</td>
<td>6</td>
<td>30</td>
<td>27-31</td>
<td>4.5</td>
<td>27 3</td>
</tr>
<tr>
<td>Guernsey</td>
<td>10</td>
<td>37</td>
<td>29-34</td>
<td>4.2</td>
<td>13 14 3 3 3 1</td>
</tr>
<tr>
<td>Renney</td>
<td>7</td>
<td>137</td>
<td>29-35</td>
<td>4.9</td>
<td>21 1 81 28 3 1 2</td>
</tr>
<tr>
<td>Renney A</td>
<td>1</td>
<td>35</td>
<td>30-34</td>
<td>5.5</td>
<td>3 13 16 1 1</td>
</tr>
<tr>
<td>Renney B</td>
<td>1</td>
<td>26</td>
<td>29-34</td>
<td>4.9</td>
<td>1 21 1 1</td>
</tr>
</tbody>
</table>

Table 2.2 Frequency of structural chromosome rearrangements (Q types) in four numerically polymorphic populations of *N. lapillus*, including results for 2 individuals from the same egg capsule (Renney A & B).
2.5 Discussion

2.5.1 NOR expression and heteromorphism

The results of this investigation show that silver-stained NORs provide useful chromosomal markers for studying numerical and structural chromosomal polymorphism in *Nucella lapillus* populations. The technique has demonstrated constancy in the karyotype and this has allowed the positive identification of three pairs of median chromosomes in the 2n = 26 form. The unequivocal identification of all or some of the six NOR-bearing chromosomes in the karyotype has advanced the analysis of 2n = 26 and numerically polymorphic spreads significantly. However, the high levels of intra- and inter-individual variation linked with this character does reduce the value of the NOR technique for detailed analysis. For example, the suggestion of conserved characters or rearrangements within some polymorphic populations (Table 2.2) would be demonstrated with more certainty if all NORs were expressed. The higher frequencies of a single C type over two C types in the Renney population e.g. Renney B (Table 2.2), may be attributable to a lower number of NORs being expressed. Attempts are presently being made to overcome this problem by means of *in situ* hybridisation using rDNA probes.

2.5.2 Structural polymorphism

Previous workers (Staiger 1954; Ahmed 1974; Page 1988) have suggested that inversion polymorphisms occur in the karyotype of *Nucella lapillus*, but this has remained speculative until the present time. Similarly, Dixon *et al.* (1994) drew attention to the presence of so-called 'rabbit-ear' acrocentrics in the 2n >26 populations that could not be explained simply in terms of centric fission events, and which are suggestive of pericentric inversions (or centric shifts). Pericentric and paracentric inversions, coupled with centric fission in some cases, are required to produce the range of structural variants described here (Fig. 2.5B); alternatively, the reverse processes may have occurred, producing
chromosome 2 from the various Q types (see later). The 'A' type can be derived from chromosome 2 by a pericentric, or possibly paracentric, inversion, although from the relative length data it would appear that there may be a loss of genetic material, suggesting that a more complex rearrangement may be involved (see John & Freeman 1975). Q type B can be produced by a fission event, but simple centric fission does not fully explain the products observed, i.e. the break-point may not be at the centromere. To derive the Q type C from the B type it would appear that another inversion event (paracentric, i.e. not involving the centromere) is necessary to account for the change in NOR position. More precise interpretation of these events is not possible at present.

The other significant finding was the discovery that different polymorphic populations were characterised by different Q-types (Table 2.2). Although relatively few individuals were examined, the data presented in Table 2 suggest a possible difference in the frequency and types of structural rearrangements between different polymorphic populations, even though the 2n values lie within the same range. The four populations analysed were geographically separated (allopatric), but similar differences may also exist between populations living in close proximity (parapatric).

2.5.3 Causes and consequences of chromosomal polymorphism

There has been considerable debate on the causes and consequences of Robertsonian polymorphism (e.g. John & Freeman 1975; Searle 1988a). Evidence exists, particularly for mammals, that Robertsonian rearrangements may affect an individual's fitness, i.e., viability, fertility or physiology. Physiological changes may be brought about by loss of genes or chromosomal breakage at important loci; although there is little evidence that these effects are significant in mammals, they could be important in intertidal marine
invertebrates. Searle (1988a) discusses the evidence for disruption at meiosis in Robertsonian heterozygotes linked to the formation of multivalents in ring or chain formation at prophase I, and the increased frequency of anaphase I non-disjunction. These events act to suppress crossing-over between homologous chromosomes, or result in sterility or sub-fertility due to gamete disruption. The fitness of individuals and populations may be directly linked to these effects, and the addition of chromosomal inversions to the Robertsonian variation already described in *Nucella lapillus* is an important step towards understanding this relationship. The inversions and other rearrangements reported here may act as isolating mechanisms preventing successful breeding between individuals of different types, i.e. suppressing exchange (crossing-over) at meiosis thus conserving large linkage groups (super-genes) and acting to create and preserve two or more types or races on the same or different shores.

It has been assumed throughout this paper that the Robertsonian phenomenon in *N. lapillus* represents an example of centric fission. In view of the complex products identified for chromosome 2, it seems reasonable to suggest that centric fusions are not important in the chromosomal polymorphism. Other evidence suggests the trend is from low numbers to high numbers rather than *vice versa*: namely the occurrence of some 2n >26 cells in otherwise 2n=26 populations (e.g. Dixon *et al.*, 1994, Fig. 4), which indicates that centromeric instability may be widely spread throughout the range of the polymorphism. This topic will be dealt with in more detail in a later paper.

It seems clear that a link between chromosome number and shore type does exist within a small part of the species' range. However, for various reasons, e.g. high intra-individual numerical variation and the fact that the same (>26) number of chromosomes can be achieved by different structural rearrangements or involve different chromosome pairs in
different individuals or populations (personal observations), it is suggested that this link is indirect. Recent evidence on the phenotypic changes in progeny of *N. lapillus* transplanted from an exposed shore to sheltered inlets (Gibbs 1993a), where the phenotypic characters typical of a sheltered shore were expressed by 2n=26 progeny, also suggests that there is no direct relationship between shell morphology or body form and chromosome number. These parameters appear to be direct adaptations to the environment or responses to other stimuli, which can be expressed within one generation, without any apparent change in karyotype. The existence and maintenance of the 2n > 26 types is likely to be linked to isolating mechanisms brought about by structural variations, as reported here, or by the environmental conditions affecting gene flow within a population or area.

Questions remain concerning the genetic status of the structurally polymorphic forms of *N. lapillus*, i.e. whether they represent polymorphism, polytypy or speciation. Further work is required to determine the distribution of the different types or races, and whether they are geographically, ecologically or reproductively isolated.

**Acknowledgements.** The authors thank Dr. J. Pasantes (University of La Coruña, Spain), Dr. B. Heimdal (Institute of Fisheries and Marine Biology, Bergen) and M. Huet (University of Brest) for supplying some of the material. We are also indebted to Dr. J. B. Searle (Dept. of Biology, University of York), Dr. C. Thiriot- Quiévreux (Université P. & M. Curie, Villefranche-sur-Mer) and Dr. P. E. Gibbs (Plymouth Marine Laboratory) for commenting on an earlier version of the manuscript.
Chapter 3. Robertsonian polymorphism in the marine gastropod, *Nucella lapillus*: advances in karyology using rDNA loci and NORs


*Published in: Chromosoma, 104 (6), 455-460.*

3.1 Abstract

Previous studies of the Robertsonian polymorphism in the Atlantic dog-whelk, *Nucella lapillus* (2n = 26 to 36), have been limited by the inability to unequivocally identify individual chromosomes in the karyotype. This species, as with many other marine invertebrates, has proven largely refractory to the standard (mammalian) chromosome banding techniques. In this study, fluorescence in situ hybridization (FISH) using a rDNA probe was applied to the metaphase chromosomes of the 2n=26 and 2n=36 forms of *N. lapillus*. The results were compared to silver-staining of the nucleolar organizer regions (NORs). The FISH technique was shown to be more sensitive and less intrinsically prone to variation than the silver-staining method. An additional NOR / rDNA locus was observed in the 2n=36 form which to date, has not been seen in any 2n=26 population. The 2n=36 karyotype is described for a south-west U.K. population which differs from that reported previously in the literature. Robertsonian metacentrics are shown to correspond to at least one sub-telocentric (not two telocentrics) in the 2n=36 form.

3.2 Introduction

Over most of its large geographic range, the dog-whelk, *Nucella lapillus* (L.) has a chromosome number of 2n=26. However, in some areas, particularly on sheltered shores
on both sides of the English Channel, the diploid chromosome number may vary between 26 and 36. The numerical variation is thought to be due to a Robertsonian polymorphism, i.e., involving simple centric fission or fusion events, affecting five pairs of metacentric chromosomes of the 2n=26 form. A proposed link between chromosome number and the degree of wave action on the shore has been widely reported (Staiger, 1954, 1957; Bantock and Cockayne, 1975; Bantock and Page, 1976). Recent evidence shows that inter- and intra-individual variation in chromosome number often occurs in polymorphic populations (Dixon et al., 1994) and that structural chromosomal rearrangements (e.g. inversions) may also be involved (Page, 1988; Pascoe and Dixon, 1994). These observations and the apparent restricted geographical range of the polymorphism suggest the proposed relationship between chromosome number and shore type (exposure), is more complicated than previously suspected.

In order to further our understanding of the chromosomal polymorphism in *N. lapillus*, identification of most or all of the chromosomes in the karyotype is necessary. We have attempted to address this problem with different chromosome labelling and banding techniques but found that many of the standard methods (e.g., C-, G- and R-banding) do not work satisfactorily with this species or other marine invertebrates. Various techniques for C-, G- and R-banding and also several fluorochrome methods for differential staining of chromosomes (e.g. Quinacrine mustard, Chromomycin A, Hoechst 33258, DAPI, Distamycin + DAPI) have all been tried without any success to date (see Appendix 1 for methods). However, the recent successful application of a silver-staining method for nucleolar organizer regions (NORs) has helped to identify three pairs of chromosomes, one of which is often involved in the polymorphism (Pascoe and Dixon, 1994). However, the high intrinsic variation in staining expression with this technique
(Howell, 1977; Hubbell, 1985) severely restricts the use of this method for detailed karyotype analysis.

In an attempt to overcome these limitations, a ribosomal DNA probe was employed using fluorescence *in situ* hybridization (FISH) to visualize the sites of the NORs as potential chromosome markers, without the variability that typifies the silver-staining method. The rDNA probe was used in this study on metaphase chromosome spreads from both a 2n=26 and a 2n=36 form of *N. lapillus*. The 2n=36 form was recently discovered in an isolated population from Mount's Bay, S. Cornwall, which is the first time a population containing 2n=36 individuals has been found during our field surveys.

The aims of the present paper are threefold; to describe a 2n=36 karyotype of *N. lapillus*, to record the first application of FISH to the metaphase chromosomes of a marine mollusc, and to compare the results of FISH and Ag-staining techniques and assess their suitability for studying chromosomal variation in *N. lapillus*.

3.3 Material and methods

Two populations were selected from the south coast of Cornwall; Whitsand Bay and Mount's Bay, which were known to exhibit low (2n=26) and high (2n=35-36) chromosome numbers respectively. Chromosome spreads were made from early veliger-stage embryos extracted from intact egg capsules collected from the shore.

The preparation of metaphase spreads and details of karyotyping and Ag-NOR staining were described in an earlier paper (Pascoe and Dixon, 1994). Chromosome spreads were karyotyped from photographic prints in the arrangement proposed by Page (1988), with the centromere position described on the basis of arm ratio following the nomenclature of Levan *et al.* (1964). The terms metacentric and telocentric are used here in preference to median and terminal.
3.3.1 **Fluorescence in situ hybridization (FISH)**

*Material:* The application of FISH to *Nucella* chromosomes followed basic protocols for non-radioactive *in situ* hybridization as applied to medium or high copy number repetitive DNA sequences in mammalian metaphase chromosomes. Recently-made slides (1 - 5 days) were found to give the best results, although slides stored at room temperature (RT) with a dessicant, or frozen (-20°C), for several weeks also gave acceptable results. Pretreatment of the metaphase spreads e.g., with RNAse or protease, was found to be unnecessary as cytoplasmic contamination on slides made from *Nucella* embryos was generally low.

3.3.2 **Probe production and labelling**

The rDNA probe used for FISH to NORs in this study was a 2.9 kb fragment spanning the 5' end of the 18S rRNA gene to the 5' end of the 28S rRNA gene from *N. lapillus* (*sp*Nl 2.9). The probe was cloned from PCR products by one of us (S.J.P.) using standard techniques (Holland *et al.*, 1993). It was labelled with biotin-11-dUTP by nick translation using the BioNick kit (Stratagene) and purified by centrifugation through a Sephadex G-50 column and eluted in 10 mM Tris pH 8, 10 mM EDTA. Resultant stock solutions (10 ng/µl) were stored at -20°C until needed.

3.3.3 **Denaturation / hybridization**

Prior to hybridization, stock probe was diluted to 2 ng/µl with hybridization solution (50% deionised formamide, 10% dextran sulphate, 40 mM sodium phosphate, 1X Denhardt's solution and 0.1% SDS in 2XSSC at pH 7). This was then denatured by boiling for 5 min and kept on ice until needed. Chromosomal DNA was denatured by immersing the slides in 70% formamide in 2XSSC at 65°C for 1 min, then transferring to cold (-20°C) 70%
ethanol, followed by gradual dehydration through 90% and absolute ethanol, before air drying. Finally, 25 µl of denatured probe mix was pipetted onto each slide, a clean coverslip (22 x 50 mm) added and the edge sealed with rubber solution adhesive. The slides were incubated overnight in a humid chamber at 37°C.

3.3.4 Detection

The rubber solution was removed and the slides rinsed in 2XSSC at 42°C to remove the coverslips. This was followed by 2 washes in 50% formamide in 2XSSC (5 min each), 2 washes in 2XSSC (5 min each) and then 30 min in TNFM detection buffer (5% non-fat milk powder and 0.05% Tween 20 in 4XSSC) at 37°C. Avidin-FITC (100µl of a 4 µg/ml solution in TNFM) was applied to each slide, then overlaid with a parafilm coverslip and the slides incubated in a humidified box at 37°C for 20 - 60 min. The slides were given 3 washes (5 min each) in TNFM at 42°C then treated with biotinylated anti-avidin (100µl of a 2 µg/ml solution in TNFM), under a parafilm coverslip for 20 min at room temperature (RT). After two (5 min) washes in TNFM at 42°C, the preparations were given another incubation with avidin-FITC (as above) for 20 min at RT followed by two rinses in TNFM at 42°C then two rinses in 4XSSC + 0.05% Tween 20 at RT.

At this stage, slides can be first dehydrated through alcohols and air-dried or immediately stained and mounted as follows. Both methods have given satisfactory results. Propidium iodide (1 µg/ml solution) and DAPI (2.5 µg/ml solution) were used as counterstains, made up in anti-fade medium, e.g., 1,4-diazobicyclo-octane (DABCO) or those available commercially. Twenty five microlitres of mountant is sufficient for each slide (22 x 50 mm coverslips). Coverslips were sealed at the edges with clear nail-varnish. Slides can be viewed immediately or stored in the dark at 4°C for several days or even weeks before viewing, although the signal does fade with time.
Fluorescently-labelled metaphase spreads were viewed and photographed, using colour print or transparency film, with a Nikon Optiphot fluorescence microscope fitted with filters blocks for FITC and DAPI (B-2A, DM510 and UV-2B, DM400 respectively).

3.4 Results

3.4.1 The 2n=36 karyotype

A 2n=36 metaphase spread from a Mount's Bay embryo is shown in Fig. 3.1A. It is characterized by a low number of large metacentrics (2) and the predominance of medium-small telocentrics and sub-telocentrics. To aid comparison when karyotyped, the 36 chromosomes were arranged in the 13 pairs/5 groups form (Fig. 3.1B) used for the more common 2n=26 karyotype (see Page 1988; Pascoe and Dixon 1994). The figure shows 5 'pairs' of chromosomes are involved in the polymorphism, i.e., those corresponding to pairs 1, 2, 3, 8 and 9 of the 2n=26 karyotype. The metacentric chromosomes involved in the polymorphism give rise to a telocentric and a sub-telocentric chromosome in the 2n=36 form, suggesting that the polymorphism is not simply Robertsonian in character, as some structural changes are probably involved. For convenience, these telocentric and sub-telocentric combinations will be referred to as 'fission products', although from an evolutionary perspective, fusion may be the likely direction for the chromosomal changes. In some spreads, both products of chromosome pairs 1 and 8 appear to have short p arms.

The 2n=35 spreads from the Mount's Bay population were found to be heterozygous for chromosome pair 1, allowing a direct length comparison of a metacentric with the corresponding fission products; no significant difference in mean relative length was observed in the five 2n=35 spreads measured (12.33% for the metacentric and 12.71% for the fission products; p=0.45, df=8, using a two-sample t-test). Relative length = chromosome length x 100 / total haploid length.
Figure 3.1 *Nucella lapillus*: A. 2n=36 metaphase spread from a Mount's Bay embryo. B. The same spread karyotyped in the 13 'pairs' format. Scale bar represents 10 μm.
3.4.2 Ag-NOR staining

In *N. lapillus*, NORs appear as darkly staining spots, positioned interstitially, on both chromatids of some chromosomes. In the \(2n=26\) form, up to six NORs were expressed on the short (p) arms of chromosome pairs 2, 7 and 10 (Fig. 3.2A). However, considerable variation in the pattern of NOR expression was observed. A detailed description of this variation has been given in a previous study (Pascoe and Dixon, 1994).
In the 2n=36 spreads examined here, chromosome 2 is involved in the numerical polymorphism and the NOR is found on the corresponding telocentric fission product (see Fig. 3.2B). However, as reported for the 2n >26 spreads in the previous study, sub-telocentric NOR-bearing chromosomes also occur frequently and give rise to an observed maximum of eight NOR sites here in the 2n=36 population, whereas the maximum recorded in the 2n=26 form was six. NOR-bearing chromosomes not conforming to the three metacentric types observed in the 2n=26 form were previously referred to as 'Q types' (Pascoe & Dixon, 1994) and three different forms were recorded: a large metacentric (type A), a telocentric (type B) and a sub-telocentric (type C). In the present study, variation in the NOR expression, both between and within individuals, was also observed in the 2n=36 spreads, the number of NORs ranged from four to a maximum of eight. In the more common arrangements, Ag-NORs were expressed on chromosome pairs 7 and 10, together with various combinations of the telocentric (type B) and sub-telocentric (type C) fission products. Figure 3.2B shows a 2n=36 karyotype with eight NORs expressed; four on pairs 7 and 10, as in normal 2n=26 spreads, and two each of the telocentric and sub-telocentric NOR-bearing chromosomes. The sub-telocentric is illustrated here as being derived from chromosome 3, and not chromosome 2 as was proposed previously (Pascoe and Dixon 1994, see discussion). The large inter- and intra-individual variation in the Ag-NOR expression pattern recorded in this study of the 2n=36 form is summarized in Figure 3.3 (A-C). Spreads from some individuals or capsules had a higher frequency of the sub-telocentric NOR chromosome, with one or both members of the pair expressed, whilst in others the telocentric type was more common. Spreads with eight Ag-NORs, i.e. those in which both pairs of sub-telocentric and telocentric chromosomes were expressed, were not common. However, on one slide 13 of the 52 spreads analysed showed this arrangement
(Fig. 3.3C) whereas in other individuals, no spreads with the full complement of eight NORs were observed (Fig. 3.3, A and B).

Figure 3.3 Histograms showing variation in silver-stained NOR expression (A-C) compared with FISH signals using the rDNA probe (D), within and between different embryos from Mount's Bay (2n=36). A and B represent individual embryos from the same egg capsule, C represents three embryos from a different capsule. D - FISH results for three embryos from same capsule as A and B. Stippled columns indicate one chromosome affected, black columns indicate both chromosomes affected. T (2) = telocentric (chromosome 2), St (3) = sub-telocentric (chromosome 3) and n values are numbers of chromosome spreads analysed.

3.4.3 Results of FISH

2n=26 spreads: Figure 3.4A shows a typical result for a 2n=26 spread from Whitsand Bay. A total of six fluorescent signals on three pairs of chromosomes are clearly visible which is consistent with the maximum number of NORs expressed with the silver-staining method. The chromosomes often appeared 'puffy' with some loss of chromosome detail after the FISH treatment. However, centromere position and relative length were usually
discernible, allowing the identification of individual chromosomes and the position of signals to be recorded. The signals were found to correspond precisely with the 'normal' (2n=26) position of silver-stained NORs, i.e., all were placed interstitially on the p arms of chromosome pairs 2, 7 and 10. On chromosome 2 the signal was towards the terminal end whilst on chromosomes 7 and 10 it was near the centromere. In most high quality spreads, the full complement of six signals were observed, although some variation in the intensity of the signal within individual spreads was apparent (see Fig. 3.4A).

