Morphological variation, population genetics and genetric relatedness in three species of Callopora.

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MORPHOLOGICAL VARIATION, POPULATION GENETICS AND GENETIC RELATEDNESS IN THREE SPECIES OF *CALLOPORA*

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The genus *Callopora* is typical of a very large number of encrusting neocheilostomate genera and can be used to demonstrate the range of autozooid morphology seen in the group. Morphometric analyses of zooid length (ZL), zooid width (ZW), ovicell length (OL) and ovicell width (OW) were conducted in order to study morphological variation in different populations of *Callopora dumerilii*, *Callopora lineata* and *Callopora rylandi* and to partition the morphological variation within and between sites and colonies for each species using a nested analysis of variance and a principal component analysis approach. In addition, the genetic structure in populations of these three *Callopora* species using the mitochondrial DNA COI gene was examined to test hypotheses concerning levels of population differentiation and intrapopulation variation. The relationships of mtDNA lineages within and between species was also investigated to clarify the phylogenetic relationships of the three species and to search for possible phylogenetic subdivisions within species.

The morphological characters zooid length and zooid width were significantly different between different sites for *Callopora lineata* and *Callopora dumerilii*, but not for *Callopora rylandi*. However, major differences for these two morphological variables appeared in all three species in between colony within site comparisons. When comparing the ovicell length variable between different sites, noteworthy differences appeared only for *Callopora rylandi*, whereas considerable differences appeared in all three sites for between colonies within site comparisons. On the other hand, non-significant differences appeared for all three species when comparing ovicell width between different sites whereas highly significant differences appeared for between colony within site comparisons. The results of principal component analysis together with the results from nested ANOVA revealed that for factor 1, which defines aspects of the overall size of the zooid, there were significant differences between sites, as well as between colonies within sites for *Callopora rylandi*. For *Callopora dumerilii* and *Callopora lineata*, it appeared that there were no significant differences between different sites whereas there were notable differences between different colonies within sites. For factor 2, which defines aspects of the shape of the organism, there were significant differences between sites as well as between colonies within sites for both *Callopora rylandi* and *Callopora dumerilii*, while for *Callopora lineata* it emerged that there were no significant differences between sites, but there were important differences between colonies within sites.

Analysis of the mitochondrial DNA population structure in these three species based on either haplotype frequencies or sequence divergence showed a large percentage of genetic variation within populations and a much smaller percentage of genetic variation among populations. However, for haplotype frequencies the among populations P values were significant for all species whereas when sequence divergence was taken into account only the P value for *Callopora rylandi* was significant. Overall nucleotide diversity was similar for *Callopora dumerilii* and *Callopora lineata* and higher than that of *Callopora rylandi*, whereas overall haplotype diversity was similar in all three species. Tajima's D and Fu's F_s test statistic appeared more negative in *Callopora rylandi* than the other species suggesting greater purifying selection or a recent population expansion. Comparisons based on d_N/d_S ratio suggested purifying selection as well. Reconstruction of phylogenetic relationships showed three major lineages which are mixed in all three species. Tests of neutrality in these lineages, which do not correspond to species, also suggested the existence of purifying selection.

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To my beloved family

Yiannis, Mersevi, Petros and Gratsiella

CHAPTER 1

GENERAL INTRODUCTION

1.0 GENERAL INTRODUCTION

1.1 Evolutionary genetics of aquatic clonal animals

The study and identification of clonal organisms as a fundamentally different and identifiable evolutionary group is due to the work of Harper (1977, 1981). By definition clonal animals are assemblages of genetically identical individuals that can function and survive on their own (Hughes 1989). Cloning is the process of multiplication that results in the production of genetically identical offspring. Therefore, members of a clone are genetically identical, although new clones may eventually arise through automixis (Brown and Hoopes 1990), mixis (Hebert 1974a) and mutations (Lynch 1985). Each genotype arising through clonal replication is termed a genet (Harper 1977) and is considered a unit of selection. A multiclonal population will therefore comprise a population hierarchy at two levels: first, the number of distinct clones i.e. genets and second the number of individuals belonging to each genet called the ramets (Kays and Harper 1974).