2n=36 spreads: In virtually all of the high chromosome number spreads (i.e., 2n=36) eight fluorescent signals were clearly visible (Fig. 3.4B). The signal-bearing chromosomes were identified as pairs 7 and 10, plus two of each of the telocentric and sub-telocentric fission products corresponding to metacentric chromosomes 2 and 3 (Figs. 3.3D, 3.4B), as shown in some spreads with silver-stained NORs (Fig. 3.2B). However, when the same spreads were silver-stained after the FISH method, some of the rDNA loci did not show as AgNORs (Fig. 3.4, C and D), demonstrating the variability and limitations of the silver-staining method due to transcriptional activity.
Figure 3.4 *Nucella lapillus*: A-C. Metaphase spreads with rDNA loci marked by fluorescent signals using FISH with a rDNA probe; A, 2n=26, six signals; B and C, 2n=36, eight signals. The less obvious signals are arrowed. D. Same spread as C, silver-stained for NORs; note that all FISH sites do not show AgNORs (*). Individual chromosomes are labelled: 7, 10, t (telocentric) and st (sub-telocentric). Scale bars represent 10 μm.
3.5 Discussion

This paper reports on the first successful application of the FISH technique to the chromosomes of a marine mollusc. Several points of relevance to the nature and significance of chromosomal polymorphism in *N. lapillus* have emerged. First, FISH is shown to have great potential for the labelling and identification of specific chromosomes in a species which has proved intractable using a variety of other 'banding' methods. As anticipated, the rDNA probe proved to be a more sensitive marker and less prone to variation than the Ag-NOR technique.

The presence of six NORs in the 2n = 26 form of *N. lapillus*, as shown previously by the Ag-NOR technique (Pascoe and Dixon 1994), was confirmed in this study using the rDNA probe. The discovery of 8 NORs/rDNA loci in at least one 2n = 36 population (i.e. Mounts Bay, S. Cornwall), appears to be a further example of polymorphism in this highly variable species (viz. shell variation, Crothers 1983; chromosomes, Staiger 1954, 1957; allozymes, Day 1990; rDNA, Patton 1995). Intra- and inter-specific variation in Ag-NORs has been reported for a wide variety of other species (e.g. Gold 1984; Thiriot-Quievreux and Insua 1992). At the intra-specific level, explanations have centred largely on variations in transcriptional activity. Other studies have reported NOR variation within species to be associated with centric fission (Babu and Verma 1985; Phillips and Ihssen 1985) and the movement (transposition) or 'jumping' of rDNA between blocks of heterochromatin on different chromosomes, with or without any associated chromosomal rearrangement (Gold 1984; Sanchez *et al.*, 1990; Hall and Parker 1995). At the phylogenetic level, a single pair of NORs appear to be a primitive (plesiomorphic) character, whereas higher NOR number and increased heteromorphism are thought to be...
more derived (apomorphic) characters (see Thiriot-Quiévreux and Insua 1992 and section 3.6).

In an earlier paper (Pascoe and Dixon 1994), it was assumed that chromosome 2 was the only large metacentric in *N. lapillus* bearing a NOR. The apparent change in NOR position relative to the centromere in polymorphic spreads was attributed to pericentric and paracentric inversion events (as an explanation of the sub-telocentric fission products). However, the findings of the present study using FISH have shown that the 'additional' NOR site occurs on chromosome 3 and not chromosome 2 as previously suspected. This finding has necessitated a revision of these earlier proposals, and it now seems likely that inversions accompanying these chromosomal rearrangements are much less common in this species than was previously indicated.

All five metacentric chromosome pairs involved in the Robertsonian polymorphism correspond to at least one sub-telocentric pair of chromosomes in the 2n = 36 form. This appears to contradict the interpretation of the simple centric fission/fusion model proposed by Staiger (1954). Visually, the combined lengths of the sub-telocentric and telocentric products often appear larger than their metacentric counterpart, but measurements have shown that these differences are not always significant. Sub-telocentric products and possible changes in chromosome length may be due to a number of different causes: a) pericentric inversions or centric shifts; b) the short arms may consist of genetically-inert heterochromatin, which may appear more visible after centric fission (see King, 1993), or appear less visible or may be lost altogether after fusion (Patton 1969; Garagna *et al.* 1995; Nanda *et al.* 1995) (no evidence of centromeric heterochromatin was found during our attempts to C-band *N. lapillus* chromosomes); c) the formation of new centromeres after
fission and the inactivation or eventual loss of centromeres after fusion (John and Freeman 1975). Further studies of this phenomenon are currently in progress.

The proposed relationship between chromosome number in *N. lapillus* and the degree of wave action on the shore (Staiger 1957; Bantock and Cockayne 1975) suggests that the 2n = 36 form is likely to inhabit extremely sheltered environments. This does not appear to be the case at Mount's Bay, a site regularly subjected to high energy conditions, but micro-ecological factors may serve to reduce the level of exposure. Clearly, the application of modern cytogenetic tools provides an excellent opportunity to elucidate the factors underlying this relationship between chromosome number and environmental conditions in a species which has less control over its internal and external environment than those (mammalian) species which have been the focus of much Robertsonian research to date (e.g. Searle 1988a, 1993).
The following sections (3.6, 3.7 and 3.8) are in addition to the above published paper and are included in this chapter as the methodology and advances in karyology are relevant here.

3.6 Application of the rDNA probe to *Ocenebra erinacea*

The above phylogenetic theory on the number of NORs within groups is supported by the application of FISH with the *Nucella* rDNA probe to chromosomes of a related muricid, *Ocenebra erinacea*. The results show rDNA loci/NORs on one pair of chromosomes only (Fig. 3.5). Phylogenetic and evolutionary studies have shown that *Nucella* is a member of the subfamily Ocenebrinae and therefore more closely related to *Ocenebra* than to species within the Rapaninae, but is clearly the derived form (Kool, 1993: Collins *et al.*, 1996).

![Figure 3.5](image)

*Figure 3.5* The application of FISH with the rDNA probe to metaphase chromosomes of *Ocenebra erinacea*. Fluorescent signals show on one chromosome pair only as confirmed in the interphase nucleus (asterisked).
3.7 Fluorescence in situ hybridisation (FISH) with a telomeric probe

3.7.1 Introduction

Telomeres are structures at the ends of chromosomes consisting of tandem repetitive DNA sequences and are known to have important roles in replication and stability of chromosomes. Increasing interest in their structure and function has developed throughout the 1990s; a broad and extensive literature has resulted (see, Kipling, 1995). Their relevance to this study is their proposed association with chromosomal breakage, rearrangements and stabilization. Meyne et al. (1990) used in situ hybridisation with the highly conserved vertebrate telomeric sequence (TTAGGG)_n to reveal the intrachromosomal distribution in 100 species of vertebrate. In many species (55) this sequence was found at non-telomeric (i.e. interstitial) sites, often in the pericentric regions of the chromosomes and this was discussed in terms of structural (fusion/fission) and evolutionary changes in chromosomes. The same sequence, (TTAGGG)_n, has been shown to occur in trypanosomes and slime-moulds (Van der Ploeg et al., 1984; Forney et al., 1987) and was found to be similar to the telomeric sequences of other simple organisms e.g. the ciliates *Tetrahymena* and *Glaucoma* (TTGGGG) and the plant *Arabidopsis* (TTAGGG)(Hastie and Allshire, 1989). Evidence of the important roles of telomeres and their relevance at interstitial sites increased (Schertan, 1990; Park et al., 1992; Schubert et al., 1992; Day, Marder & Morgan, 1993; Rossi et al., 1993) alongside improved visualisation techniques employing FISH (Ijdo et al., 1991; Balajee et al., 1994). At the time of this study, a probe for (TTAGGG)_n was being applied to marine invertebrates, i.e. polychaetes, by Jha et al. (1995). More recently, Slijepcevic (1998) discussed the involvement of telomeres in Robertsonian (Rb) fusion and proposed at least three possible mechanisms which can lead to Rb fusion in mammals, one or two of which are known to act in karyotype evolution.
Consequently, I applied the FISH technique with a probe for the vertebrate telomere sequence in the hope that it would also prove homologous to the telomeres in *N. lapillus*, and possibly identify the sites of fusion/fission relating to the Robertsonian rearrangements and also aid the identification of individual chromosomes in the karyotype.

### 3.7.2 Material and methods

The probe used was a 800bp fragment (Bam H1 – Bg 72) of (TTAGGG)$_n$, kindly provided by the Department of Genetics, University of Cambridge. Methods for FISH using this probe were essentially the same as those described earlier for the ribosomal probe (see 3.3.1). Metaphase chromosomes prepared using *N. lapillus* embryos from Whitsand Bay (2n=26) and Mount’s Bay (2n=35-36) were hybridised with the above probe.

![Figure 3.6](image)

**Figure 3.6** The application of FISH with a telomeric probe (TTAGGG)$_n$ to metaphase chromosomes of *Nucella lapillus* (2n >26). Fluorescent signals show at each telomere and a few interstitial sites (arrowed)
3.7.3 Results and Discussion

Moderate success was obtained in that most chromosomes showed fluorescent signals at the end of each chromatid and in some cases at interstitial sites also (Fig. 3.6). There was some suggestion that interstitial sites shown in the 2n=26 spreads might be on metacentric chromosomes and therefore could mark locations of fusion/fission events. In the few trials made here, the signals were often rather faint which made detailed comparisons and analyses of the two karyotypes inconclusive. However, in support of this theory, Nomoto et al. (2001) have recently confirmed the (TTAGGG)$_n$ sequence in molluscan telomeres and suggest that those observed at interstitial sites coincide with presumed points of fusion in Robertsonian rearrangements.

Further development and refinement of either the probe homology/compatability or the detection methods are required before the sites of telomeric sequences in \textit{Nucella} can be discussed in relation to the Robertsonian polymorphism and other structural rearrangements. Time and funding restrictions did not allow this research to be continued, but considerable potential exists with this method to further our knowledge of the structural, and possibly evolutionary, chromosomal changes in this species. For example, recent studies have shown that the use of DNA probes in molecular cytogenetics, particularly with the FISH technique, can provide useful insights into karyotype variation and evolution in several other groups of organisms (Humans/primates/mammals – Rettenberger \textit{et al.}, 1995; FergusonSmith, 1997; Kasai \textit{et al.}, 2000; Muller \textit{et al.}, 2000; Finelli \textit{et al.}, 1999. Muntjac/cattle – Fronicke \textit{et al.}, 1997. Fish – Martins & Galetti, 1999; Inafuku \textit{et al.}, 2000. Plants – Tagashira & Kondo, 2001).
3.8 Proposal for Nomenclature

In order to facilitate description and classification of the karyotypes found in *N. lapillus*, I propose a system of nomenclature based initially on the scheme used by Searle (1986b) for the Robertsonian polymorphism of the common shrew. Searle used the abbreviations M, H and A to indicate whether the variable elements (chromosome arms) are in a homozygous metacentric, heterozygous or homozygous twin-acrocentric state, respectively. Although in the case of *Nucella* there is not a true ‘twin-acrocentric’ state as one or both of the fusion elements are sub-telocentric or even sub-metacentric, I propose to follow the same scheme (Fig. 3.7). I also dispense with the ‘M’ category by assuming this to be the ‘normal’ or ‘fixed/derived’ form. Therefore, in a polymorphic karyotype, only chromosome pairs that are heterozygous (H) or homozygous ‘acrocentric’ (A) are named, all others are assumed to be in the homozygous metacentric state of the 2n = 26 form. For example, a karyotype designated H1,8,9; A2,3 would have a diploid number of 2n = 33 with chromosome pairs 1, 8 and 9 having one of the pair in the acrocentric state, i.e. heterozygous for the polymorphism and pairs 2 and 3 having both chromosomes as two fusion elements, i.e. homozygous (Fig. 3.8). The presence of inversions also requires notation. This will be made when the designated chromosome differs in appearance from that observed in the 2n = 26 form by giving the chromosome pair number followed by the letter ‘I’ (for inversion), e.g. 5I, or 55I when both chromosomes of pair 5 show the inversion. The inversions observed in this study will be described in Chapter 4.
Figure 3.7  Nomenclature shown in idiogram form for chromosome pair 2. Scale Bar = 5\(\mu\text{m}\).

Figure 3.8  Idiogram showing form of the polymorphic chromosomes in a karyotype designated H 1,8,9; A 2,3
Chapter 4. Geographical variation in the karyotype of *Nucella lapillus*

4.1 Introduction

Geographical variation in chromosome number and arrangement features in many evolutionary studies on both animal and plant species (White, 1978; Sharma & Sharma, 1983, 1984; King 1993). The examples are far too numerous to review here but there are some classic cases in which Robertsonian fusion/fission events occur that are relevant to this study. Rodents have been widely studied and provide several examples of Robertsonian variation leading to chromosomal races, sub-species or full speciation. Yosida (1973, 1980) has recorded and debated the effects of Robertsonian fusion and fission events in the karyotype evolution of the black rat (*Rattus* spp., 2n=38 to 42) throughout its wide distribution. The house mouse (*Mus musculus*, 2n=22 to 40) has been extensively studied as over 40 Robertsonian races have been recorded in Europe and North Africa, and 60 are known throughout the world (Winking et al., 1988; Hauffe and Searle, 1998). Both wild-caught and laboratory strains make convenient subjects for heterozygosity, fertility and hybrid zone studies (Capanna, 1982; Hauffe and Searle, 1998). However, as single Rb fusions, multiple independent Rb fusions, multiple Rb fusions with monobrachial homologies and reciprocal translocations are all known to occur in the mouse, the system is perhaps too complex to make comparisons with our current knowledge of the variation in *Nucella lapillus*. The common shrew (*Sorex araneus*), the house musk shrew (*Suncus murinus*, 2n=30 to 40) and mole-rats (*Spalax* spp., 2n=38 to 62) are further examples of small mammals in which the geographical variation in Rb karyotype has been well studied (Searle, 1986a, 1988b; Yosida, 1982; Nevo et al, 1994). The other classic mammalian cases often cited are within the genus *Lemur* and muntjac deer (*Muntiacus* spp.). In lemur, 29 fusion rearrangements distinguish six species and
seven subspecies (Rumpler & Dutrillaux, 1976). Muntjac, or barking deer, show a remarkable difference in chromosome number between morphologically similar species. A subspecies of the Indian muntjac (Muntiacus muntjak vaginalis) has the lowest diploid number known in any vertebrate (2n=6 in the female, 2n=7 in the male) whereas the Chinese muntjac (Muntiacus reevesi) has 2n=46 chromosomes. This is thought to be brought about by a series of tandem fusions and Rb fusions (Liming et al., 1980), so the similarities to the polymorphism in Nucella, which mainly consists of multiple independent Rb fusions, may be limited.

Some genera or species of orthopteroid insects (John, 1983), fish (Ojima, 1983) and reptiles (Bickham, 1984) also show possible links between Rb fusion/fission, geographical distribution and evolution. Whether karyotype variation is a cause or an effect of geographical distribution is often the question discussed in evolutionary studies. The common features in many of these groups with high karyotypic variability are (a) low vagility, (b) small deme size or environments with limited niches, and (c) high prolificity (Arnason, 1972). Although Nucella may not be classed as highly prolific, on the other counts it would perhaps serve as the extreme example.

In early studies on N. lapillus, the Robertsonian polymorphism was thought to be restricted to the Channel coasts of England and France (Staiger, 1954; Mayr, 1969; Hoxmark, 1970; Bantock and Cockayne, 1975). Subsequent studies recorded the polymorphism in two other areas of the UK, namely Pembrokeshire and the Firth of Clyde (W. Scotland) and also suggested the presence of inversion polymorphisms in some populations (Bantock & Page, 1976; Page, 1988). No further data on the wider geographical variation have been published until those of Dixon et al. (1994, see Chapter 1).

One of the intriguing anomalies to date is that Page (1988) identified the chromosomes involved in the Robertsonian polymorphism as being in groups A, B and C, presumably
two, one and two pairs respectively. My attempt at karyotyping her 2n = 36 spread in the format used here suggests a H1,3;A2,5,8,9 arrangement (Fig 4.1) which immediately implies that 6 pairs of chromosomes are involved in the phenomenon.

The 2n = 36 karyotype found in this study (Fig. 4.2, rearranged slightly from that shown earlier as Fig. 3.1) has the form A1,2,3,8,9. So, are there 6 (or more) pairs of metacentrics in the 2n = 26 form involved in the Robertsonian polymorphism, and are there also pericentric inversions in addition to this?

Figure 4.1 *Nacella lapillus*: 2n = 36 karyotype from Lulworth Cove, Dorset.
In this Chapter, an attempt has been made to record and analyse the karyotypes and/or chromosome numbers from 70 sites throughout the geographic range of this species. The results give a good insight into the huge variation that exists, and extend our knowledge of the Robertsonian and other polymorphisms in this species. The following points are addressed:

a. Whether the karyotype varies for a given chromosome number.

b. Whether the theory of only 5 chromosome pairs being involved in the polymorphism is correct (Bantock & Cockayne, 1975).

c. Whether all 243 (i.e. $3^5$) possible types (Bantock & Page, 1976) are likely to occur.

d. Whether there are any geographical patterns or micro-geographical factors which influence the karyotype.

Figure 4.2  $2n = 36$ karyotype from Mount’s Bay, Cornwall.
e. Whether there is any correlation between numerical and inversion polymorphisms?

4.2 Material and Methods

Sampling of *N. lapillus* specimens (mainly embryonic) was made on an opportunistic basis between 1992 and 1999, from sites throughout the large geographic range. A strategic sampling programme was not implemented as no direct funding was available for this project. Early veliger stage embryos were favoured for the production of metaphase chromosome spreads as quantity and quality were higher and cytoplasm contamination was lower than in adult testicular tissue. Recently-laid egg capsules were maintained in tidal tanks until embryos developed to this stage. Methods for tissue preparation and slide-making are given in Chapter 2 (2.3.1).

Chromosome numbers were recorded for several individual embryos and/or adults from all populations (see Table 4.1) and a number of the better quality spreads were karyotyped as described in Chapter 2 (2.3.2). The number of developing embryos in each egg capsule was also recorded for most populations (see Fig. 6.1).

Several factors can lead to apparent variation in number and form of chromosomes during these techniques and hence great care is needed to avoid misinterpretation when analysing karyotypes. Experience and familiarity with the material in combination with the few positive chromosome markers achieved in this study, usually resulted in a clear karyotype. Unusual or new forms remained speculative until others of the same type were found and evidence through marked chromosomes gave support to the proposed new karyotype.
4.3 Results

4.3.1 Numerical variation

The results of the survey are summarised in Table 4.1. Sampling sites around the UK are listed in counter-clockwise order from Lands End eastwards and include several offshore islands (Burgh Island, Isle of Wight, Orkney, Coll, and Isle of Man). The Channel Islands are included later with samples from other countries (Norway, Ireland, France, Spain, Portugal and 3 sites from Maine, USA). The survey shows that the numerical polymorphism in this species is extremely widespread and may follow some general geographic patterns. An overview of the geographic variation is illustrated in Fig 4.3 which highlights the main new findings.
Table 4.1 Summary data for *Nucella lapillus* - Geographical variation in chromosome numbers and karyotype. Confirmed karyotypes are separated by a colon. (continued overleaf)
<table>
<thead>
<tr>
<th>Site</th>
<th>Position</th>
<th>Embryos (Adults)</th>
<th>No. of Chromosome (polymorphic chromosomes)</th>
<th>Karyotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Animals Examined</td>
<td>Spreads counted</td>
<td>Spreads Karyotyped number</td>
</tr>
<tr>
<td>St. Andrews</td>
<td>56° 20.7' N, 02° 47.5' W</td>
<td>5</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Burray (Gims Holm)</td>
<td>58° 52.8' N, 02° 54.3' W</td>
<td>5</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>Hoy</td>
<td>58° 54.95' N, 03° 18.8' W</td>
<td>5</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>Kyle Rhea</td>
<td>57° 13.2' N, 05° 39.5' W</td>
<td>(4)</td>
<td>(1)</td>
<td>1</td>
</tr>
<tr>
<td>Coll</td>
<td>56° 37.1' N, 06° 32.6' W</td>
<td>10</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>St. Helens</td>
<td>54° 40.2' N, 03° 33.1' W</td>
<td>5</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>IOM, Port St. Mary</td>
<td>54° 03.9' N, 04° 43.7' W</td>
<td>15</td>
<td>120</td>
<td>10</td>
</tr>
<tr>
<td>Gower</td>
<td>51° 34.3' N, 04° 06.2' W</td>
<td>5</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Clovelly</td>
<td>51° 00.3' N, 04° 24.0' W</td>
<td>10</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>Bude</td>
<td>50° 49.9' N, 04° 33.8' W</td>
<td>25 (13)</td>
<td>130 (11)</td>
<td>30+</td>
</tr>
<tr>
<td>Northcott</td>
<td>50° 50.6' N, 04° 33.8' W</td>
<td>10</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Widemouth Bay</td>
<td>50° 46.8' N, 04° 33.2' W</td>
<td>15</td>
<td>135</td>
<td>30+</td>
</tr>
<tr>
<td>Polzeath</td>
<td>50° 34.8' N, 04° 55.7' W</td>
<td>3 (13)</td>
<td>40 (13)</td>
<td>30+</td>
</tr>
<tr>
<td>Sennen</td>
<td>50° 04.5' N, 05° 41.5' W</td>
<td>2</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Norway-Svartholmane</td>
<td>60° 14.0' N, 05° 14.0' E</td>
<td>8</td>
<td>53</td>
<td>30+</td>
</tr>
<tr>
<td>Norway-Snaitholmen</td>
<td>60° 16.3' N, 05° 14.0' E</td>
<td>6</td>
<td>100</td>
<td>30+</td>
</tr>
<tr>
<td>Galway</td>
<td>53° 15.0' N, 09° 03.0' W</td>
<td>2</td>
<td>24</td>
<td>7</td>
</tr>
<tr>
<td>Guernsey, St. Peter Pt.</td>
<td>49° 27.2' N, 02° 31.6' W</td>
<td>15 (2)</td>
<td>40 (3)</td>
<td>30+</td>
</tr>
<tr>
<td>Sark, Dixcart Bay</td>
<td>49° 25.3' N, 02° 21.5' W</td>
<td>5</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Greve St. Michel</td>
<td>48° 41.0' N, 03° 35.5' W</td>
<td>4</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Roscoff 1 North</td>
<td>48° 43.7' N, 03° 59.0' W</td>
<td>(4)</td>
<td>(4)</td>
<td>4</td>
</tr>
<tr>
<td>Roscoff 2 West</td>
<td>48° 42.3' N, 04° 03.5' W</td>
<td>5</td>
<td>50</td>
<td>30+</td>
</tr>
<tr>
<td>Ile de Siec</td>
<td>48° 19.7' N, 04° 36.3' W</td>
<td>7</td>
<td>46</td>
<td>15</td>
</tr>
<tr>
<td>Brest 20 (22)</td>
<td>48° 16.7' N, 04° 19.5' W</td>
<td>10</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Brest 07 (35)</td>
<td>47° 50.4' N, 04° 20.9' W</td>
<td>7</td>
<td>95</td>
<td>30+</td>
</tr>
<tr>
<td>Pte. De la Torche</td>
<td>47° 24' N, 08° 11' W</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Spain (NW, Centrofia)</td>
<td>37° 00' N, 08° 56' W</td>
<td>10</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>Portugal (SW, Zavial)</td>
<td>44° 25' N, 08° 22' W</td>
<td>10</td>
<td>27</td>
<td>10</td>
</tr>
<tr>
<td>USA 1 Otter Cove SP</td>
<td>44° 15' N, 08° 15' W</td>
<td>2</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>USA 2 Otter Point</td>
<td>44° 13' N, 08° 14' W</td>
<td>8</td>
<td>130</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 4.1 Summary data for *Nucella lapillus* - Geographical variation in chromosome numbers and karyotype. (continued from previous page)
Figure 4.3 A summary of the distribution of chromosome number variation in *N. lapillus* at a selection of the sites examined in the N. Atlantic. Red sectors in the pie diagrams relate to the mean number of chromosomes exhibiting the Robertsonian polymorphism, each chromosome is represented by 36° (see Appendix 2, Fig. A2.3). e.g.
The highest chromosome numbers (2n = 36) are found in populations from SW Cornwall and NW Brittany on directly opposing shores of the English Channel. Populations with intermediate diploid values i.e. within the range 2n = 28 and 2n = 35, have been recorded in several areas: e.g. at sites along the South coast of the UK, including one site in the far SW of Cornwall (Sennen Cove), in the Channel Islands, near Brest, and on the West coasts of Ireland and Scotland. However, as found by Bantock & Cockayne (1975), these populations are interspersed with those which exhibit only the 2n = 26 karyotype. Samples from the North Kent coast and northwards along the UK East coast are sparse but show only 2n = 26 karyotypes until the Orkney Islands, north of Scotland where spreads of 2n = 27 to 32 are recorded. The polymorphism is found to a greater or lesser degree throughout the West coast of the UK and Ireland but again as recorded by Bantock & Cockayne (1975), the North coast of the SW peninsula exhibits only the monomorphic, 2n = 26 form. In the other countries, towards the extremes of the species’ range, the polymorphism is recorded here for the first time in Norway and the USA; however, only 2n = 27 and/or 28 spreads were observed. Sites sampled in Spain and Portugal, as recorded previously (Chapter 1), exhibited only 2n = 26 karyotypes.

4.3.2 Karyotypic variations and inversions

The identification of most of the chromosomes, through the labelling techniques described earlier, is a considerable aid to analysing the karyotypic variations found in this study. However, the intrinsic variability of metaphase chromosomes in these techniques as well as the inevitable occasional loss and gain of chromosomes during slide-making renders this analysis a time-consuming and arduous task. The previously proposed inversion polymorphisms (in pairs 4 or 5 and 8 or 9; Page, 1988) have also been found here in some populations and further complicate analysis and interpretation. The simplest and perhaps most logical approach is to deal with the lowest chromosome numbers first.
4.3.2.1 2n = 26 form

The 2n=26 karyotype (Figs. 1.2 and 2.1) is both common and consistent throughout the eastern Atlantic (Norway to Portugal) as reported and illustrated in Chapters 1 and 2 (Dixon et al., 1994; Pascoe and Dixon, 1994). Only a few sites have produced spreads that show slight deviations from the 'standard' form of this karyotype. This phenomenon has not been studied in detail as it seems to be neither common nor consistent in any population. The variation observed involves change of form in one or more of chromosome pairs 4, 5, and 8. Pairs 4 and 5 are of similar size, normally being clearly metacentric and sub-metacentric respectively; at times the four chromosomes are represented by one of each of these types and two which are intermediate between the classes, i.e. an arm ratio of around 0.6. Through observations of numerous karyotypes, this is tentatively interpreted as pair 4 being slightly less metacentric than usual and pair 5 being heterozygous for an inversion leaving one chromosome as clearly metacentric. This condition is not uncommon in 2n >26 spreads, as shown in several of the following figures (e.g. Fig. 4.6), but can lead to problems and uncertainties in karyotyping. In addition to this, but not necessarily concurrent, the chromosomes of pair 8 sometimes appear virtually telocentric, as observed in approximately 10% of the spreads from Slaattholmen and North Foreland (Figs. 4.4 and 4.15). These 'abnormal' chromosomes are interpreted as the product of inversions, as suggested by Page (1988), or possibly more complex rearrangements, e.g. translocations. These findings in some respects help to explain the immense difficulties cytogeneticists have had with the karyology of this species, but in other ways can be an aid to karyotype analysis in some populations which do not immediately conform to known types.
4.3.2.2 \(2n = 27\) Karyotypes

Populations with individuals having this chromosome number are relatively common and widespread. One question is answered immediately: not all \(2n = 27\) karyotypes are the same. Five different types are shown in Figures 4.5 to 4.11. One type is found on the South and East coasts of Kent, where several populations have some individuals with the H9 arrangement (Fig 4.5). This is occasionally accompanied by an inversion in one chromosome of pair 5 which appears as a metacentric (Fig. 4.6). Undoubtedly pair 9 is frequently involved in the polymorphism, but these 27s maybe a slightly special case and are discussed again in Chapter 6.

Figure 4.4 \(2n = 26, 881\) karyotype from Slaattholmen, Norway.
Figure 4.5  $2n = 27$, H9 karyotype from Dumpton Gap, Kent

Figure 4.6  $2n = 27$, H9, 5Į* karyotype from Dumpton Gap, Kent
Figure 4.7  $2n = 27$, H4 karyotype from Maine, USA.

Figure 4.8  $2n = 27$, H4 karyotype from Slaattholmen, Norway.
Figure 4.9  $2n = 27$, H4 karyotype from the Isle of Man

Figure 4.10  $2n = 27$, H3 ($551^*$) karyotype from the Isle of Wight.
Several populations exhibit $2n = 27$ types in the H4 arrangement. This has not been recorded previously and is quite common in the populations where it occurs, e.g. Maine (USA), Slaattholmen (Norway), Isle of Man and some in Kent (Figs. 4.7, 4.8 and 4.9).

H3 also occurs, but not frequently and is shown here with pair 5 in the metacentric form, i.e. homozygous for the inversion (Fig. 4.10) in a spread from Horse Ledge, Isle of Wight.

In the animals sampled from Cuckmere Haven, on the Sussex coast, H5 spreads are common. These comprise a normal submetacentric 5, a smaller submetacentric and a small subtelocentric (Fig. 4.11). N.B. It is possible to interpret this arrangement as H4 (55I), but further labelling techniques to identify these chromosomes would be required to clarify this.

![Figure 4.11](image)

Figure 4.11  $2n = 27$, H5 karyotype from Cuckmere Haven, Sussex.
In some populations exhibiting the $2n = 27$ form, the homozygous arrangements, i.e. $2n = 26$ or 28, were also found, but in others the individuals karyotyped were monomorphic for the $2n = 27$ form (see Chapter 6).

Chromosome counts from cells of most polymorphic individuals often vary around a modal value, and karyotyping is usually essential to confirm the diploid number is correct and that the variation is not due to loss or gain of chromosomes during slide making. However, in a few cases several spreads from the same individual or population were found that appeared to be aneuploid, i.e. a $2n = 27$ form which karyotypes as 13 pairs of chromosomes in the 5 main groups but leaves an extra sub-telocentric chromosome, similar in form to pair 11 (Fig. 4.12).

![Figure 4.12](image-url) 2n = 27, aneuploid* spread (type X) from Old Stairs Bay, Kent.
This was recorded in a population at Old Stairs Bay (Kent); 46 spreads were analysed from 6 embryos, of which 30 were ‘normal’ $2n = 26$ karyotypes and 16 were $2n = 27$. Of these 16, eight karyotyped as H9 and eight as ‘type X’ (aneuploid), seven of which were from the same individual embryo. Molecular markers or paints for the relevant chromosomes would be needed to confirm if these are examples of true aneuploidy or another karyotype rearrangement (e.g. translocations). At present, this type must remain an enigmatic form.

So, even in the lowest polymorphic number there is considerable variation, including chromosomes that have not been previously recorded as polymorphic (pair 4), and also evidence of inversions as well as Robertsonian rearrangements in the same chromosomes (pair 5), and possible evidence of aneuploidy (type X).

### 4.3.2.3 $2n = 28$ variations

As chromosome number increases, karyotyping spreads with certainty is more difficult, particularly when the large metacentric chromosomes of group A are involved. A spread from Slaattholmen, Norway illustrates this (Fig. 4.13); it is classed as A3 based on size and arm ratios of the others in group A, but there are other possibilities. The $2n = 27$ spreads from the same population were classed as H4 (Fig. 4.8), but if this spread was designated A4, the others in the group would differ to some extent from their ‘normal’ size and arm ratio (see Table 2.1). The same would apply if this was classed as A2. The A4 spread from Dover (Fig. 4.14) is less ambiguous. Usually when chromosomes from group C are involved (smaller metacentrics, pairs 8 and 9) there are fewer problems with karyotyping, e.g. A9 spreads (Fig. 4.15) and similarly with H4,9 forms (Fig. 4.16).

### 4.3.2.4 $2n = 29-30$

Permutations increase further in populations/cells with 29 or 30 chromosomes. In Figs. 4.17 to 4.22 it is evident that several arrangements are possible with given chromosome
pairs being hetero- or homozygous for the polymorphism. Inversions also appear in some of these populations (e.g. in both chromosomes of pair 4 in Fig. 4.19).

4.3.2.5 $2n = 31$ to $34$

Figs. 4.23 to 4.32 illustrate some of the wide range of polymorphic karyotypes in this numerical range. Most of the karyotypes here have been confirmed through evidence from the chromosomal markers developed in this study, others remain speculative. The major difficulty involves pairs 4 and 5 which are of similar size and often have inversions that render these pairs indistinguishable. The only population found with putative karyotypes showing both pairs 4 and 5 to be polymorphic is from St. Peter Port, Guernsey (Fig. 4.26). These have been studied with the aid of AgNOR markers (Table 2.2) but nevertheless further evidence is needed to confirm these arrangements.

4.3.2.6 $2n = 35$ to $36$

Populations with this diploid range have been recorded at only three sites in this study. Mount's Bay, Cornwall and Roscoff, N. Brittany both show the same arrangement with chromosome pairs 1,2,3,8 and 9 involved and in $2n = 35$ spreads, pair 1 appears to be heterozygous (Figs. 4.33 to 4.36). At Looe, S. Cornwall, the $2n = 36$ karyotype is the same (A1,2,3,8,9) but the $2n = 35$ spreads have chromosome 2 in heterozygous form. The $2n = 36$ spread of Page (1988) from Lulworth Cove (Fig. 4.1; H1,3; A2,5,8,9) differs from those recorded here and spreads karyotyped in this study from Lulworth or nearby Kimmeridge did not show pair 5 to be involved in the polymorphism.
Figure 4.13  $2n = 28$, A3 karyotype from Slaattholmen, Norway.

Figure 4.14  $2n = 28$, A4 karyotype from Dover, Kent.
Figure 4.15 2n = 28, A9, 81* karyotype from North Foreland, Kent.

Figure 4.16 2n = 28, H4,9 karyotype from Folkestone, Kent.
Figure 4.17  $2n = 29, \text{H}9$; A3 karyotype from Jennycliff, Plymouth Sound

Figure 4.18  $2n = 29, \text{H}1,8,9$ karyotype from Newlyn, S. Cornwall
Figure 4.19  \(2n = 29, H3,8,9; 44I^*\) karyotype from Western Combe Cove, S. Devon

Figure 4.20  \(2n = 29, H5,8,9\) karyotype from Rade de Brest, N.W. France
Figure 4.21  $2n = 30$, A8,9 karyotype from Horse Ledge, Isle of Wight

Figure 4.22  $2n = 30$, H1,8; A9 karyotype from Thurlestone, S. Devon
Figure 4.23  $2n = 31$, H9; A3,8 karyotype from Western Combe Cove, S. Devon

Figure 4.24  $2n = 31$, H9; A3,8 karyotype from Rade de Brest, N.W. France.
Figure 4.25  $2n = 31$, $H1,3,4,8,9$ karyotype from Hanover Point, Isle of Wight.

Figure 4.26  $2n = 31$, $H1,2,4$; $A5$ karyotype from St. Peter Port, Guernsey
Figure 4.27  $2n = 32$, A1,3,8 karyotype from Renney Rocks, S. Devon

Figure 4.28  $2n = 32$, H1,5; A8,9 karyotype from Thurlestone, S. Devon
Figure 4.29  $2n=32$, H1,2,3,4,8,9 karyotype from Penlee Point, Cornwall

Figure 4.30  $2n = 33$, H1,8,9; A2,3 karyotype from Renney Rocks, S. Devon
Figure 4.31  $2n = 34, H1,3,9; A2,8$ karyotype from Renney Rocks, S. Devon

Figure 4.32  $2n = 34, H1,3; A2,8,9$ karyotype from Renney Rocks, S. Devon
Figure 4.33  2n = 35, H1; A2,3,8,9 karyotype from Mount's Bay, Cornwall

Figure 4.34  2n = 35, H1; A2,3,8,9 karyotype from Mount's Bay, Cornwall
Figure 4.35  $2n = 35$, H1; A2,3,8,9 karyotype from Roscoff, N. Brittany

Figure 4.36  $2n = 36$, A1,2,3,8,9 karyotype from Roscoff, N. Brittany
4.4 Discussion

The results demonstrate a large variation in chromosome number and karyotype configuration, including inversions or possibly translocations in some chromosomes. There also appears to be variation in relative size of individual pairs of chromosomes, particularly in pairs 4 and 5. Silver-stained NORs, FISH using a rDNA probe and secondary constrictions in some Giemsa stained preparations, the positions of which all coincide (Pascoe and Dixon, 1994; Chapter 2), have allowed identification of chromosome pairs 2, 7, and 10 in most spreads. Pair 3 can also be identified using these methods in some polymorphic populations (Pascoe et al., 1996; Chapter 3). However, even after becoming familiar with the many possible variations and analysing hundreds of karyotypes, it still remains impossible to be absolutely certain of the correct identification and arrangement of all the chromosomes of this species.

The 2n = 27 forms, where only one chromosome of one pair exhibits this polymorphism, are relatively common throughout the wide geographic range and show a remarkable heterogeneity. Chromosomes from three or four different pairs can be polymorphic in this type (i.e. 3,4,5 or 9) and in the analysis of these lower number spreads (2n = 26-28) inversions in pairs 4 and 5 became apparent which aided interpretation of higher number spreads.

The questions raised in the introduction can now be addressed:

(a). Does the karyotype vary for a given chromosome number? Yes, as mentioned above, even in the low number forms (27-28) three or four different chromosome pairs can be polymorphic. In higher number forms, although there is likely to be limited variation for any given diploid number in one population or area, the total possible permutations are...
significantly higher. The inversions (paracentric and/or pericentric) observed also add to the karyotypic variation in some diploid numbers.

(b). Are only 5 chromosome pairs involved in the Robertsonian polymorphism (Bantock & Cockayne, 1975)? Initially, when the 2n = 36 spread illustrated by Bantock and Page (1976) was karyotyped in the format used here, and compared to the 2n = 36 karyotype from Mount's Bay, I was sceptical that both were 'true'. I had considerable evidence for the configuration of the latter (A1,2,3,8,9; Fig. 4.2) having karyotyped and measured 12 spreads and observed in excess of 150 others. However, the former, showing 4 large metacentric chromosomes (H1,3; A2,5,8,9) has been represented by the same (rearranged) spread in three publications (Bantock & Cockayne, 1975; Bantock and Page, 1976; Page, 1988), although Bantock and Cockayne (1975) state that the population was monomorphic and at least five cells from at least four individuals adults were scored. Material from Lulworth Cove, Bantock and Page's site, and nearby Kimmeridge failed to provide evidence that chromosome 5 was polymorphic. However, in due course, populations elsewhere were found showing pair 5 to be involved; these included some on the South coast of England (e.g. Cuckmere Haven) as well as Sennen Cove, Brest and Guernsey, and illustrated conclusively that more than 5 pairs of chromosomes can be polymorphic in *N. lapillus*. Additional evidence presented here shows that pair 4 can also be represented by the 'acrocentric' state (i.e. smaller sub-telocentric or sub-metacentric chromosomes), although without additional labelling techniques this must remain speculative as this configuration could possibly be accounted for by a series of inversions. Therefore, despite the maximum diploid number recorded remaining at 36, it would appear that 7 pairs of chromosomes (in the 2n = 26 form) have probably been independently involved in the reduction of chromosome number in this species. This assumes that centric fusion has been the only event in the (evolutionary) process, but some configurations may possibly be
the result of fission events in the opposite direction, e.g. as Finelli et al. (1999) found when comparing humans (2n = 46) with the African Green monkey (2n = 60). Further work with molecular labelling, i.e. using DNA probes specific to other chromosomes or reciprocal chromosome painting with other members of the family, may help elucidate this debate/theory.

(c & d). Are the proposed 243 (i.e. $3^5$) different karyotypes likely to occur, are there any geographical patterns evident and do micro-geographical factors appear to influence the karyotype? It would seem now that this maximum could be raised to 2187 (i.e. $3^7$), but this does not affect the answer. I feel, from the range of karyotypes exhibited in this extensive survey, that a relatively small proportion of the possible permutations are likely to exist. Estimating a number is difficult; evidence for over 40 types is given here but less than a hundred is probably realistic. Although the polymorphism is found to some extent across almost the whole geographic range, some geographical trends are apparent. This is not surprising in a species with such limited dispersal, but the similarities and contrasts in different areas are surprising. For example, chromosome 4 often appears in acrocentric form in low number spreads from populations on the South and East coasts of Kent, and also those studied from the Isle of Man, Norway and the USA, four extremely distant areas. The acrocentric forms of chromosomes 1, 2, 3 and 8 appear to be restricted mainly to populations from the western end of the Channel (Mount’s Bay to the Isle of Wight and Guernsey to Roscoff) although populations from the Brest area and the islands off Scotland have polymorphic karyotypes involving most of these chromosome pairs.

Evidence from humans and other mammals show that certain Robertsonian translocations are much more frequent than others, i.e. some chromosomes are more often involved in the fusion/fission events, e.g. in humans rob(13q14q) and rob(14q21q) are the most common
and account for 85% of all Rbs (Shaffer & Lupski, 2000). Page et al. (1996) used FISH to localise the breakpoints in 56 non-homologous Rb translocations in humans, and found them to be highly variable in seven types of the less common Rbs, whereas most of the rob(13q14q) and rob(14q21q) analysed showed breakpoints in the same location. They suggest the mechanism of formation might be different in these forms. This non-random distribution of Rbs in humans is discussed further by Shaffer & Lupski (2000) again suggesting a specific mechanism or underlying genomic architecture or sequence that promotes the exchange between certain acrocentrics. Related studies have been made on the pericentric organisation at the fusion point of mouse Rbs compared to that of telocentrics (Garagna et al., 1995, 2001), latterly using chromosome-orientation FISH (CO-FISH), primed in situ labelling (PRINS) and conventional FISH. The structure and orientation of major and minor satellite DNA was found to be the same with respect to both the centromeres and telomeres, thus when Rbs occur, satellite DNA families are organised head to tail and overall DNA polarity is maintained. The suggestion is that chromosomes that do not share the same satellite DNA are not involved in Rb translocations.