1.1.1 Variety and classification of aquatic clonal animals

A wide range of clonal animals inhabit freshwater, estuarine and marine environments. However, the genetic information available for each group differs clearly (Hughes 1989). From an evolutionary perspective, the most comprehensively studied organisms are cladocerans in fresh water habitats (Hebert 1987; Mort 1991) and sea anemones in the marine environment (Shick 1991). Other marine animals that

have been used for studies include rotifers (King 1972, 1980; Snell and Hawkinson 1983; Zhao and King 1989), ostracods (Havel and Hebert 1989; Chaplin and Ayre 1989; Havel *et al.*, 1990a,b), ascidians (Sabbatin 1979; Grosberg 1987; 1988a,b) corals, (Hughes *et al.*, 1992) and bryozoans (Jackson 1985; Hughes 1989, 1992). However, cladocerans and sea anemones are the most researched species because of their display of miscellaneous cloning mechanisms (Hughes 1989) as well as the availability of comprehensive and detailed ecological data available on natural populations (Hebert 1978; Peters and Bernardi 1987; Shick 1991).

Aquatic clonal animals may be classified according to certain common reproductive and morphological principles. First, reproduction may involve gametes, so-called gametic cloners, or propagules derived from the soma, the agametic cloners with no involvement of a germ line (Hughes 1989). Second, a fundamental division occurs between those species with solitary ramets e.g. *Daphnia* and those which are interconnected, or colonial e.g. ascidians. Detached solitary ramets are mobile and may actively swim or drift over considerable distances, whereas colonial species are usually sessile, even though their reproductive propagules may disperse (Hughes and Cancino 1985). Third, clonal life histories may be entirely amictic, as in obligate cloners, or may exhibit short periods of mictic reproduction, either with a frequency that is genetically fixed or on a facultative basis (Hughes 1989).

1.1.2 Why study clonal animals?

Using clonal animals as model systems in evolutionary studies demonstrates their value as important investigative tools of evolution (Hughes 1989). Using clonal organisms affords a clear practical advantage because of the possibility of using replicated genotypes in controlled experiments. In addition, many of these groups of organisms are dominant numerically in some communities (e.g. corals); therefore one reason to study clonal organisms is that they are ecologically important. Besides that, extensive population genetic data are available for some of these groups. The extent and structure of genetic variation in many populations of a variety of organisms including clonal animals is reviewed by Carvalho (1994).

The characteristics of clonal animals that make them valuable model systems in evolutionary studies can be summarised as follows. Genet longevity is much greater compared to that for unitary individuals. This is attributable to the theoretically infinite production of ramets, until mixis intervenes. Extended longevity and an associated large genet size, particularly where mixis is rare, as in some corals, may provide variants for within-genet or somatic selection (Hughes *et al.*, 1992). However, the roles of somatic mutations depend on the role of inheritance, which differs essentially between gametic and agametic cloners (Hughes 1989). The scale of longevity differs noticeably between these two types of cloners. For example, a parthenogenetic cladoceran or rotifer exhibits the same lifespan as its unitary equivalent, whereas many sponges and corals generally live for one to many centuries (Hughes and Jackson 1980). In addition, reproductive rates in gametic cloners are in general high due to the two-fold advantage of parthenogenesis (Maynard-Smith

1978). A high population growth rate gives the possibility of rapid changes in genotype frequency and therefore increases the possibility of gene fixation. In addition, it may increase genet fitness through the evolution of enhanced fecundity (Hughes and Cancino 1985).