Slijepcevic (1998) has also addressed the mechanisms involved with Rb fusion in relation to telomeric sequence patterns and proposed that there may be, theoretically, at least three ways that Rbs can be formed in mammals. Only one has been confirmed as operational in karyotype evolution to date, i.e. chromosome breakage within minor satellite sequences (Nanda et al., 1995; Garagna et al., 1995), but possible evidence for another (telomere inactivation) is shown by the presence of interstitial telomeric sites in the pericentromeric regions of several vertebrate species (Meyne et al., 1990). Techniques and approaches such as these could help to elucidate some of the unresolved issues in the Rbs of N. lapillus and explain the geographical variations in karyotypes.
In terms of the numerical variation, there appears to be a general trend of reduction in chromosome number in the Channel from West to East, i.e. the higher numbers (2n = 35-36) are exhibited in populations nearer the western end (Mount’s Bay, Looe and Roscoff) whereas those towards the eastern end (Kent) seem restricted to 2n = 27-28. No other major clines are evident in the limited samples recorded here. High chromosome numbers are found in various distant places e.g. Scotland, Ireland and the Channel Islands, and in general these sites tend to be relatively sheltered, but overall there is no obvious firm link to exposure/wave action although micro-habitat may be significant. The differences in chromosome numbers and karyotypes on the South and East coasts of the UK perhaps suggest a different origin linked to geological or glacial events e.g. the areas will have been isolated in the past due to ice sheets and/or much lower sea levels (Briggs, 1970; Cambridge and Kitching, 1982; Crothers, 1983). General theories on an explanation or cause of the polymorphism, stemming from this study and previous work, are outlined and discussed in Chapter 6.

e. Is there any correlation between numerical and inversion polymorphisms? This is probably difficult to address with the limited data available. Possible inversions have been recorded here in populations from Norway (2n = 26-27: pairs 5 and 8), USA, Kent and the Isle of Wight (pair 5), Western Combe Cove, Devon (pair 4) and North Foreland, Kent (pair 8). Other studies (Bantock and Page, 1976; Page, 1988) have recorded inversions in populations from Sussex (pair 4 or 5 and 8 or 9) and Somerset (pair 8 or 9). These findings and the fact that any inversion is not consistent throughout a population (or individual) suggest that most of the inversions appear in those types with lower chromosome numbers (2n < 30) and are not fixed in the population. Further discussion is given in chapter 6.
Chapter 5. Karyotypes and Genome Size in Muricid gastropods

“Evolution is essentially a cytogenetic process” (White, 1978)

5.1 Introduction

In previous chapters, the karyotype of *N. lapillus* (L.) is shown to be highly variable through both a Robertsonian numerical polymorphism and other structural rearrangements. It is also strikingly different numerically from other members of the family, as most of those recorded to date have a diploid chromosome number in the range 2n = 60 to 70 and *Ocinebrina aciculata* (Lamarck, 1822) has been recorded here as 2n=86 (see Results: Table 5.1).

This marked reduction in chromosome number in the Atlantic dog-whelk, *Nucella lapillus*, is thought to have occurred since its ancestral form dispersed into the North Atlantic from the NE Pacific, via an Arctic route, during a period of global warming, < 5 MYA (Briggs, 1970). More recent evidence on the evolutionary history of northern hemisphere *Nucella* confirms this theory but suggests that dispersal was via the Eurasian Arctic i.e. links *N. lapillus* to a western rather than an eastern Pacific clade (Collins *et al.*, 1996). Their evidence supports; (1) a model of speciation in *Nucella* driven by cycles of climatic amelioration and deterioration that began during the Miocene, (2) an early participation by *Nucella* in the trans-Arctic interchange, (3) that *N. lapillus*, with reduced chromosome number, is clearly the derived form and is consistent with White’s (1978) observations on chromosome evolution in other clades. Other theories suggest the karyotypes of specialised species, including those that occupy varied as opposed to stable environments, consist generally of smaller and fewer chromosomes than those of the less specialised relatives (Swanson *et al.*, 1981).
Rb fusion certainly seems to be the more common evolutionary direction in mammals and Darwinian gradualism (relatively small changes in karyotype) is favoured in the chromosomal evolution of many other groups. However, two recent papers (Kolnicki, 2000; Godfrey & Masters, 2000) have reviewed and possibly resurrected the 'Karyotypic Fission theory' of Todd (1970), in which he proposed that wholesale fission of all the metacentrics in a complement as one event plays a major role in chromosomal evolution and explains the dramatic differences in diploid numbers of closely related species. This was dismissed by White (1973) as preposterous and 'equivalent to a belief in miracles'. However, on the basis of recent cellular and molecular evidence, Kolnicki (2000) postulates the 'Kinetochore reproduction theory' (an update of Todd's theory), includes Nucella lapillus as an example of Rb fission and cites Pascoe et al. (1996). It is certainly an interesting alternative that I am reluctant to accept in view of the above evidence, but currently lack conclusive reasons to reject it. Therefore, a simplistic overview of karyotype evolution in Nucella might be that the ancestral type was likely to have a high number of chromosomes, nearer the 2n=70 of the Pacific forms. The North Atlantic form evolved from this by a reduction towards the 2n=36 type and more recently (in geological time; see Cambridge and Kitching 1982) has undergone a further reduction towards the 2n=26 form, mainly by centric fusions.

In this study, I attempt to address the question of how this evidence and theory relates to the karyotypes of living muricids and the long-standing debate on the evolution of genome size, i.e. nuclear DNA content.

Here, the genome size of 8 muricid species from the N. Atlantic and NE. Pacific (Nucella lapillus, N. canaliculata (Duclos, 1832), N. emarginata (Deshayes 1839), N. lamellosa (Gmelin 1791), Ocenebra erinacea (L.), Murex (Hexaplex) brandaris L.,
Murex (*Hexaplex*) *trunculus* L., and *Thais haemastoma* (L.) are compared to determine whether the reduction in chromosome number in *N. lapillus* has been accompanied by a reduction in nuclear DNA content compared to the congenerics in the Pacific and the other members of its family. Also, the karyotypes and total haploid chromosome lengths of the 2n=26 and 2n=36 forms of *N. lapillus* and those of three other species (not previously published), were determined to see how chromosome length varies with chromosome number and if it relates to DNA content in this family.

Figure 5.1 Shells of the muricid species in this study. Scale Bar = 50 mm.
5.2 Material and methods

For this study, specimens of *N. lapillus* were collected from Bude (N. Cornwall, 2n=26) and Mount’s Bay (S. Cornwall, 2n=36), and those of *Ocenebra erinacea* from Plymouth Sound, Looe (S. Cornwall) and near Brest (Brittany, France). Fixed specimens of *N. canaliculata, N. emarginata,* and *N. lamellosa* were sent from Bamfield Marine Station (Vancouver Island) and those of *Murex brandaris, M. trunculus, Ocinebrina aciculata* and *Thais haemastoma* were collected from southern Portugal. In those species karyotyped, metaphase chromosomes were obtained from embryos collected at the same sites. Shells of some of the above species are shown in Fig. 5.1.

5.2.1 Karyotyping

Metaphase spreads were produced as described in Chapter 2 (see 2.3.1) In karyotyping the other species of muricid, namely, *Ocenebra erinacea* (the sting winkle, 2n=70, NW Europe to NW Africa), *Thais haemastoma* (2n=70, sub-species distribution, E and W. Atlantic and E. Pacific), and *Murex trunculus* (2n=70, Mediterranean and warm temperate E. Atlantic), chromosomes were divided into groups based on chromosome type (centromere position) after Levan *et al.* (1964), i.e. classed as metacentric, sub-metacentric, sub-telocentric and telocentric (arm ratios (p/q) of 1 - 0.6, 0.6 - 0.33, 0.33 - 0.14, and 0.14 - 0 respectively), and then in descending order of size within each group. The karyotypes of both 2n = 26 and 36 forms of *N. lapillus* have been described earlier but here are arranged into the groups described above. N.B. the 2n = 36 form of *N. lapillus* is the only species having true telocentric chromosomes.
Chromosome arm lengths were measured from photographic enlargements using a Kontron IBAS Image analysis system. Total lengths (p + q), arm ratio (p/q) and relative haploid lengths (p+q / total haploid length) were determined for each chromosome allowing idiograms to be constructed.

5.2.2 Genome Size

Nuclear DNA content or C-values i.e. pg DNA in the haploid genome, were determined in the 8 species mentioned above by derivation from the regression of pg DNA v. optical density in the Feulgen reaction using reference molluscan species (*Littorina littorea*, *Mytilus edulis* and *Nucella lapillus*) in which the DNA content is known (Hinegardner, 1974). Gills from all species were fixed in cold (4°C) Carnoy’s for at least 1h, gently agitated or teased in 60% acetic acid to produce a single-cell suspension and then dropped onto warm microscope slides. Batches of slides comprising each of the species to be compared were then stained as follows (modified method of Itikawa & Ogura, 1954):

Rinse in 1N HCl for 1 min.

Treat with 1N HCl at 60°C for 8 min

Wash in distilled water.

Stain in Schiff’s reagent for 45 min at 20°C.

Wash well in tap water.

Rinse in Sulphite (0.5% Potassium sulphite and 0.05N HCl in distilled water) for a few seconds, followed by rinsing in distilled water, blot and air dry.

Density of the Feulgen-positive nuclear material was measured with a Vickers M85 scanning microdensitometer. At least 50 nuclei were measured in each species.
### 5.3 Results

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Purpura (Nucella) lapillus</em></td>
<td>13 - 18</td>
<td>Staiger 1950a, 1951</td>
</tr>
<tr>
<td><em>Purpura (Mancinella) bronni</em></td>
<td>30</td>
<td>Nishikawa 1962</td>
</tr>
<tr>
<td><em>Purpura (Mancinella) clavigera</em></td>
<td>30</td>
<td>Nishikawa 1962</td>
</tr>
<tr>
<td><em>Purpura (Mancinella) luteostoma</em></td>
<td>30</td>
<td>Nishikawa 1962</td>
</tr>
<tr>
<td><em>Thais (Nucella) lamellosa</em></td>
<td>35</td>
<td>Ahmed and Sparks 1970</td>
</tr>
<tr>
<td><em>Thais (Nucella) lima</em></td>
<td>35</td>
<td>Ahmed and Sparks 1970</td>
</tr>
<tr>
<td><em>Thais (Nucella) emarginata</em></td>
<td>35</td>
<td>Ahmed and Sparks 1970</td>
</tr>
<tr>
<td><em>Thais (Nucella) canaliculata</em></td>
<td>35</td>
<td>Ahmed and Sparks 1970</td>
</tr>
<tr>
<td><em>Ocinebra erinaceus</em> (syn. <em>Ocenebra erinacea</em>)</td>
<td>35</td>
<td>Staiger 1950b, 1951</td>
</tr>
<tr>
<td><em>Ocinebra japonica</em></td>
<td>35</td>
<td>Ahmed and Sparks 1970</td>
</tr>
<tr>
<td><em>Ocinebrina aciculata</em></td>
<td>43</td>
<td>This study</td>
</tr>
<tr>
<td><em>Thais haemastoma</em></td>
<td>35</td>
<td>This study</td>
</tr>
<tr>
<td><em>Murex brandaris</em></td>
<td>28</td>
<td>Lams 1934 *</td>
</tr>
<tr>
<td><em>Murex trunculus</em></td>
<td>11 or 12</td>
<td>Schitz 1920 *</td>
</tr>
<tr>
<td></td>
<td>14 + X-O mechanism 35</td>
<td>Tuzet 1930 *</td>
</tr>
<tr>
<td></td>
<td>14 + X-O mechanism 35</td>
<td>Staiger 1950b, 1951</td>
</tr>
<tr>
<td><em>Nassa nitida</em></td>
<td>14 + X-O mechanism</td>
<td>Tuzet 1930 *</td>
</tr>
<tr>
<td><em>Chicoreus asianus</em></td>
<td>34</td>
<td>Nishikawa 1962</td>
</tr>
<tr>
<td><em>Bedevina birileffi</em></td>
<td>32?</td>
<td>Nishikawa 1962</td>
</tr>
<tr>
<td><em>Ceratostoma (Purpura) foliatum</em></td>
<td>35</td>
<td>Ahmed and Sparks 1970</td>
</tr>
<tr>
<td><em>Fusitriton oregonensis</em></td>
<td>35</td>
<td>Ahmed and Sparks 1970</td>
</tr>
</tbody>
</table>

Table 5.1 Published data on chromosome number in the Muricidae including confirmation or new records from this study (in bold). *Cited in Nishikawa, 1962.
5.3.1 Karyotypes

5.3.1.1 Nucella lapillus

Karyotypes of the 2n=26 and 2n=36 forms of N. lapillus are shown here rearranged into groups by chromosome type and then in decreasing size (Figs. 5.2 and 5.3) and their respective chromosome measurement data are given in Tables 5.2 and 5.3. These have been described fully in Chapter 2, but essentially show the reduction in chromosome number from 2n=36 to 26 via Robertsonian fusions in chromosome pairs 1, 2, 3, 8 and 9. In the 2n=26 form the karyotype consists of 10 pairs of metacentric chromosomes ranging from around 10μm to 1.6μm in total length, three of which tend to be close to the sub-metacentric limit (arm ratio < 0.6), two pairs of sub-metacentrics of around 6.4 and 4.8μm in length, and one pair of sub-telocentric chromosomes at about 3.6μm in length.

In the 2n=36 form found in this study (A1,2,3,8,9), 5 of the metacentric pairs shown in the 2n=26 karyotype now comprise 5 sub-metacentrics, 2 sub-telocentrics and 3 telocentrics, although their precise classification into these types may vary slightly in different spreads. Hence the whole karyotype (n=18) consists of 4M (7.3 – 1.6 μm), 6SM (6.4 – 2.5μm), 4ST (5 – 3 μm) and 4T (4 – 2.3 μm).
Figure 5.2 *Nucella lapillus* $2n = 26$ form, grouped by centromere position or arm ratio and in descending size (Scale bar = 10 $\mu$m).

Figure 5.3 *Nucella lapillus*: $2n = 36$ spread grouped by chromosome type and size (Scale bar = 10 $\mu$m).
<table>
<thead>
<tr>
<th>Chromosome Pair No.</th>
<th>Total Length (μm)</th>
<th>Relative Length (%)</th>
<th>Arm Ratio</th>
<th>NOR np %</th>
<th>Chromosome Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± 2SE)</td>
<td>(Mean ± 2SE)</td>
<td>(Mean ± 2SE)</td>
<td>(Mean ± 2SE)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9.72 ± 0.60</td>
<td>13.93 ± 0.22</td>
<td>0.796 ± 0.021</td>
<td>73.5 ± 0.662</td>
<td>m</td>
</tr>
<tr>
<td>2</td>
<td>8.72 ± 0.52</td>
<td>12.51 ± 0.19</td>
<td>0.838 ± 0.020</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>3</td>
<td>7.90 ± 0.49</td>
<td>11.32 ± 0.21</td>
<td>0.663 ± 0.028</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>4</td>
<td>7.32 ± 0.46</td>
<td>10.48 ± 0.16</td>
<td>0.734 ± 0.028</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>5</td>
<td>5.14 ± 0.30</td>
<td>7.39 ± 0.17</td>
<td>0.775 ± 0.033</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>6</td>
<td>4.39 ± 0.26</td>
<td>6.30 ± 0.14</td>
<td>0.597 ± 0.031</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>7</td>
<td>4.15 ± 0.25</td>
<td>5.95 ± 0.09</td>
<td>0.614 ± 0.025</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>8</td>
<td>3.67 ± 0.20</td>
<td>5.29 ± 0.12</td>
<td>0.720 ± 0.025</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>9</td>
<td>2.26 ± 0.11</td>
<td>3.27 ± 0.11</td>
<td>0.785 ± 0.027</td>
<td>m</td>
<td>m</td>
</tr>
<tr>
<td>10</td>
<td>1.61 ± 0.07</td>
<td>2.35 ± 0.09</td>
<td>0.622 ± 0.031</td>
<td>m</td>
<td>tro</td>
</tr>
<tr>
<td>11</td>
<td>6.38 ± 0.40</td>
<td>9.16 ± 0.24</td>
<td>0.384 ± 0.018</td>
<td>m</td>
<td>tro</td>
</tr>
<tr>
<td>12</td>
<td>4.80 ± 0.27</td>
<td>6.91 ± 0.13</td>
<td>0.382 ± 0.020</td>
<td>m</td>
<td>tro</td>
</tr>
<tr>
<td>13</td>
<td>3.57 ± 0.19</td>
<td>5.13 ± 0.12</td>
<td>0.256 ± 0.013</td>
<td>m</td>
<td>tro</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>69.63</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 Total chromosome lengths, relative lengths and arm ratios for *N. lapillus* from three 2n=26 populations: Whitsand Bay (13 spreads from testes), Polzeath and West Runton (15 spreads each, from embryos).

<table>
<thead>
<tr>
<th>Chromosome Pair No.</th>
<th>Total Length (μm)</th>
<th>Relative Length (%)</th>
<th>Arm Ratio</th>
<th>Chromosome Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± 2SE)</td>
<td>(Mean ± 2SE)</td>
<td>(Mean ± 2SE)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.86 ± 0.42</td>
<td>9.76 ± 0.19</td>
<td>0.85 ± 0.01</td>
<td>m</td>
</tr>
<tr>
<td>2</td>
<td>5.92 ± 0.36</td>
<td>7.33 ± 0.15</td>
<td>0.81 ± 0.03</td>
<td>m</td>
</tr>
<tr>
<td>3</td>
<td>4.29 ± 0.20</td>
<td>5.33 ± 0.09</td>
<td>0.73 ± 0.03</td>
<td>m</td>
</tr>
<tr>
<td>4</td>
<td>2.54 ± 0.14</td>
<td>3.16 ± 0.10</td>
<td>0.84 ± 0.04</td>
<td>m</td>
</tr>
<tr>
<td>5</td>
<td>7.07 ± 0.38</td>
<td>8.78 ± 0.14</td>
<td>0.37 ± 0.01</td>
<td>sm</td>
</tr>
<tr>
<td>6</td>
<td>5.73 ± 0.23</td>
<td>7.15 ± 0.20</td>
<td>0.40 ± 0.02</td>
<td>sm</td>
</tr>
<tr>
<td>7</td>
<td>4.99 ± 0.29</td>
<td>6.20 ± 0.16</td>
<td>0.38 ± 0.04</td>
<td>sm</td>
</tr>
<tr>
<td>8</td>
<td>4.80 ± 0.26</td>
<td>5.96 ± 0.13</td>
<td>0.45 ± 0.03</td>
<td>sm</td>
</tr>
<tr>
<td>9</td>
<td>2.67 ± 0.13</td>
<td>3.33 ± 0.10</td>
<td>0.51 ± 0.05</td>
<td>sm</td>
</tr>
<tr>
<td>10</td>
<td>1.61 ± 0.11</td>
<td>1.99 ± 0.06</td>
<td>0.52 ± 0.04</td>
<td>sm</td>
</tr>
<tr>
<td>11</td>
<td>5.42 ± 0.37</td>
<td>6.70 ± 0.14</td>
<td>0.26 ± 0.02</td>
<td>st</td>
</tr>
<tr>
<td>12</td>
<td>4.05 ± 0.21</td>
<td>5.04 ± 0.12</td>
<td>0.24 ± 0.03</td>
<td>st</td>
</tr>
<tr>
<td>13</td>
<td>3.87 ± 0.20</td>
<td>4.81 ± 0.13</td>
<td>0.30 ± 0.04</td>
<td>st</td>
</tr>
<tr>
<td>14</td>
<td>3.84 ± 0.27</td>
<td>4.75 ± 0.11</td>
<td>0.25 ± 0.02</td>
<td>st</td>
</tr>
<tr>
<td>15</td>
<td>4.83 ± 0.26</td>
<td>6.00 ± 0.06</td>
<td>0.00 ± 0.00</td>
<td>t</td>
</tr>
<tr>
<td>16</td>
<td>4.41 ± 0.20</td>
<td>5.49 ± 0.10</td>
<td>0.00 ± 0.00</td>
<td>t</td>
</tr>
<tr>
<td>17</td>
<td>4.28 ± 0.25</td>
<td>5.31 ± 0.11</td>
<td>0.00 ± 0.00</td>
<td>t</td>
</tr>
<tr>
<td>18</td>
<td>2.35 ± 0.16</td>
<td>2.91 ± 0.07</td>
<td>0.00 ± 0.00</td>
<td>t</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>80.52</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3 Total chromosome lengths, relative lengths and arm ratios for *Nucella lapillus* (2n = 36, embryos, n=7) from Mount’s Bay and Roscoff. To conform to the arrangement for other muricids, the chromosomes have been grouped by centromere position or arm ratio and in descending size.
5.3.1.2 *Ocenebra erinacea*

Chromosomes of *Ocenebra erinacea* are shown in Fig. 5.4 and the karyotype (Fig. 5.5) comprises 35 pairs which divide into 5 M (8.1 – 2.3μm), 20 SM (6 – 2.5μm) and 10 ST (5.0 – 3.3μm) one or two of which may classify as telocentric. No significant differences were seen in karyotypes from the 3 sites where this species was collected.

Lengths and arm ratios of the chromosomes are given in Table 5.4.

![Chromosomes of *Ocenebra erinacea* (2n = 70) from Looe, S. Cornwall.](image-url)
Figure 5.5 Karyotype of *Ocenebra erinacea* (2n = 70) from Looe, S. Cornwall (Scale bar = 10 μm).
<table>
<thead>
<tr>
<th>Chromosome Pair No.</th>
<th>Total Length (µm) Mean ± 2SE</th>
<th>Relative Length (%) Mean ± 2SE</th>
<th>Arm Ratio Mean ± 2SE</th>
<th>Chromosome Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.14 ± 0.92</td>
<td>5.85 ± 0.18</td>
<td>0.81 ± 0.04</td>
<td>m</td>
</tr>
<tr>
<td>2</td>
<td>6.75 ± 0.70</td>
<td>4.87 ± 0.30</td>
<td>0.91 ± 0.05</td>
<td>m</td>
</tr>
<tr>
<td>5</td>
<td>5.90 ± 0.53</td>
<td>4.26 ± 0.25</td>
<td>0.60 ± 0.09</td>
<td>m</td>
</tr>
<tr>
<td>3</td>
<td>4.38 ± 0.64</td>
<td>3.14 ± 0.25</td>
<td>0.83 ± 0.06</td>
<td>m</td>
</tr>
<tr>
<td>4</td>
<td>2.30 ± 0.17</td>
<td>1.67 ± 0.15</td>
<td>0.85 ± 0.03</td>
<td>m</td>
</tr>
<tr>
<td>6</td>
<td>5.07 ± 0.57</td>
<td>3.65 ± 0.27</td>
<td>0.49 ± 0.09</td>
<td>sm</td>
</tr>
<tr>
<td>7</td>
<td>4.75 ± 0.35</td>
<td>3.43 ± 0.13</td>
<td>0.51 ± 0.07</td>
<td>sm</td>
</tr>
<tr>
<td>8</td>
<td>4.46 ± 0.39</td>
<td>3.22 ± 0.19</td>
<td>0.50 ± 0.07</td>
<td>sm</td>
</tr>
<tr>
<td>9</td>
<td>4.21 ± 0.53</td>
<td>3.02 ± 0.09</td>
<td>0.50 ± 0.06</td>
<td>sm</td>
</tr>
<tr>
<td>10</td>
<td>4.33 ± 0.54</td>
<td>3.12 ± 0.23</td>
<td>0.55 ± 0.10</td>
<td>sm</td>
</tr>
<tr>
<td>11</td>
<td>4.29 ± 0.44</td>
<td>3.09 ± 0.16</td>
<td>0.48 ± 0.06</td>
<td>sm</td>
</tr>
<tr>
<td>12</td>
<td>4.16 ± 0.46</td>
<td>3.00 ± 0.19</td>
<td>0.51 ± 0.07</td>
<td>sm</td>
</tr>
<tr>
<td>13</td>
<td>3.96 ± 0.30</td>
<td>2.86 ± 0.16</td>
<td>0.51 ± 0.04</td>
<td>sm</td>
</tr>
<tr>
<td>14</td>
<td>3.69 ± 0.36</td>
<td>2.68 ± 0.28</td>
<td>0.53 ± 0.07</td>
<td>sm</td>
</tr>
<tr>
<td>15</td>
<td>3.47 ± 0.19</td>
<td>2.52 ± 0.20</td>
<td>0.50 ± 0.10</td>
<td>sm</td>
</tr>
<tr>
<td>16</td>
<td>3.49 ± 0.26</td>
<td>2.54 ± 0.27</td>
<td>0.57 ± 0.09</td>
<td>sm</td>
</tr>
<tr>
<td>17</td>
<td>3.31 ± 0.41</td>
<td>2.39 ± 0.26</td>
<td>0.47 ± 0.12</td>
<td>sm</td>
</tr>
<tr>
<td>18</td>
<td>3.27 ± 0.40</td>
<td>2.36 ± 0.16</td>
<td>0.53 ± 0.14</td>
<td>sm</td>
</tr>
<tr>
<td>19</td>
<td>3.15 ± 0.70</td>
<td>2.24 ± 0.31</td>
<td>0.37 ± 0.06</td>
<td>sm</td>
</tr>
<tr>
<td>20</td>
<td>3.05 ± 0.51</td>
<td>2.19 ± 0.23</td>
<td>0.37 ± 0.04</td>
<td>sm</td>
</tr>
<tr>
<td>21</td>
<td>2.80 ± 0.25</td>
<td>2.03 ± 0.16</td>
<td>0.54 ± 0.10</td>
<td>sm</td>
</tr>
<tr>
<td>22</td>
<td>2.70 ± 0.31</td>
<td>1.94 ± 0.06</td>
<td>0.37 ± 0.05</td>
<td>sm</td>
</tr>
<tr>
<td>23</td>
<td>2.58 ± 0.29</td>
<td>1.86 ± 0.18</td>
<td>0.50 ± 0.07</td>
<td>sm</td>
</tr>
<tr>
<td>24</td>
<td>2.27 ± 0.36</td>
<td>1.63 ± 0.16</td>
<td>0.59 ± 0.05</td>
<td>sm</td>
</tr>
<tr>
<td>25</td>
<td>2.45 ± 0.46</td>
<td>1.75 ± 0.21</td>
<td>0.42 ± 0.08</td>
<td>sm</td>
</tr>
<tr>
<td>26</td>
<td>5.02 ± 0.87</td>
<td>3.58 ± 0.31</td>
<td>0.29 ± 0.03</td>
<td>st</td>
</tr>
<tr>
<td>27</td>
<td>4.76 ± 0.99</td>
<td>3.39 ± 0.40</td>
<td>0.32 ± 0.05</td>
<td>st</td>
</tr>
<tr>
<td>28</td>
<td>4.20 ± 0.63</td>
<td>3.01 ± 0.22</td>
<td>0.31 ± 0.01</td>
<td>st</td>
</tr>
<tr>
<td>29</td>
<td>4.04 ± 0.58</td>
<td>2.90 ± 0.19</td>
<td>0.29 ± 0.04</td>
<td>st</td>
</tr>
<tr>
<td>30</td>
<td>4.05 ± 0.46</td>
<td>2.91 ± 0.09</td>
<td>0.30 ± 0.04</td>
<td>st</td>
</tr>
<tr>
<td>31</td>
<td>3.83 ± 0.62</td>
<td>2.74 ± 0.23</td>
<td>0.29 ± 0.06</td>
<td>st</td>
</tr>
<tr>
<td>32</td>
<td>3.79 ± 0.54</td>
<td>2.71 ± 0.15</td>
<td>0.26 ± 0.06</td>
<td>st</td>
</tr>
<tr>
<td>33</td>
<td>3.60 ± 0.58</td>
<td>2.58 ± 0.20</td>
<td>0.28 ± 0.05</td>
<td>st</td>
</tr>
<tr>
<td>34</td>
<td>3.55 ± 0.65</td>
<td>2.53 ± 0.26</td>
<td>0.29 ± 0.03</td>
<td>st</td>
</tr>
<tr>
<td>35</td>
<td>3.30 ± 0.62</td>
<td>2.36 ± 0.26</td>
<td>0.33 ± 0.04</td>
<td>st</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>139.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.4  Total chromosome lengths, relative lengths and arm ratios for *Ocenebra erinacea* (embryos, n=6) from Looe (3) and Brest (3). The chromosomes have been placed into three groups by centromere position or arm ratio and in descending size.
5.3.1.3 *Thais haemastoma*

Chromosomes of *Thais haemastoma* (2n=70, Fig. 5.6) karyotype as follows: 10 M (4.3 – 1.7μm), 18 SM (5.6 – 2.2μm) and 7 ST (4.4 – 2.4μm) (Fig. 5.7). Chromosome measurement data are given in Table 5.5.

![Chromosomes of Thais haemastoma (2n = 70) from Portugal.](image)

**Figure 5.6** Chromosomes of *Thais haemastoma* (2n = 70) from Portugal.
Figure 5.7 Karyotype of *Thais haemastoma*, 2n = 70, from Portugal (Scale bar = 10 μm).
<table>
<thead>
<tr>
<th>Pair No.</th>
<th>Total Length (µm)</th>
<th>Relative Length (%)</th>
<th>Arm Ratio</th>
<th>Chromosome Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± 2SE</td>
<td>Mean ± 2SE</td>
<td>Mean ± 2SE</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.25 ± 0.50</td>
<td>4.01 ± 0.18</td>
<td>0.85 ± 0.09</td>
<td>m</td>
</tr>
<tr>
<td>2</td>
<td>3.52 ± 0.48</td>
<td>3.32 ± 0.12</td>
<td>0.91 ± 0.13</td>
<td>m</td>
</tr>
<tr>
<td>3</td>
<td>3.24 ± 0.34</td>
<td>3.06 ± 0.14</td>
<td>0.94 ± 0.11</td>
<td>m</td>
</tr>
<tr>
<td>4</td>
<td>3.03 ± 0.56</td>
<td>2.85 ± 0.28</td>
<td>0.87 ± 0.08</td>
<td>m</td>
</tr>
<tr>
<td>5</td>
<td>2.67 ± 0.51</td>
<td>2.51 ± 0.17</td>
<td>0.93 ± 0.08</td>
<td>m</td>
</tr>
<tr>
<td>6</td>
<td>2.44 ± 0.45</td>
<td>2.29 ± 0.14</td>
<td>0.85 ± 0.10</td>
<td>m</td>
</tr>
<tr>
<td>7</td>
<td>2.40 ± 0.60</td>
<td>2.24 ± 0.33</td>
<td>0.81 ± 0.12</td>
<td>m</td>
</tr>
<tr>
<td>8</td>
<td>2.04 ± 0.54</td>
<td>1.91 ± 0.32</td>
<td>0.87 ± 0.10</td>
<td>m</td>
</tr>
<tr>
<td>9</td>
<td>5.64 ± 0.60</td>
<td>5.31 ± 0.10</td>
<td>0.48 ± 0.06</td>
<td>sm</td>
</tr>
<tr>
<td>10</td>
<td>3.79 ± 0.52</td>
<td>3.56 ± 0.04</td>
<td>0.53 ± 0.07</td>
<td>sm</td>
</tr>
<tr>
<td>11</td>
<td>3.64 ± 0.48</td>
<td>3.43 ± 0.10</td>
<td>0.43 ± 0.13</td>
<td>sm</td>
</tr>
<tr>
<td>12</td>
<td>3.59 ± 0.51</td>
<td>3.38 ± 0.09</td>
<td>0.54 ± 0.16</td>
<td>sm</td>
</tr>
<tr>
<td>13</td>
<td>3.25 ± 0.57</td>
<td>3.06 ± 0.21</td>
<td>0.51 ± 0.16</td>
<td>sm</td>
</tr>
<tr>
<td>14</td>
<td>3.38 ± 0.46</td>
<td>3.18 ± 0.04</td>
<td>0.48 ± 0.12</td>
<td>sm</td>
</tr>
<tr>
<td>15</td>
<td>3.24 ± 0.61</td>
<td>3.04 ± 0.20</td>
<td>0.51 ± 0.11</td>
<td>sm</td>
</tr>
<tr>
<td>16</td>
<td>3.00 ± 0.48</td>
<td>2.82 ± 0.11</td>
<td>0.52 ± 0.08</td>
<td>sm</td>
</tr>
<tr>
<td>17</td>
<td>2.87 ± 0.35</td>
<td>2.70 ± 0.04</td>
<td>0.55 ± 0.05</td>
<td>sm</td>
</tr>
<tr>
<td>18</td>
<td>3.05 ± 0.42</td>
<td>2.87 ± 0.11</td>
<td>0.53 ± 0.07</td>
<td>sm</td>
</tr>
<tr>
<td>19</td>
<td>2.85 ± 0.41</td>
<td>2.68 ± 0.11</td>
<td>0.54 ± 0.06</td>
<td>sm</td>
</tr>
<tr>
<td>20</td>
<td>2.66 ± 0.27</td>
<td>2.51 ± 0.10</td>
<td>0.51 ± 0.04</td>
<td>sm</td>
</tr>
<tr>
<td>21</td>
<td>2.71 ± 0.30</td>
<td>2.56 ± 0.11</td>
<td>0.53 ± 0.09</td>
<td>sm</td>
</tr>
<tr>
<td>22</td>
<td>2.61 ± 0.32</td>
<td>2.46 ± 0.05</td>
<td>0.54 ± 0.16</td>
<td>sm</td>
</tr>
<tr>
<td>23</td>
<td>2.56 ± 0.15</td>
<td>2.42 ± 0.16</td>
<td>0.55 ± 0.05</td>
<td>sm</td>
</tr>
<tr>
<td>24</td>
<td>2.38 ± 0.32</td>
<td>2.24 ± 0.04</td>
<td>0.55 ± 0.10</td>
<td>sm</td>
</tr>
<tr>
<td>25</td>
<td>2.29 ± 0.45</td>
<td>2.15 ± 0.16</td>
<td>0.56 ± 0.03</td>
<td>sm</td>
</tr>
<tr>
<td>26</td>
<td>2.24 ± 0.23</td>
<td>2.12 ± 0.10</td>
<td>0.55 ± 0.14</td>
<td>sm</td>
</tr>
<tr>
<td>27</td>
<td>1.95 ± 0.21</td>
<td>1.84 ± 0.04</td>
<td>0.59 ± 0.08</td>
<td>sm</td>
</tr>
<tr>
<td>28</td>
<td>1.72 ± 0.35</td>
<td>1.62 ± 0.27</td>
<td>0.58 ± 0.06</td>
<td>sm</td>
</tr>
<tr>
<td>29</td>
<td>4.42 ± 0.36</td>
<td>4.18 ± 0.19</td>
<td>0.26 ± 0.02</td>
<td>st</td>
</tr>
<tr>
<td>30</td>
<td>3.65 ± 0.13</td>
<td>3.46 ± 0.33</td>
<td>0.20 ± 0.03</td>
<td>st</td>
</tr>
<tr>
<td>31</td>
<td>3.50 ± 0.27</td>
<td>3.31 ± 0.23</td>
<td>0.22 ± 0.01</td>
<td>st</td>
</tr>
<tr>
<td>32</td>
<td>3.26 ± 0.28</td>
<td>3.09 ± 0.33</td>
<td>0.27 ± 0.03</td>
<td>st</td>
</tr>
<tr>
<td>33</td>
<td>3.14 ± 0.34</td>
<td>2.96 ± 0.05</td>
<td>0.32 ± 0.08</td>
<td>st</td>
</tr>
<tr>
<td>34</td>
<td>2.83 ± 0.30</td>
<td>2.67 ± 0.09</td>
<td>0.33 ± 0.02</td>
<td>st</td>
</tr>
<tr>
<td>35</td>
<td>2.36 ± 0.22</td>
<td>2.22 ± 0.09</td>
<td>0.31 ± 0.03</td>
<td>st</td>
</tr>
</tbody>
</table>

| Total   | 106.18           |  |  |  |

Table 5.5 Total chromosome lengths, relative lengths and arm ratios for *Thais haemastoma* (embryos, n=3) from the Algarve, Portugal. Chromosomes are grouped by centromere position (arm ratio) and in descending size.
5.3.1.4 *Murex (Hexaplex) trunculus*

A metaphase spread from *Murex (Hexaplex) trunculus* (2n=70, Fig. 5.8) karyotypes as shown in Fig. 5.9 and consists of 14 M (6.6 – 2.3μm), 15 SM (4.8 – 2.2μm) and 6 ST (3.5 – 2.7μm) pairs. Table 5.6 shows lengths and arm ratios of the chromosomes.

Figure 5.8 Chromosomes of *Murex (Hexaplex) trunculus*, 2n = 70, from Portugal.
Figure 5.9 Karyotype of *Murex (Hexaplex) trunculus*, 2n = 70, from Portugal (Scale bar = 10 μm).
<table>
<thead>
<tr>
<th>Chromosome Pair No.</th>
<th>Total Length (μm)</th>
<th>Relative Length (%)</th>
<th>Arm Ratio</th>
<th>Chromosome Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.60</td>
<td>5.50</td>
<td>0.96</td>
<td>m</td>
</tr>
<tr>
<td>2</td>
<td>5.83</td>
<td>4.86</td>
<td>0.75</td>
<td>m</td>
</tr>
<tr>
<td>3</td>
<td>5.00</td>
<td>4.16</td>
<td>0.81</td>
<td>m</td>
</tr>
<tr>
<td>4</td>
<td>4.37</td>
<td>3.64</td>
<td>0.79</td>
<td>m</td>
</tr>
<tr>
<td>5</td>
<td>3.87</td>
<td>3.22</td>
<td>0.71</td>
<td>m</td>
</tr>
<tr>
<td>6</td>
<td>3.53</td>
<td>2.94</td>
<td>0.74</td>
<td>m</td>
</tr>
<tr>
<td>7</td>
<td>3.47</td>
<td>2.89</td>
<td>0.70</td>
<td>m</td>
</tr>
<tr>
<td>8</td>
<td>3.50</td>
<td>2.91</td>
<td>0.75</td>
<td>m</td>
</tr>
<tr>
<td>9</td>
<td>3.03</td>
<td>2.53</td>
<td>0.90</td>
<td>m</td>
</tr>
<tr>
<td>10</td>
<td>2.90</td>
<td>2.42</td>
<td>0.74</td>
<td>m</td>
</tr>
<tr>
<td>11</td>
<td>2.90</td>
<td>2.42</td>
<td>0.81</td>
<td>m</td>
</tr>
<tr>
<td>12</td>
<td>2.73</td>
<td>2.28</td>
<td>0.91</td>
<td>m</td>
</tr>
<tr>
<td>13</td>
<td>2.63</td>
<td>2.19</td>
<td>0.68</td>
<td>m</td>
</tr>
<tr>
<td>14</td>
<td>2.33</td>
<td>1.94</td>
<td>0.75</td>
<td>m</td>
</tr>
<tr>
<td>15</td>
<td>4.77</td>
<td>3.97</td>
<td>0.59</td>
<td>sm</td>
</tr>
<tr>
<td>16</td>
<td>4.50</td>
<td>3.75</td>
<td>0.50</td>
<td>sm</td>
</tr>
<tr>
<td>17</td>
<td>3.97</td>
<td>3.30</td>
<td>0.59</td>
<td>sm</td>
</tr>
<tr>
<td>18</td>
<td>3.93</td>
<td>3.28</td>
<td>0.57</td>
<td>sm</td>
</tr>
<tr>
<td>19</td>
<td>3.83</td>
<td>3.19</td>
<td>0.58</td>
<td>sm</td>
</tr>
<tr>
<td>20</td>
<td>3.60</td>
<td>3.00</td>
<td>0.42</td>
<td>sm</td>
</tr>
<tr>
<td>21</td>
<td>3.47</td>
<td>2.89</td>
<td>0.44</td>
<td>sm</td>
</tr>
<tr>
<td>22</td>
<td>3.33</td>
<td>2.78</td>
<td>0.43</td>
<td>sm</td>
</tr>
<tr>
<td>23</td>
<td>2.97</td>
<td>2.47</td>
<td>0.48</td>
<td>sm</td>
</tr>
<tr>
<td>24</td>
<td>2.83</td>
<td>2.36</td>
<td>0.57</td>
<td>sm</td>
</tr>
<tr>
<td>25</td>
<td>2.80</td>
<td>2.33</td>
<td>0.56</td>
<td>sm</td>
</tr>
<tr>
<td>26</td>
<td>2.37</td>
<td>1.97</td>
<td>0.54</td>
<td>sm</td>
</tr>
<tr>
<td>27</td>
<td>2.37</td>
<td>1.97</td>
<td>0.48</td>
<td>sm</td>
</tr>
<tr>
<td>28</td>
<td>2.30</td>
<td>1.92</td>
<td>0.44</td>
<td>sm</td>
</tr>
<tr>
<td>29</td>
<td>2.23</td>
<td>1.86</td>
<td>0.46</td>
<td>sm</td>
</tr>
<tr>
<td>30</td>
<td>3.47</td>
<td>2.89</td>
<td>0.28</td>
<td>st</td>
</tr>
<tr>
<td>31</td>
<td>3.37</td>
<td>2.80</td>
<td>0.26</td>
<td>st</td>
</tr>
<tr>
<td>32</td>
<td>3.30</td>
<td>2.75</td>
<td>0.32</td>
<td>st</td>
</tr>
<tr>
<td>33</td>
<td>2.90</td>
<td>2.42</td>
<td>0.30</td>
<td>st</td>
</tr>
<tr>
<td>34</td>
<td>2.90</td>
<td>2.42</td>
<td>0.32</td>
<td>st</td>
</tr>
<tr>
<td>35</td>
<td>2.67</td>
<td>2.22</td>
<td>0.33</td>
<td>st</td>
</tr>
<tr>
<td>Total</td>
<td><strong>120.57</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.6 Total chromosome lengths, relative lengths and arm ratios for *Murex (Hexaplex) trunculus*, 2n = 70, (embryo, n=1) from Portugal. The chromosomes are grouped by centromere position (arm ratio) and in descending size.
5.3.1.5 *Ocinebrina aciculata*

Metaphase spreads were obtained from embryos (capsules) collected from Ria Formosa, southern Portugal. The diploid number, which has not been recorded previously, was confirmed as \(2n=86\) from three separate spreads, one of which is shown in Fig. 5.10 and is karyotyped into the three chromosome groups in Fig. 5.11. The karyotype consists of 16M (9.5 – 2.0\(\mu\)m), 24SM (5.3 – 1.6\(\mu\)m) and 3ST (4.0 – 2.8\(\mu\)m) chromosome pairs, and has a total haploid length of approximately 150\(\mu\)m.

![Metaphase spread of *Ocinebrina aciculata* (2n=86) from Ria Formosa, Portugal. Scale bar = 10\(\mu\)m.](image)

Figure 5.10  Metaphase spread of *Ocinebrina aciculata* (2n=86) from Ria Formosa, Portugal. Scale bar = 10\(\mu\)m.
Figure 5.11 Karyotype of *Ocinebrina aciculata* (2n=86) from Ria Formosa, Portugal. (Scale bar = 10μm).
Total haploid chromosome lengths determined from similarly condensed chromosomes measured from karyotypes of all the above species (except *O. aciculata*) including the two forms of *N. lapillus* are given in Table 5.8, where number and length of chromosomes are compared to genome size.

### 5.3.2 Genome size

The results of Feulgen densitometry on the three species of known genome size, *Nucella lapillus* (26), *Mytilus edulis*, and *Littorina littorea* (Hinegardner, 1974), are summarised in Table 5.7. The calculated regression (through the origin) is $pg \, DNA = 0.274 \times \text{density}$. This regression was used to determine genome size of the following muricid species (see Table 5.8 and Fig. 5.12):

5 (6) species studied from N. Atlantic: *Nucella lapillus* (26), *Nucella lapillus* (36) 
*Ocenebra erinacea*, *Thais haemostoma* 
*Murex trunculus*, *Murex brandaris*

3 species from the NE Pacific: *Nucella canaliculata*, *N. emarginata*, *N. lamellosa*
<table>
<thead>
<tr>
<th>Species</th>
<th>Chromosome number</th>
<th>Mean Relative Density</th>
<th>Known DNA/ haploid genome (pg)</th>
<th>Counts (No. of nuclei)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nucella lapillus</em></td>
<td>26</td>
<td>10</td>
<td>2.8</td>
<td>120</td>
</tr>
<tr>
<td><em>Mytilus edulis</em></td>
<td>28</td>
<td>6.22</td>
<td>1.6</td>
<td>110</td>
</tr>
<tr>
<td><em>Littorina littorea</em></td>
<td>34</td>
<td>3.64</td>
<td>1</td>
<td>50</td>
</tr>
</tbody>
</table>

Regression: \( \text{pg DNA} = 0.274 \times \text{density} \)

**Table 5.7** Results of Feulgen densitometry on gill nuclei of three molluscan species of known genome size to determine regression.

<table>
<thead>
<tr>
<th>Species</th>
<th>2n</th>
<th>Mean Total Haploid Chromosome length ± 2SE (n=No. of spreads)</th>
<th>Mean Relative Density</th>
<th>DNA / haploid genome (pg) (Mean ± 2SE)</th>
<th>Counts (No. of nuclei)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nucella lapillus</em></td>
<td>26</td>
<td>69.6 ± 4.12 (n=43)</td>
<td>10</td>
<td>2.74 ± 0.051</td>
<td>120</td>
</tr>
<tr>
<td><em>Nucella lapillus</em></td>
<td>36</td>
<td>80.5 ± 4.11 (n=7)</td>
<td>9.48</td>
<td>2.6 ± 0.033</td>
<td>50</td>
</tr>
<tr>
<td><em>Ocenebra erinacea</em></td>
<td>70</td>
<td>139.1 ± 14.76 (n=6)</td>
<td>9.51</td>
<td>2.61 ± 0.043</td>
<td>50</td>
</tr>
<tr>
<td><em>Thais haemostoma</em></td>
<td>70</td>
<td>106.2 ± 13.25 (n=3)</td>
<td>7.62</td>
<td>2.09 ± 0.027</td>
<td>50</td>
</tr>
<tr>
<td><em>Murex brandaris</em></td>
<td>70</td>
<td>10.34</td>
<td>2.83 ± 0.033</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td><em>M. trunculus</em></td>
<td>70</td>
<td>120.6 (n=1)</td>
<td>8.26</td>
<td>2.26 ± 0.029</td>
<td>50</td>
</tr>
<tr>
<td><em>N. canaliculata</em></td>
<td>70</td>
<td>9.27</td>
<td>2.54 ± 0.057</td>
<td></td>
<td>160</td>
</tr>
<tr>
<td><em>N. emarginata</em></td>
<td>70</td>
<td>10.05</td>
<td>2.75 ± 0.055</td>
<td></td>
<td>120</td>
</tr>
<tr>
<td><em>N. lamellosa</em></td>
<td>70</td>
<td>8.88</td>
<td>2.43 ± 0.045</td>
<td></td>
<td>120</td>
</tr>
</tbody>
</table>

**Table 5.8** Chromosome number, haploid chromosome length (where determined) and genome size (C value = pg DNA in haploid genome) derived from the Feulgen reaction, in 9 muricid species.
Figure 5.12 Correlation between relative density of interphase nuclei in the Feulgen reaction and Genome Size (pg DNA) derived from three reference species (red). The other species (green) are placed on the regression line based on mean relative density measurements.

Figure 5.13 Relationships between chromosome length and genome size.
The most striking observations when comparing karyotypes and genome sizes in the species studied are as follows:

a. *N. lapillus* (2n=26) has a greater proportion of metacentric chromosomes, the lowest chromosome number and length, but almost the highest DNA content compared to the other species.

b. In the 2n=70 species, there appears to be a correlation between total chromosome length and genome size, as although there is considerable variation in total haploid length (106 to 139 μm) the ratio of chromosome length : DNA is very similar in all three (51 to 53; Fig. 5.13). However, total haploid lengths of the 2n=70 species are around 50 to 100% greater than that of *N. lapillus* (70 μm), but genome size (2.1 to 2.6 pg DNA) is usually significantly less than *N. lapillus* (2.74 pg). [The published figure for *N. lapillus* is 2.8 pg (Hinegardner, 1974).]

c. *Ocenebra erinacea*, from the genus most closely related to *Nucella* (Kool, 1993), has the highest total haploid length measured (139 μm), twice that of *Nucella lapillus* (26) and it has virtually twice the number of chromosomes as *Nucella lapillus* (36) yet a similar genome size (2.6 pg).

d. The 2n=36 form of *N. lapillus* has a 14% greater haploid length but 7% less DNA than the 2n=26 form (Fig. 5.13).

e. The only species with a genome size not significantly different from *N. lapillus* (2n=26) is *N. emarginata* (2.75 pg) and the only species with a larger genome size is *Murex* (*Hexaplex*) *brandaris* (2.83 pg), but neither of these species have been karyotyped.
5.4 Discussion

Evolutionary studies suggest that *N. lapillus* is the derived form within the genus/group (Collins *et al.*, 1996) and is more closely related to *Ocenebra* than the other genera, i.e. *Thais* or *Murex* (*Hexaplex*) (Kool, 1993). The comparative values from this study for the less derived/less specialised congenerics and other muricids suggest the general evolutionary trend has been towards an increase in genome size and a decrease in chromosome number and haploid length. To achieve this, i.e. packaging more DNA into a smaller size, changes in the coiling and/or folding of the DNA must occur. Sumner (1990) has provided some figures for Packing Ratio (total length of DNA/length of chromosomes) in humans and mammals. The haploid genome of humans amounts to about 3 pg DNA which is equivalent to approximately 1 metre of linear DNA. The total haploid length for humans is around 100 μm so the Packing Ratio is 10000. In the muricids studied here, *N. lapillus* (26) has a packing ratio of 13410, that of the 2n = 36 form is 10766 and those of *Ocneebra erinacea*, *Thais haemastoma* and *Murex trunculus*, are 6254, 6491 and 6246 respectively.

So it would appear that in *N. lapillus*, evolution has brought about a reduction in chromosome number and haploid length but an increase in genome size and therefore packing ratio. The proportion of metacentrics in the karyotype has also increased through Robertsonian fusions. This raises the following questions: (a) Why should selection favour these changes, (b) have similar trends occurred in other groups, and (c) what form and function does the additional DNA have?

There has been considerable debate on the evolution of genome size i.e. nuclear DNA content, in many eukaryote groups, and its relationship with the evolution of chromosomes and karyotype has invoked increasing interest as our knowledge of the subject accumulates (White, 1978; Swanson *et al.*, 1981; Cavalier-Smith, 1985a & b;
John & Miklos, 1988; King, 1993). It is questionable that the data here are sufficiently robust and extensive to enter this debate but some tentative observations or hypotheses are possible based on some of the current theories.

Cavalier-Smith (1985a, Chapter 1) introduces the subject under the title ‘the evolutionary significance of genome size’. The DNA C-value paradox (Thomas, 1971), i.e. the lack of relationship between the number of genes and the DNA C-values in different eukaryotes, is discussed and the types and function of non-genic, secondary DNA are described and related to genome size, e.g. junk DNA, constitutive heterochromatin, selfish DNA, B-chromosomes. The relevant points or proposals are:
a. Several correlations exist between genome size and quantitative characters but these are felt to be “the result of indirect or developmental and/or evolutionary correlations; but two phenotypic characters, namely chromosome volume and nuclear volume, do seem to be directly and causally determined by a combination of the sheer amount of DNA together with the tightness or looseness of its folding.”
b. Evolution of DNA sequences and chromosome numbers are separate problems from genome size evolution. For a given genome size, variations in chromosome number change the size of a chromosome; therefore if selection on chromosome size was more powerful than selection on numbers, then chromosome numbers might evolve incidentally as a result of selection for particular genome sizes or chromosome sizes.
c. On cell volume and genome size, Cavalier-Smith (1985b) suggests; ‘The absence of a clear correlation between chromosome number and genome size means that on average chromosome size is directly proportional to genome size.’ The addition or deletion of non-coding DNA during the evolution of different genome sizes is discussed and the work of Narayan (1983) is cited which may be relevant to the
muricid data here. It shows that in legumes of the genus *Lathyrus*, as genome size increases in related species the same total amount of DNA is added to each chromosome. A close correlation is also seen between total chromosome volume and nuclear DNA content for 24 species of *Lathyrus*. The results for the 2n = 70 muricid species here suggest a similar pattern in that the chromosomes of *Ocenebra erinacea* are generally larger than *Murex trunculus* which in turn are larger than those of *Thais haemastoma*, but the *N. lapillus* karyotypes have obviously evolved in a distinctly different way (see Table 5.8).

Genome size variation in several animal groups show similar trends and phenomena. Most molluscan subclasses have average C-values close to 1.6 pg, but archeogastropods are lower than neogastropods which average 3.2 pg (Hinegardner, 1974). Patterson (1969) found that most neogastropods have high chromosome numbers. These findings both suggest that polyploidy may have played a role in the evolution of neogastropods. Bivalves seem to have reduced DNA in evolved or specialised forms, whereas neogastropods show the opposite (Hinegardner, 1974), as indicated in this study.

Several studies have shown evidence of differential packing of DNA between species. Sella *et al.* (1993) show no correlation between genome size and karyotype length in species of *Ophryotrocha* (Polychaeta); two species with two or three times the `normal` DNA do not differ greatly in karyotype length. Pellicciari *et al.* (1986) show that three species of planarians (*Dugesia* spp.) have different DNA content per unit length of karyotype and Raina and Bisht (1988) in a study of 56 species of *Vicia* (another legume), show that the density of packing of DNA increases with amount of DNA and suggest this is at least partly due to a disproportionate increase in heterochromatin relative to the euchromatin component of DNA. However, in other
groups, significant correlations between DNA content and total chromosome length have been found, e.g. in 23 species of *Aedes* mosquitoes (Rao & Rai, 1987). The role, form and function of heterochromatin addition has been debated for many years (see King, 1993) and its involvement in the fixation of negatively heterotic rearrangements (e.g. Rb) or the process of speciation has been discounted by some authors e.g. Miklos *et al.* (1980). However, the debate continues, as Redi *et al.* (2001) review the current understanding of the functions and effects of heterochromatin in evolution and development.

Suggestions of further work to extend and resolve some of the outstanding issues here include: (a) Confirming the genome size values with alternative methods, e.g. flow cytometry, as the Feulgen reaction is known to have some limitations (Bennett and Smith, 1976). (b) Comparisons of more species within the group; e.g. an investigation of the chromosomal homologies between *Ocenebra erinacea* (2n=70, haploid length = 139μm) and *Ocinebrina aciculata* (2n=86, haploid length = 150μm) in order to ascertain if Robertsonian rearrangements also occur in this sub-group might prove interesting. (c) More detailed analysis of the DNA content in the extreme and intermediate forms of *N. lapillus*. (d) An attempt to identify the additional DNA in the 2n=26 karyotype of *N. lapillus*. Is it secondary (non-coding/heterochromatin) or are there more genes (euchromatin) present? (e) If possible, ascertain if this polymorphism represents a form of chromosomal speciation. Some comments of King (1993) may be pertinent here:

a. Defining a species – 'appears to be no more resolvable today than it was two hundred years ago. Indeed it could be argued that the situation is deteriorating.'

b. 'It is no exaggeration to say that the history of science is littered with discarded species concepts'.
Chapter 6. The possible causes, consequences and correlations of the chromosomal polymorphism in *Nucella lapillus*.

6.1 Introduction

Robertsonian rearrangements occur in many different animal groups often with differing life histories, and behavioural or ecological traits; consequently many theories on the causes and consequences have arisen and the literature on this subject, and on chromosome change/evolution in general, is plethoric (e.g. see John & Freeman, 1975; White, 1978; Sharma & Sharma, 1983; Sites & Moritz, 1987; Searle, 1988a,b; King, 1993; Hauffe & Searle, 1998; Shaffer & Lupski, 2000). They are reported to be the most common chromosomal rearrangement in humans (1 in 1000 individuals; see Shaffer and Lupski, 2000), the most effective process in chromosomal evolution leading to speciation in mammals, and they have profound effects on human health (through trisomies) and fertility in many domesticated animals (see Garagna et al., 2001).

Presumably Robertsonian fusions or fissions (Rbs), or indeed most chromosomal changes, occur initially as random mutations, although they may be more common at certain ‘fragile’ sites. Such mutation events are more likely to persist or become fixed in species that have low vagility, low powers of dispersal and small deme size, as suggested in the stasipatric speciation model of White (1978). How and whether these become fixed in populations depends on the consequences of the rearrangement. If the Robertsonian fusion/fission is balanced there may be little or no effect on reproduction, fertility or fitness as reported in some Rbs of sheep (Bruere & Ellis, 1979) and humans (Collinson et al., 1997). In wild populations these rearrangements would persist as a polymorphism, particularly if they confer some selective advantage in heterozygous and/or homozygous form, if not they may be lost. However, in many cases studied Rbs are shown to be negatively heterotic with possibly severe reduction in viability or fertility of heterozygotes.
due to difficulties in meiotic segregation, e.g. in shrews (see Searle, 1988a, 1988b and section 2.5.3), mice (Hauffe & Searle, 1998) and humans (Conn et al., 1998). Reproduction can be affected in several ways, e.g. disruption at meiosis in Robertsonian heterozygotes linked to the formation of multivalents in ring or chain formation at prophase I, and the increased frequency of anaphase I non-disjunction. These events can act to suppress crossing-over between homologous chromosomes, or result in sterility or sub-fertility due to gamete disruption.

There are many examples of Robertsonian rearrangements leading to polymorphism, e.g. in humans, polytypy (differences in races or subspecies) e.g. house mice and shrews, and speciation, e.g. lemurs (see Rumpler et al., 1986), mole rats (Nevo et al., 1994), great apes/human (Yunis & Prakash, 1982), some teleost families (Arkhipchuk, 1995; Ueno & Takai, 2000) and penaeid shrimps (Chow et al., 1990), to name a few. Further cases are described by Sharma & Sharma (1983) and King (1993). According to King (1987), RBs will only lead to speciation if the rearrangement is negatively heterotic (or potentially so) and post-mating reproductive isolation is formed. Gregorius and Hertzog (1989) suggest that this is not generally valid and offer theoretical evidence that speciation can still occur if the RB produces a balanced meiotic system with normal segregation. The debate on the various models of chromosomal evolution and speciation is long-standing and on-going (Sites & Moritz, 1987; King 1993)

The rearrangements reported here in *N. lapillus* may have led to reproductive isolation through chromosomal incompatibilities preventing successful breeding between individuals of different karyotypes. This would allow different ‘chromosomal races’ to persist but does not explain the presence of so many intermediate types in some populations. Another theory involves the suppression of exchange (crossing-over) around the centromeres at meiosis in Rb heterozygotes, thus conserving large linkage groups.
This, as Searle (1988a) suggests, may allow these regions of the chromosome to evolve independently in both homozygotes leading to two types or races that are adapted to different ecological niches. The heterozygotes may become well adapted to intermediate environments, fitter in all habitats, or at a disadvantage in general, compared to the homozygotes. The addition of chromosomal inversions to the Robertsonian fusion variations already described in *Nucella lapillus* is likely to increase any possible effects on fitness (positively or negatively). However, previous authors (Staiger, 1957; Mayr, 1969) have claimed that the extreme karyotypic forms of *N. lapillus* are fully interfertile, the only evidence being the Robertsonian nature of the polymorphism, and the presence of both intermediate types (26<2n<36) and trivalents in meiosis. In light of our increased knowledge of the karyotypic variations, including inversions, this is obviously a claim that requires testing.

In true Robertsonian polymorphism there should be no alteration to the genetic material and therefore, no direct physiological effects. However, it is generally agreed that chromosome breakage is involved and consequently genetic material may be lost or gained and linkage groups will be affected, all of which could have a physiological effect on an individual. Searle (1988a) discusses this issue and Nevo *et al.* (1994) describe an example of a correlation between diploid chromosome number and aridity stress in the speciation and adaptive radiation of the mole-rat in Asia Minor. This may be similar to the Rb polymorphism in *N. lapillus*, where inversions and variation in overall chromosome lengths and genome size are observed, which may well have an important effect during selective processes in the varied environmental conditions experienced by littoral invertebrates such as the dog-whelk. Studies on the steroid metabolism in this species have shown that metabolic pathways can be very finely balanced (Gibbs *et al.*, 1988; Spooner *et al.*, 1990) and it is quite conceivable that chromosomal changes that affect gene
activity or linkage might have fairly profound physiological or developmental consequences.

The degree and geographic pattern of Robertsonian variation in any species is dependent on many factors and Searle (1988a) discusses how the frequency of these rearrangements may be influenced by selection if they (1) affect fitness (e.g. fertility) or (2) are likely to have unequal transmission from the heterozygotes compared to the standard chromosomes.

In recent studies of human Rbs, evidence supports transmission ratio distortion in favour of the Rb in offspring of heterozygous female carriers through the preferential segregation of chromosomes during the first meiotic division (Pardo-Manuel de Villena & Sapienza, 2001). Other examples of this 'meiotic drive', leading to fixation of negatively heterotic rearrangements are given and discussed by King (1993).

Therefore, in a study such as this, looking at karyotypic and geographic patterns in this phenomenon one should hope to gain evidence of some bias or possible relationship to fitness. In this chapter I attempt a brief overview of previous evidence on the causes, consequences and correlations of the polymorphism in *N. lapillus* and provide some new data that may add to the debate. I present the available data on fecundity, fertility, chromosome numbers and karyotypes in some of the populations studied here to determine whether there are any obvious relationships between them and whether the polymorphism is balanced or favours particular rearrangements.

If the questions posed in chapter 1 are reviewed, some remain to be addressed and are relevant here:

1. Is there selection within a population for certain karyotypes or chromosome number, e.g. comparing embryos with adults?
2. Is the polymorphism neutral within populations, i.e. are karyotypes biased towards homozygotes or heterozygotes?

3. How strong is the link between chromosome number and environment/shore type?

Another question could be added:

4. Is there any evidence for a correlation between karyotype and phenotypic or physiological traits that could affect fitness?

6.2 Fecundity and Fertility

6.2.1 Number of embryos.

In processing material for chromosome preparations the number of developing embryos in each capsule was recorded. The number of capsules analysed from each site ranged from 2 to 76. This is the only measure of comparative fecundity attempted in this study and may give an insight into any major differences in the fertility of the adults, i.e. production of viable gametes/zygotes, or fitness of the embryos, i.e. failure to develop. These data are expressed graphically in Figure 6.1, and show no clear relationship between chromosome number and number of embryos per egg capsule. Personal field observations would suggest that many more capsules are laid by 2n = 26 populations on exposed shores than those in sheltered environments, e.g. at Bude and Whitsand Bay, both open sandy beaches exposed to considerable wave action, extensive mats containing thousands of capsules are often seen around crevices and overhangs in the rocks at certain times of the year (Figs. 6.2, 6.3). However, these populations tend to have much higher densities than those in sheltered areas and breeding aggregations comprising hundreds of animals are not uncommon (Fig. 6.3). Hence the number of capsules produced per female may not differ widely.
Figure 6.1 Mean number of developing embryos per egg capsule versus the mean diploid chromosome number (approximate in some cases) of the population.

A quantitative measure of this difference has not been made but is likely to be related to the larger animal size and better food supply on some exposed shores. Extensive studies have been made previously on fecundity and other productivity parameters in various populations of *N. lapillus* on both sides of the Atlantic; considerable variation is evident between different populations and areas (Feare, 1970; Hughes, 1972; Spight & Emlen, 1976; Crothers, 1985 and Etter, 1989). Hughes (1972) estimated that females from Halifax, Nova Scotia, produced a mean of 46.6 capsules per year, whereas Fretter and Graham (1985) noted that females from the northernmost European populations (White Sea) lay 20 to 30 capsules per season, but animals from more temperate Atlantic regions may lay around five times this number. Interestingly, in a comparison of two populations from Massachusetts, Etter (1989) found that the mean number of capsules laid by females from an exposed population (21.4) and the mean number of hatchlings emerging from each (19.51) were both around twice that of females from a protected site (12.4 and 8.32). However, hatchlings from the exposed site were considerably smaller in size.
Figure 6.2 Whitsand Bay, SE Cornwall, an example of a high energy shore (shown here in relatively calm conditions).

Figure 6.3 Breeding aggregation of *Nucella lapillus* at Whitsand Bay with numerous recently laid egg capsules and others at various stages of development.
6.2.2 Breeding experiments

Adults from three populations exhibiting low (2n=26, Whitsand Bay), medium (2n=28 to 34, Renney Rocks) and high chromosome numbers (2n=35 to 36, Mount's Bay) were collected and separated into sexes for breeding crosses. One of the problems in making crosses in this species is that the females store sperm in the bursa copulatrix for a considerable time after mating. The sperm is then used to fertilize eggs in the capsule gland prior to encapsulation. It is not known how long sperm from a previous mating remains viable in female *N. lapillus*; in *N. emarginata* a period of up to two months is suggested (Palmer, 1984). However, in the hope of avoiding fertilisation by stored sperm, sexed animals from each population were maintained in separate cages for at least one month before females and males were mixed. Due to lack of breeding success, four attempts (over a 4 year period) were made to cross females and males from the three populations in all the possible combinations, i.e. 9 crosses in all, including 3 controls using females and males from the same field populations. In each cross, approximately 20 animals of each sex were mixed and maintained with a food supply of mussels in separate cages within tidal tanks for up to three months. All cages were checked for egg capsules on a frequent basis (at least weekly).

The results of these experiments were spectacular in their paucity! On many occasions laboratory-maintained *N. lapillus* have produced egg capsules in similar seawater systems but in these experiments capsules were laid in only three crosses from the total of 36. In the first trial, 10 capsules were produced by the WW (Whitsand females and males) cross, all of which proved fertile and developed normally, and 5 capsules were laid in the WM (Whitsand females and Mount’s Bay males) cross, which all appeared to be infertile and failed to develop. In the fourth attempt, after two months a total of 77 capsules were laid in the WW cross; 74 developed normally, 3 were infertile. In addition to this, 87 egg
capsules were produced in a cage holding a stock of Whitsand females that had been separated from any males for at least two months; embryos developed in 58 of the capsules and 29 appeared infertile.

No viable offspring were produced by any of the crosses between the three chromosomal types, but unfortunately and frustratingly, the paucity of results renders the study inconclusive. The reasons for the lack of capsule production remain a mystery, but those produced by the separated stock females after possibly more than two months since the last mating, indicate that sperm can remain viable in the bursa copulatrix of the female for a considerable time and emphasises the difficulties with breeding studies in this species. This also suggests that females do not require direct or recent stimuli from males or mating to produce egg capsules.

6.3 Is the polymorphism neutral?

In an attempt to ascertain if the polymorphism is neutral/balanced with respect to homo- and heterozygotes, one polymorphic population (Renney Rocks) was selected for a detailed study. Variation within and between individual embryos and adults from a breeding aggregation were to be compared. However, karyotyping the prepared spreads proved extremely difficult as many did not conform to the arrangements already found and often appeared to be aneuploid, comprising two or three supernumerary chromosomes. Time constraints have rendered this detailed study impossible. Nevertheless, several populations and individuals have been studied in sufficient numbers and detail to allow some analysis of this aspect, but intra-individual variation in chromosome number and karyotype is common in some populations which makes analysis and firm conclusions difficult. Data from some populations are shown below in Figures 6.4 to 6.12.
6.3.1 Numerical variation within and between individual embryos

Analyses of low or high chromosome number populations (2n < 29 and 2n > 34) are relatively easy because karyotyping is usually less complex. Figures 6.4 to 6.6 summarise the numerical variation in karyotypes of embryos from the Isle of Man, Norway and Mount’s Bay. Some embryos are monomorphic for one diploid value (Figs. 6.4B, 6.5C and E) and embryos from the same capsule can exhibit different monomorphic values, (Figs. 6.6C and D). Most single embryos in these populations have spreads with more than one diploid value but there is no apparent pattern in the ratios of each. Combined chromosome numbers for all the embryos analysed from each population are spread over 3 values e.g. 26, 27, and 28, but again no clear overall bias is evident (Figs. 6.4D, 6.5G and 6.6F). The 2n>36 (+) spreads from Mount’s Bay were a few showing 37 chromosomes, one in excess of the normal 2n=36 karyotype (A1,2,3,8,9) which may be just ‘contamination’ from another spread during slide-making or possibly aneuploidy/trisomy.

![Graphs showing frequency of diploid chromosome numbers](Figure 6.4 Frequency of diploid chromosome numbers in embryos from Port St. Mary, Isle of Man. A and B. – single embryos from the same capsule. C. – group of embryos (n=5) from another capsule. D. – totals for all embryos.)

151
Figure 6.5  Frequency of diploid chromosome numbers in embryos from Slaattholmen, Norway. A and B, C and D, E and F, – pairs of embryos each from the same capsule. G, – totals for all embryos.
Figure 6.6 Frequency of diploid chromosome numbers in embryos from Mount's Bay, Cornwall. A and B. groups of embryos from two separate capsules. C and D. single embryos from the same capsule. E. single embryo from a different capsule. F. totals for all embryos.
In highly polymorphic populations e.g. Renney Rocks, Cawsand and Thurlestone (Figs. 6.7, 6.8 and 6.9) monomorphic embryos have not been found, individuals often exhibit spreads over a range of four or five diploid values, and again no distinct patterns are shown. The diploid range for all embryos analysed in these populations (e.g., Figs. 6.7D, 6.8H and 6.9F), and indeed for some individuals (e.g. Fig 6.7B, 6.8A, B and 6.9A, B), is quite extensive, but again no obvious pattern or bias is shown in the limited number of animals analysed here. However, in these, confirmation of all karyotypes has not been feasible so possible errors cannot be ruled out. For example, the 2n=26 and 27 spreads shown in Fig. 6.7D were not confirmed by karyotyping and could be accounted for by loss of chromosomes during slide-making.

Figure 6.7 Frequency of diploid chromosome numbers in embryos from Renney Rocks, S. Devon. A, B, C – three single embryos from the same capsule. D – totals for all embryos.
Figure 6.8 Frequency of diploid chromosome numbers in embryos from Cawsand, SE Cornwall. A and B - single embryos from the same capsule C - F – four single embryos from the same capsule. G. – group of embryos (n=8) from a different capsule. H. – totals for all embryos.
Figure 6.9  Frequency of diploid chromosome numbers in embryos and adults from Thurlestone, S. Devon. A and B - single embryos from the same capsule. C and D - single embryos from the same capsule. E. - group of embryos (n=5) from a different capsule. F. - totals for all embryos (n=9). G. - a single adult (testis). H. - group of 20 adults combined.
6.3.2 Variation between embryos and adult males

Few data are available for this comparison; the adults at Thurlestone (Figs. 6.9 G, H) show more variation and a wider range than the embryos, and those at Dumpton Gap (Figs. 6.10 E, F) show a difference, both showing a majority of 2n=27 spreads but embryos have few 2n=26 and numerous 2n=28 spreads whereas adults have numerous 2n=26 and few 2n=28. However, the embryos analysed from Dumpton are unlikely to be the offspring of the adults used, which may explain this difference.

6.3.3 Variations within and between nearby populations

Figure 6.10 shows frequency distributions for chromosome numbers in some of the populations studied on the Kent coast. Most histograms relate to two or more embryos, and some show a clear bias for one diploid value (Figs. 6.10A, C and G) whilst others have a more even distribution (Figs. 6.10B and E). All populations north of North Foreland, i.e. on the north coast of Kent, are monomorphic for the 2n=26 karyotype.

Populations from Maine, USA (Fig. 6.11) all exhibit some cells at 2n=26 and some at 2n=27; those at Otter Point (high energy shore) and Otter Cove Salt Pond (low energy) are biased towards 2n=26 (about 85%) whereas at La Moine (low energy) similar numbers of each occur.

Although featuring few spreads from relatively few embryos from three populations on the Isle of Wight, Figure 6.12 emphasises again the large numerical variation that often occurs in populations of N. lapillus over small geographic distances. Another even more dramatic difference is shown between populations on the leeward side of Burgh Island (2n = 30-31) and the adjacent mainland coast at Bigbury (2n = 26), with only about 200 metres of open sand separating them (see Table 4.1).
Figure 6.10  Frequency of diploid chromosome numbers in embryos and adults from sites in Kent. A. - 2 embryos (same capsule) from Folkestone. B. - 10 embryos (from 3 capsules) from Dover. C. - 2 embryos (same capsule) from St. Margaret’s Bay. D. - 2 embryos (different capsules) from Old Stairs Bay. E. - 4 embryos (from 2 capsules) from Dumpton Gap. F. - group of adults (n=26) from Dumpton Gap. G. - 2 embryos (same capsule) from North Foreland. H. - typical frequency for embryos from Palm Bay, Margate or Hampton.
Figure 6.11 Frequency of diploid chromosome numbers in embryos from sites in Maine, USA. A. – 6 embryos from two capsules, La Moine. B. – 2 embryos from Otter Cove Salt Pond. C. – group of embryos (n=8) from two capsules, Otter Cove. D. – totals for all three sites.

6.3.4 Statistical analysis

Ideally, the chromosomal variation between individuals and between adults and offspring should be tested statistically to ascertain whether the polymorphism is neutral, i.e. to compare observed frequencies of heterozygotes and homozygotes with the Hardy-Weinberg ratio and possibly estimate the fitness of different karyotypes. This has not been attempted as I feel it is impossible to make these analyses when intra-individual variation is so common and complex.

This mosaicism, which appears so often in many polymorphic populations of *N. lapillus*, is worthy of further investigation to determine if all tissues within an individual exhibit it equally, and if it is as common in adults as in embryos. To facilitate statistical analysis in
future, simpler and/or automated methods of determining 'chromosomal genotype' or karyotype should be sought. Labelling with FISH to distinguish the metacentric and 'A' forms of a selected chromosome pair, which could then be scored visually or via flow cytometry, may be feasible. However, finding a detectable sequence difference in the Robertsonian chromosomal forms would be time-consuming, expensive and perhaps impossible with the methods available at present.

Figure 6.12 Frequency of diploid chromosome numbers in embryos from sites on the Isle of Wight. A. – 4 embryos from Hanover Point. B. – 2 embryos from Horse Ledge. C. - 2 embryos from Ethel Point.
6.4 Correlations: Environment/Wave action, Phenotype and Physiology

It seems clear that a link between chromosome number or karyotype and shore type does exist within a small part of the species' range. However, for various reasons, e.g. high intra-individual numerical variation and the fact that the same (>26) number of chromosomes can be achieved by different structural rearrangements or involve different chromosome pairs in different individuals or populations (see Chapter 4), I suggest that this link is probably indirect.

As discussed earlier (section 3.5), shores with high chromosome number populations sometimes appear to be quite exposed (e.g. Mount's Bay and Renney Rocks). Although I have not attempted to classify the shore type/exposure in any quantitative way, e.g. using Ballantine's (1961) scale, field observations at many sites during all weather conditions suggest that micro-habitat may have a key role in reducing the effective exposure to severe wave action and thus decreasing the likelihood of animals being swept from the rocks and dispersed. Interestingly, but not surprisingly, the shores found to have 2n = 36 individuals/karyotypes are similar in topography and microhabitat; they are fairly open stretches of coast with large outcrops of rock protecting areas of relatively small boulders (e.g. see Fig 6.13). This would serve to reduce deme size and gene flow and may be one explanation for the persistence of these karyotypic forms.
Figure 6.13 Views of Renney Rocks, SW Devon. A. The reef faces south-west and is often exposed to severe swells and wave action. B. Behind the main outcrop an extensive area of small boulders provides a more sheltered micro-habitat.
One factor that may be relevant to the occurrence of chromosomal polymorphism in *N. lapillus* and not in related muricids is the possible difference in embryonic development at hatching, notably in the size and function of the velum. For example, *N. lapillus*, *Ocenebra erinacea* and most of the other muricids in this study are reported to hatch as young snails or 'crawlaways', where the velum has been partially or totally resorbed, whereas *Thais haemastoma* and several other species hatch as veligers (Pelseneer, 1910; Spight, 1975, 1976). However, the precise stage of development at hatching is known to vary with latitude and habitat in some species (Spight, 1975; Gallardo, 1979). Personal observations on the embryos of *N. lapillus* and *O. erinacea* at hatching, show that although the velum is much reduced in the former, it often remains as a large active organ in *O. erinacea*, allowing a free-swimming phase in this species (Fig. 6.14). Other traits such as drifting in the water column via mucus threads have also been recorded in some muricids (e.g. *N. emarginata*; Martel & Chia, 1991). Powers of dispersal, deme size and gene flow are likely to be affected by these differences and these in turn will affect the maintenance of chromosomal polymorphisms through isolating mechanisms.
Some studies on *N. lapillus* have proposed links between shell type and chromosome number (Staiger, 1957; Kirby et al., 1994a), but Hoxmark (1971) did not find support for this in Norwegian populations and Crothers (1975) found no apparent correlation in southern England. I feel this perhaps depends on which parameters are analysed and in how much detail (e.g. size/weight, thickness, shape of the opening), the location of the population or merely which populations are compared. It is possible that this trait may just covary with other parameters. Recent evidence on the phenotypic changes in progeny of *N. lapillus* transplanted from an exposed shore to sheltered inlets (Gibbs 1993a), where the
phenotypic characters typical of a sheltered shore were expressed by 2n=26 progeny, also suggests that there is no direct relationship between shell morphology or body form and chromosome number. These parameters appear to be direct adaptations to the environment or responses to other stimuli (e.g. predators; Crothers, 1983), which can be expressed within one generation, without any apparent change in karyotype.

Correlations between physiological factors and chromosome number have also been proposed, and in some cases the evidence seems reasonably strong, e.g. with allozymes in populations of SW England, Day and Bayne (1988) found an absolute locus association between *Mdh-1* and *Est-3* at the only site they analysed which was likely to have 2n > 26 karyotypes. As these individuals may possess trivalents which would result in effectively highly linked loci, they suggested that this locus association might relate to the chromosomal polymorphism. However, no correlation was found between allozyme variation and either shell morphology or geographical distance. Further interesting results were found when relating allozyme variation to microgeography and over greater geographic distance (Day, 1990; Day *et al.*, 1993); in these studies putatively high chromosome number populations were found to possess a suite of alleles at four allozyme loci (*Est-3, Lap-2, Mdh-1* and *Pep-2*) which were rare or absent in populations thought to be 2n=26. Kirby *et al.* (1994b, 1997) also studied links between both physiological and mitochondrial DNA variation in *N. lapillus* and allozymic, phenotypic and karyotypic variation. Differences in response to hyperosmotic stress were shown in populations with different shell shape, and two distinct MtDNA haplotypes were found which appeared to exhibit coincident step clines with variation in allozymes, phenotypes and karyotypes. In a subsequent study, Kirby (2000) argues that the polymorphism in mitochondrial *Mdh* and the clines in *N. lapillus* represent the presence of two persistent coadapted gene complexes, which may serve as multitrait co-evolving genetic solutions to environmental variation. Undoubtedly these extensive studies have substantial credibility but all lack convincing evidence of any direct correlation between these factors and chromosome number or karyotype, mainly because few, if any, individuals from the populations studied were examined karyotypically. Most populations were assigned a diploid value from data of
other studies from a decade or more earlier, some of which I have shown in this study may not be correct (e.g. populations from Norway and the USA). Although it is regrettable that these studies did not include precise karyotypic analysis, they indicate that with the increased knowledge of karyology, future emphasis on linking these factors with specific chromosomal rearrangements might prove fruitful.

6.5 Dumpton Syndrome

Further evidence of a possible genetic link with phenotype (i.e. a morphological abnormality) came to light during this study when karyotypes of populations on the Kent coast were analysed. In surveys of dog-whelk populations in SE England, the effects of TBT pollution were monitored by analysing the degree of imposex in this species (Gibbs, et al., 1991b). During these analyses, curious anomalies were observed in both female and male genital tracts. In the population at Dumpton Gap, a site with moderately high TBT concentrations at the time, some females exhibited no evidence of imposex development and surprisingly, some males were found to lack a penis. Further investigation confirmed this ‘aphally’ phenomenon (coined ‘Dumpton Syndrome’, Gibbs, 1993b) in approximately 10% of the male population and that it also resulted in underdevelopment of the whole genital tract including retardation of spermatogenesis. Laboratory breeding experiments with sub-samples from this population suggested the trait was inherited by the next generation (Gibbs, 1993b). Speculative conclusions were that this syndrome was a genetic disorder, which in terms of Darwinian fitness has disastrous consequences for the afflicted males, as contributing to the gene pool without a penis presents a major problem. However, it has a beneficial effect on females in a TBT-polluted environment as it confers a degree of immunity from the sterilizing effect of imposex thus allowing them to breed normally whilst unaffected females in the population are prone to sterilization.

Preliminary findings on this phenomenon initiated a study of karyotypes in this population; I present the key results here as part of this study. Metaphase spreads were prepared from testes of both normal and ‘Dumpton Syndrome’ (DS) males from the field site and from
the breeding experiments. Although data are limited, three DS males from the field produced a total of twenty seven 2n=27 spreads and three 2n=26 spreads; of these, 17 karyotyped as H9 and 12 exhibited the heterozygous inversion in chromosome pair 5 (SI, see Figs. 4.5 and 4.6). The two DS progeny from a F2 backcross produced just five 2n=27 spreads, three of which karyotyped as H9 and one exhibited SI. Only one other population in Kent, without a confirmed observation of DS, produced any spreads showing the inversion (viz. St. Margaret’s Bay) and three populations exhibited the H9 karyotype (Oldstairs and North Foreland, in Kent and Port St. Mary on the Isle of Man).

Although the evidence for a link between Dumpton Syndrome and the inversion in chromosome 5, possibly combined with the H9 karyotype, is not conclusive it cannot be ruled out as a possibility. Relatively small changes or structural rearrangements in chromosome complement are known to have profound developmental effects in other groups e.g. trisomy 21 and intersex in man and other mammals (Haqq & Donahoe, 1988; Singh et al., 1994). This speculation is supported by the discovery of DS in other populations (Brest (Huet et al., 1996) and Norway (Gibbs, personal observations)) and my observations of inversions and/or Rb rearrangements in pair 5 in the same populations, although no direct link with affected individuals has been made. If future work extends to more detailed karyology and gene expression/activity, this and similar links may be worth investigating.

6.6 Discussion

Responses to the questions posed at the beginning of the chapter are offered below:

1. Is there selection within a population for certain karyotypes or chromosome number, e.g. comparing embryos with adults?

In most populations, individuals appear to be restricted in their range of chromosome number and karyotype; some populations, as shown here, have much greater intra- and inter-individual variation than others. Possible differences may exist between adults and
embryos of the same population (e.g. Fig. 6.9), which is an important issue in this study where mostly embryonic material has been analysed. In other animals intra-individual variation in chromosome number or mosaicism is often linked to Rbs but is usually restricted to just 2 consecutive diploid values, e.g. 30 and 31 or 28 and 29 in specimens of the marine perciform fish, *Uranoscopus scaber* (Vitturi et al., 1991) and from prenatal diagnosis in humans (Hsu et al., 1996). Here in *N. lapillus* the diploid range often extends to 5 or 6 values (Figs. 6.5, 6.6, 6.7) which can be explained by the multiple Rbs often exhibited in this species. Mosaic embryos are often not viable depending on the proportion of cells with a 'normal' chromosome complement (Conn et al., 1998), and in this study, as early veliger-stage embryos were used, phenotypic or developmental abnormalities may not have been observed. Differences in chromosome numbers of adults and embryos may reflect this in the embryos having a wider range of values and many not surviving to become adults, e.g. in spontaneous abortions of human embryos with chromosomal anomalies (Evans, 1988). However this does not appear to be the case in *N. lapillus* at Thurlestone (Fig. 6.9 F, H) as adults appear to have a wider range than embryos. It would seem that Rb polymorphism in this species does not impair viability, which suggests that the Rbs are balanced structural rearrangements resulting in a normal phenotype (Conn et al., 1998). More detailed investigations of this subject were planned but the difficulties with breeding this species and detailed analysis of highly polymorphic populations have precluded conclusive evidence on this matter. Suggested future work includes a detailed investigation of meiosis of each sex and synaptonemal complexes in various populations (Searle, 1986a; Wallace and Searle, 1990) which may show possible fertility anomalies. Also, attempts at *in vitro* fertilization with ripe gametes may allow faster and more revealing results on cross-fertility than breeding studies.
2. Is the polymorphism neutral within populations, or are the karyotypes biased towards homozygotes or heterozygotes?

Individuals in low or high chromosome number populations are often monomorphic but in the populations as a whole I have found no clear evidence that the polymorphism is biased towards homo- or heterozygotes. As intra- and inter-individual variation is high in most polymorphic populations, a much larger sample size would be required to test this thoroughly, and without automation of the karyotyping (e.g. with the Leica ‘Chantal’ system) this would require an inordinate amount of time. Perhaps this is a problem that cannot easily be answered through simple cytogenetics.

3. and 4. How strong is the link between chromosome number and environment/shore type? (and) Is there any evidence for a correlation between karyotype and phenotypic or physiological traits that could affect fitness?

The debate on karyotypic correlations with other parameters continues; although data given here allow further hypotheses, the need for careful karyotyping is clear if future work hopes to advance this subject. The majority of Rbs in *N. lapillus* may be balanced and are less likely to result in isolation mechanisms or physiological changes relating to fitness than inversions or other more radical changes in karyotype. Ideally, better molecular techniques, e.g. chromosome painting to reveal monobrachial homologies, and a link to gene expression, need to be developed to resolve this enigmatic problem.

So what are the causes and consequences of this phenomenon? The collective evidence suggests that the form of this polymorphism has arisen and is maintained by mutation and stochastic factors e.g. the founder effect and genetic drift, and through selective events acting at evolutionary, environmental and possibly physiological levels. Evolutionary processes seem to have been in the direction of reduction in chromosome number,
favouring larger metacentrics with more tightly packed DNA to allow an increase in genome size. *N. lapillus* has extremely low vagility and powers of dispersal in most habitats, which allow mutational changes to become fixed in populations and may create the widely differing karyotypes observed within small geographic ranges. The existence and maintenance of the different chromosomal types or races is likely to be linked to isolating mechanisms brought about by structural variations, as reported here, or by the environmental conditions affecting gene flow within a population or area.

There is no clear evidence (apart perhaps from DS) that any rearrangements affect fecundity or fertility of individuals or populations, but different populations may not be fully inter-fertile. However, I have not been able to demonstrate post-mating reproductive isolation between populations in this study. The possible links between karyotype and variation in physiology (allozymes) and phenotype appear worthy of further investigation but first precise confirmation of correlations with specific chromosomal rearrangements would be needed in a few populations.

It was hoped that this study would provide data that would help resolve some of the debate and theories on Robertsonian polymorphism in *N. lapillus*. However, as in most, or probably all, of the previous studies on the subject, conclusive evidence for a particular correlation or explanation is lacking, as there often appear to be exceptions to any proposed rule and most hypotheses require further investigation to prove. Therefore, it still remains unclear whether the numerous different karyotypic forms of this highly polymorphic species represent just chromosomal polymorphism, polytypy, or speciation in progress. Nevertheless, the work presented here has advanced the knowledge of the polymorphism in this species in several ways, each chapter describes significant additions to previous data and theories, and it has clarified the direction and focus future studies should take to resolve the remaining questions.
Summary by quotations etc.:

“One cannot hope to do anything significant or original in science unless one accepts the inevitability of substantial error along the way.”

*Wonderful Life – the Burgess Shale and the Nature of History.*

‘Evolution is not a force but a process, not a cause but a law.’


A fire-mist and a planet,
A crystal and a cell,
A jellyfish and a Saurian,
And caves where cavemen dwell;
Then a sense of law and beauty,
And a face turned from the clod-
Some call it Evolution,
And others call it God.

*W.H. Carruth. Each in his own tongue. (1908)*

The mind of most Scientists is like a glutton with poor digestion.

*Vauvenargues*

The struggling for knowledge has a pleasure in it like that of wrestling with a fine woman.

*Lord Halifax*

One who knows how to stop at where he cannot know has reached the limit of knowledge.

*Chuang-tse*

I didn’t begin to learn anything until after I had finished my studies.

*Anatole France*
Appendix 1. - Banding methods – trials and notes

Several different banding techniques were employed on chromosome preparations from the dog-whelk, *Nucella lapillus*, throughout the course of this study. The material used was metaphase chromosome spreads prepared from embryos or adult testes as described in Chapters 1 and 2, and dried onto glass slides (except where stated). Where possible, human metaphase spreads were used as controls. Protocols and results are listed below. Some methods provided limited success, e.g. trypsin C-banding and restriction enzymes, but in general, these techniques did not provide sufficiently consistent or clear results to allow the identification of homologous chromosomes.

1.1 Trypsin (basic method, Seabright, 1972)

Ten ml of distilled water are added to a vial of Bactotrypsin (Difco) to give a 5% solution. This is diluted further to make a 0.1% solution in distilled water. Slides bearing metaphase spreads are covered with 16 - 20 drops of 1X Earle’s balanced salt solution followed by 8 - 10 drops of 0.1% trypsin solution (10 secs - 1 min; 30 - 40 secs usually). The solution is then discarded and the slide quickly washed in phosphate buffer (pH 6.8) and stained in 6% Giemsa solution.

The following modifications were tried:

Trypsin solutions (0.01% - 5%), at temperatures of 18°C - 37°C, which were dissolved in distilled water, 0.85% sodium chloride or phosphate buffer pH 6.8.

The best results were obtained using a 0.1% solution of trypsin in distilled water, for 10 - 30 secs, at 21°C. This improved the visualisation of secondary constrictions in most spreads.

Pre-treating the cells with 2 x SSC, 5 min - 1 h at room temperature (RT) - 65°C or, HCl
(0.1 - 5N) 5 min - 1 h, RT - 65°C, produced the same effect as trypsin on untreated cells, but in less time and at lower concentrations. There was, however, no significant improvement in the end result. In summary, the trypsin method produced no clear banding pattern, but some chromosomal structural features were made more apparent.

The following pre-treatments were tried with each of the following four methods (1.2 to 1.5):

None
Carnoy's solution (3 parts ethanol: 1 part acetic acid)
Hydrogen peroxide
Hydrogen peroxide followed by Carnoy's
Carnoy's followed by hydrogen peroxide
45% acetic acid

1.2 2 x SSC (ASG technique of Evans et al., 1971)

a) Incubation in 2 x SSC, RT - 65°C, 30 min - 2 h, stained in Giemsa in phosphate buffer. The best results were obtained with temperatures of 60 -65°C, for 1 - 2 h. This gave a granular appearance to the chromatids which superficially resembled banding; close analysis subsequently failed to identify a sufficient number of distinct, diagnostic bands to make the method workable. N.B. lower temperatures gave homogeneous staining with no suggestion of 'banding'.

The following modifications were tried:

b) Incubation in 2 x SSC pH 12 (adjusted with sodium hydroxide), RT - 65°C, 30 min - 2 h, stained with Giemsa in phosphate buffer. Similar results to the previous method, 60 - 65°C for 2 h gave no staining.
c) Incubation 1 - 10 min in 2 x SSC pH 12 at RT followed by 1 - 2 h in 2 x SSC at 65 °C (i.e. a combination of the two previous methods) produced no improvement in staining properties, some chromosomes showed a granular appearance.

d) Incubation for 30 min - 2 h in 2 x SSC (RT - 65 °C) followed by 30 min - 2 h incubation in borate buffer (see next method for details) pH 9.2 (RT - 65 °C). No banding was observed but good staining was achieved with all treatments up to 1 h.

1.3 Borate buffer method

a) Aged slides (at least one-week old) are incubated in a Coplin jar containing preheated borate buffer at 65°C (pH 9.2; dissolve 14.2 g of sodium sulphate (Na₂SO₄) and 1.91 g sodium borate (Na₂B₄O₇) in 1000 ml distilled water) for 10 - 20 min (absolute incubation time varied in individual cases).

b) Rinse slides in distilled water and air dry.

c) Place slides on a flat rack over a sink. Three ml of diluted borate buffer (1:1, 1:2, 1:3)* and 1 ml of Gurr’s R66 giemsa stock are quickly mixed and poured onto each slide and left for 1 - 2 min or until the desired stain intensity has been reached.

d) The slides are briefly rinsed in tap water and air dried before microscopic examination.

*Note: 1:1 diluted borate buffer (1 part buffer to 1 part distilled water) should be used with well aged slides; 1:2 or 1:3 diluted borate buffer works better with fresh slides.

The following modifications were tried:

a) Incubation, 10 - 20 min, in borate buffer pH 9.2 at 65°C, washed in distilled water and air dried. Stained in Giemsa: borate buffer: distilled water:

1:3:3

10 - 120 secs

1:3:6
The best staining was achieved after 60 secs, but paler than the normal Giemsa stain. Chromosomes were swollen but not seen to be banded.

b) Incubation in borate buffer pH 9.2, stain in Giemsa in phosphate buffer

RT – 65°C, 30 min - 2h

The chromosomes were more darkly stained but not banded.

c) Incubation in HCl, followed by incubation in borate buffer. Stained in Giemsa in phosphate buffer

HCl, 0.1N - 5N, RT – 65°C, 1 min - 1 h:
Borate buffer, 30 min - 2 h, RT – 65°C.

This minor modification sometimes worked well in producing 'chromomere-type' banding patterns, e.g. when using HCl, 5N, RT, 5 min: Borate buffer, 60°C, 1 h. However, this was a difficult method to repeat since small variations in temperature resulted in either no staining or over-staining which made sub-structural details indiscernible.

1.4 C-banding methods

1.4.1 (Sumner, 1972)

Methods: HCl, 0.1N - 5N, 5 min - 2 h, RT – 65°C;

Ba(OH)₂, 5% filtered, RT -65°C, 1 min - 30 min;

2 x SSC, 10 min - 2 h, RT – 65°C.

Chromosome staining intensity is dependent upon the Ba(OH)₂ step; 55°C for 5 min gave
pale staining chromosomes; 55°C for 1 min resulted in darkly staining chromosomes. Weaker Ba(OH)$_2$ treatments gave normal chromosomes, whereas stronger treatments resulted in abnormal nuclei and chromosomes (viz., no banding, precipitation of stain, uneven staining).

1.4.2 **following Kohno et al. (1991)**

Modifications (some of mine also) on method of Sumner (1972)

Slides (stored at RT and -20°C) are treated with 1N HCl for 5 min, rinsed in DW, treated with Ba(OH)$_2$ at 55°C for 5 min, rinsed DW, incubated in 2XSSC at 55°C for 15 min, given a long rinse in DW followed by staining in 4% Giemsa for 20 + 20 min (stained twice).

1.4.3 **method of Jha, personal communication**

Slides (stored at RT for 4 days) are treated with 0.2N HCl for 30 min at 20°C, rinsed in DW, treated with Ba(OH)$_2$ at 32°C for 1.5 - 3 min, dipped in 0.2N HCl (10 secs) and rinsed in DW. Dry slides then incubated in 2XSSC at 60°C for 90 min, rinse thoroughly in DW followed by staining in 5% Giemsa for 30 min.

No real banding seen with any of these C-banding methods, chromosomes tended to appear rather fluffy.

1.5 **Alkali-Giemsa staining methods**

Incubation in NaOH (sodium hydroxide) 0.001M - 1 M, followed by staining in Giemsa, 1 - 20%.

Concentrated solutions of NaOH led to a loss of chromosomes. Weaker solutions produced pink staining chromosomes and no banding. The Giemsa-9 and Giemsa-11 techniques
were tried and these each yielded pink-staining chromosomes with no discernible bands. The staining was paler than that of chromosomes with Giemsa of lower pH. Staining was tried over the range 1 - 30 min. No banding patterns were detected using these methods and incubations of more than 10 min yielded degraded preparations.

1.6 R-banding from Kohno et al., 1991.

The method of Dutrillaux et al. (1973) was used with some modification (Kuro-o et al., 1986). Slides were stained with Hoechst 33258 50µg/ml in PBS for 15 min, rinsed in DW, mounted in PBS and exposed to UV light for 30 min. Rinsed in DW, incubated in 2XSSC at 60°C for 30 min, rinsed in DW and stained with Giemsa, 3% (pH 6.8) for 4 min. Slides were rinsed again and dried. PBS(-) used throughout is Ca and Mg free.

No convincing bands were seen.


a. Rinse in PBS pH 7.0

b. Stain with 1/100 dilution of stock solution of Hoechst 33258 (50 µg/ml) in McIlvaine's buffer for 10 min

c. Rinse in buffer for 10 min, then in DW

d. Drain and mount in DABCO (antifade).

**Results – see below.

1.8 C-banding with Acridine Orange (AO) (see Martinez-Lage et al., 1994 )

a. Incubate in 1N HCl for 5 min at RT

b. Then in 5% Ba (OH)₂ for 5 min at 60°C, followed by 15 min in 2XSSC at 60°C.

c. Stain in 0.01% AO in Sorensen's buffer pH 6.5 for 5 min.

d. Wash and mount in buffer.
1.8.1 *Acridine Orange alone - trial*

a. Rinse in buffer before and after AO.

b. Mount.

**Results – see below.

1.9 Chromomycin for GC-rich heterochromatin

Following Schweizer's (1976) protocol:

a. Preincubate in McIlvaines (citric acid – NaPO$_4$) buffer, pH 6.9 – 7.0 containing 10mM MgCl$_2$ for 10 – 15 min.

b. Stain in above buffer containing 0.12 mg/ml chromomycin A3*, 5 – 10 min (also tried 1h)

c. Wash and mount in McIlvaines and seal with rubber solution.

*Stock is 0.25 mg/ml, so dilute 1:1 for use.

**Results – see below.

1.10 DAPI for AT-rich heterochromatin

This is often done in combination with Actinomycin D or Distamycin A.

For DAPI alone use 0.2 – 0.4 μg/ml in McIlvaines diluted from stock at 0.2 mg/ml in DW.

1.10.1 *DAPI alone.*

a. Stain in DAPI solution at 0.2 – 0.4 μg/ml in McIlvaines for 5 – 10 min.

b. Rinse briefly and mount in McIlvaines and seal.
1.10.2 Actinomycin D and DAPI

a. AMD is made up in small volume of methanol then diluted to 0.25 mg/ml in McIllvaines pH 6.9 – 7.0
b. Preincubate slides in McIlvaine’s buffer for < 5 min.
c. Stain with AMD for 15 – 20 min, then rinse in buffer.
d. Then stain with DAPI at 0.33 µg/ml for 5 – 10 min, rinse in buffer and mount.

1.10.3 Distamycin A and DAPI

a. Stain with freshly made 0.1 – 0.2 mg/ml DMA in McIlvaines pH 7.0 for 5 – 15 min.
b. Rinse briefly in buffer then stain with 0.2 – 0.2 µg/ml DAPI in McIlvaines for 5 – 15 min.
c. Rinse and mount in McIlvaine’s buffer.

Above treated slides were viewed: no obvious banding was observed. With DMA + DAPI there may be some evidence of enhanced secondary constrictions on chromosomes 2 and 3 as well as 7 and 10.


a. Soak in McIlvaine’s buffer pH 7.0 for 5 – 10 min.
b. Stain in QM at 50 µg/ml in McIlvaines buffer for 20 – 30 min at 20°C.
c. Mount in anti-fade medium.

**Slides treated with the above methods (1.7 to 1.11) were checked under appropriate fluorescence – no obvious banding was observed in any of the protocols.**
1.12 Restriction Enzymes

Methods followed Martinez-Lage et al. (1994).

3 Restriction Enzymes (RE), supplied by Boehringer Mannheim UK Ltd, were tried in appropriate buffers (from supplier) at 0.5 U/µl at 37°C for 6h

Dilutions:

<table>
<thead>
<tr>
<th>RE (2.5µl)</th>
<th>Buffer (5µl)</th>
<th>DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu I</td>
<td>A</td>
<td>42.5µl</td>
</tr>
<tr>
<td>Hae III</td>
<td>M</td>
<td>42.5µl</td>
</tr>
<tr>
<td>Hinf I</td>
<td>H</td>
<td>42.5µl</td>
</tr>
</tbody>
</table>

Above RE solutions (50µl) were dropped onto slides of chromosome spread preparations and covered with a coverslip and then placed in a moist chamber at 37°C for 6h. Slides were rinsed in DW to remove coverslip and stained with 4% Giemsa for 5-10 min.

Results: generally, chromosomes were well preserved and 2° constrictions were usually visible in all treatments. In Hinf I treatments some spreads appeared to have a banding effect but was it not consistent and would be difficult to use for identification.

Additional trials:

SAL I and Eco R I both in H buffer

5µl of RE + 5µl of buffer + 40µl of DW gives 1.0 U/µl.

Left overnight in humid chamber (20h)

In some spreads some evidence of bands and dark 'feet' were seen but these were not consistent.
1.13 Trial of PRINS (Primed in situ Synthesis) technique.

Trials were made with a Chromosome Print Kit (Advanced Biotechnologies), using a Human Alu specific sequence.

This has several advantages over FISH: i.e. rapid, preserves morphology, safer.

I used the protocol from the supplier, Programmable Heat Block method.

Chromosomes looked reasonably well preserved but no convincing signals were seen (a few possibles, but very feint).

1.14 Scanning Electron Microscopy (SEM) studies

Metaphase chromosomes may be Giemsa banded (G-banded) by a variety of techniques which have revolutionized chromosome analysis. The most widely used procedure involves exposure of metaphase spread chromosomes to a dilute trypsin solution (see above). G-banding produces a consistent differential staining pattern along the chromosome arms in human chromosomes, allowing individual chromosome identification. Observations of G-banded chromosomes in the SEM by Harrison and co-workers (e.g. Harrison et al., 1981) have revealed a series of indentations along the chromosome arms which are much more obvious and numerous than those in unbanded chromosomes. There is a direct positional relationship between these circumferential grooves and the G-positive bands observed using the light microscope in human metaphases. It was decided, therefore, to examine some trypsin-treated metaphase chromosome spreads from the dog-whelk using SEM to see if bands were clearer at this level and whether other features e.g. centromeres, were visible to aid the study of chromosomal polymorphism.
Method for preparing *Nucella* chromosomes for SEM analysis
(based on Harrison *et al.*, 1985):

a) Take testis through normal procedure for light microscopy preparation as far as storage in Carnoy's fixative (see Chapter 2).

b) As usual, tease tissue in 60% acetic acid to form fine suspension, then drop onto No. 0 glass coverslips and dry in the normal way. The preparations are then viewed using phase contrast microscopy and good spreads marked in some way.

c) The coverslip is then scored with a diamond pencil and broken around the good spreads (=10 mm squares) and treated in the following way:

d) Fix in 3% glutaraldehyde in 0.1M Sorensens phosphate buffer (pH 7.4) for at least 30 min.

e) Rinse in the above buffer.

f) Part fix in 1% OsO$_4$ in same buffer for 10 min.

g) Rinse 3 times in distilled water.

h) 5 min in freshly prepared, filtered, 1% (saturated) solution of thiocarbohydrazide in distilled water.

i) Rinse 3 times in distilled water.

j) Fix again for 10 min in 1% OsO$_4$ in distilled water.

k) Repeat steps g) to j) - this builds up Os-TCH layers, providing an electron dense, conductive coating and consequently a better image.

l) Dehydrate in graded acetone series (20 - 100%).

m) Critical point dry with liquid CO$_2$.

n) View in SEM either a) uncoated or b) with thin gold coating (for additional contrast). In practice uncoated specimens gave poor image so a thin gold coating is recommended.
Although some success was achieved (Fig. App1.1), improvement in the technique is needed before the SEM method can be used routinely to study dog-whelk chromosomes. There was however some evidence of circumferential grooves in trypsin-treated material which indicated the effect may have been similar to that seen in human cells after G-banding.

![Figure A1.1 Scanning electron micrograph of *Nucella lapillus* chromosomes. Scale bar = 10μm.](image)

Figure A1.1 Scanning electron micrograph of *Nucella lapillus* chromosomes. Scale bar = 10μm.
Appendix 2. The metaphase spreads of *Nucella lapillus* - shown karyotyped in Chapter 4.

Figure A 2.1  $2n=26$ from West Runton, Norfolk

Figure A 2.2  $2n = 36$ from Mount's Bay, Cornwall
Figure A 2.3 Full legend for chromosome number variation in *N. lapillus* at sites examined in the N. Atlantic, as shown in Figure 4.3

Figure A 2.4 2n = 26, (88I) from Slaattholmen, Norway.
Figure A 2.5  $2n = 27$, (H9) from Dumpton Gap, Kent

Figure A 2.6  $2n = 27$, (H9, S1) from Dumpton Gap, Kent
Figure A 2.7  $2n = 27$, (H4) from Maine, USA.

Figure A 2.8  $2n = 27$, (H4) from Slaattholmen, Norway
Figure A 2.9  $2n = 27$, (H4) from the Isle of Man

Figure A 2.10  $2n = 27$, (H3, 55I) from the Isle of Wight
Figure A 2.11  2n = 27, (H5) from Cuckmere Haven, Sussex

Figure A 2.12  2n = 27, aneuploid (type X) from Old Stairs Bay, Kent
Figure A 2.13  $2n = 28$, (A3) from Slaattholmen, Norway

Figure A 2.14  $2n = 28$, (A4) from Dover, Kent
Figure A 2.15  $2n = 28$, (A9) from North Foreland, Kent

Figure A 2.16  $2n = 28$, (H4,9) from Folkestone, Kent
Figure A 2.17  $2n = 29, (H9; A3)$ from Jennycliff, Plymouth Sound

Figure A 2.18  $2n = 29, (H4,8,9)$ from Pudcombe Cove, S. Devon
Figure A 2.19  $2n = 29$, (H3,8,9; 44I) from Westcombe Cove, S. Devon

Figure A 2.20  $2n = 29$, (H5,8,9) from Rade de Brest, N.W. France
Figure A 2.21  2n = 30, (A8,9) from Horse Ledge, Isle of Wight

Figure A 2.22  2n = 30, (H1,8; A9) from Thurlestone, S. Devon
Figure A 2.23  $2n = 31$, (H9; A3,8) from Westcombe Cove, S. Devon

Figure A 2.24  $2n = 31$, (H9; A3,8) from Rade de Brest, N.W. France
Figure A 2.25  $2n = 31$, $(H1,3,4,8,9)$ from Hanover Point, Isle of Wight

Figure A 2.26  $2n = 31$, $(H1,2,4; A5)$ from St. Peter Port, Guernsey
Figure A 2.27  $2n = 32$, (A1,3;8) from Renney Rocks, S. Devon

Figure A 2.28  $2n = 32$, (H1,5; A8,9) from Thurlestone, S. Devon
Figure A 2.29  $2n = 32$, (H1,2,3,4,8,9) from Penlee Point, Cornwall

Figure A 2.30  $2n = 33$, (H1,8,9; A2,3) from Renney Rocks, S. Devon
Figure A 2.31  $2n = 34$, (H1,3,9; A2,8) from Renney Rocks, S. Devon

Figure A 2.32  $2n = 34$, (H1,3; A2,8,9) from Renney Rocks, S. Devon
Figure A 2.33  $2n = 35$, (H1; A2,3,8,9) from Mount’s Bay, Cornwall

Figure A 2.34  $2n = 35$, (H1; A2,3,8,9) from Mount’s Bay, Cornwall
Figure A 2.35  $2n = 35$, (H1; A2,3,8,9) from Roscoff, N. Brittany

Figure A 2.36  $2n = 36$, (A1,2,3,8,9) from Roscoff, N. Brittany
References


208


Staiger, H. (1957). Genetical and morphological variation in *Purpura lapillus* with respect to local and regional differentiation of population groups. Colloque International de Biologie Marine Station, Roscoff; L'Année Biologique, 33, 251-258.


Copyright Statement

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the author's prior consent.