Clonal life-histories, especially where cloning and mixis are combined, may result in an effective population size that is significantly smaller compared to the census population size. This is mainly attributed to factors such as skewed sex ratios and genet fecundities (Stoddart 1984), overlapping generations (Hughes and Connell 1987), self fertilisation and inbreeding (Grosberg 1987) and periodic catastrophes (Hughes *et al.*, 1992). The net effect of a small effective population size will be to increase the chances of founder effect and genetic drift, producing locally divergent subpopulations. However, such effects depend on the relative contributions of sexual and amictic recruitment (Hebert 1974a,b). Effects of inbreeding may arise more frequently in colonial forms, as dispersal is often apparently low (Jackson 1985).

Other characteristics of clonal animals include an association between overall heterozygosity and genet fitness (Hebert *et al.*, 1982), which will be perpetuated clonally, sometimes producing heterozygote excesses, particularly where mixis is rare. In addition, a phase of cloning avoids disruption of co-adapted gene complexes, which when combined with periodic mixis, may facilitate response to directional selection (Templeton 1979) and therefore favour local adaptation. The extended longevity of some genets, together with their likely accumulation of heterozygosity, may increase at mixis through the release of hidden genetic variance being made available to selection (Lynch and Gabriel 1983). Also separation of genets into many

spatially or temporally dispersed ramets significantly decreases the risk of genet mortality, thus extending their lifespan. Risks of genet mortality differ between solitary and colonial clones (Hughes 1989). This is due to the fact that aggregation of modules will render genets more vulnerable to localised mortality. Indeed, the risk of genet mortality is the primary selective force that shapes the geometry and integration of ramets in aquatic clonal animals (Jackson *et al.*, 1985).

1.2 The application of molecular techniques in population and evolutionary genetics

1.2.1 Techniques for studying protein variation

The principle of population genetics is the study of the frequency of occurrence of alleles within and between populations across ranges of geographic distribution under the influence of the four evolutionary forces: natural selection, genetic drift, mutation and migration. It also takes account of population subdivision and population structure in space. As such, it attempts to explain such phenomena as adaptation and speciation. Population genetics is a vital ingredient in the modern evolutionary synthesis. Its primary founders were Wright, Haldane and Fisher, who also laid the foundations for the related discipline of quantitative genetics.

The first major advance in our understanding of genetic variation in natural populations began in the mid-1960s with the advent of protein electrophoresis. Protein electrophoresis provided the first tool for measuring genetic variation at the molecular level in species and for detecting gene flow across the species boundary (e.g. Brown 1995; Gardner 1996; Jiggins *et al.*, 1997; Spaak 1997; Berrebi *et al.*, 2000). The study

of variation in amino acid sequences of proteins by electrophoresis allowed an immediate assessment of genetic variation in a wide variety of species (Lewontin *et al.*, 1974).

There is a direct relationship between genes (DNA base pair sequences) and proteins (amino acid sequences). Proteins have an electrical charge and migrate in an electrical field at different rates depending upon their charge, size and shape. A single amino acid substitution can affect migration rate and thus can be detected by electrophoresis.

The advantages of protein electrophoresis are many. Genetic variation at a large number of nuclear loci can be studied with relative ease, speed and low cost. In addition, the genetic basis for variation of protein loci can often be inferred directly from electrophoretic patterns because of the codominant expression of isozyme loci, the constant number of subunits for the same enzyme in different species and consistent patterns of tissue-specific expression of different loci. Furthermore, it is relatively easy for different laboratories to examine the same loci and use identical allelic designations so that data sets from different laboratories can be combined (White and Shaklee 1991). However, this technique is limited as it cannot detect genetic changes that do not affect the amino acid sequence of a protein subunit. Thus, silent substitutions within codons or genetic changes in non-coding regions within genes cannot be detected with protein electrophoresis. Furthermore, this technique is limited by the amount of tissue present in the organism studied. For example, Corte-Real *et al.*, (1994) mentioned the difficulties of using larvae with this technique. In addition, this technique usually requires that tissue taken for analysis is stored in ultra-cold freezers or liquid nitrogen in the field. This requirement, however, sometimes

cannot be met (Thorpe and Sole-Cava 1994). While protein electrophoresis has now been largely taken over by DNA based analyses, allozymes still provide a cost-effective tool for screening variation in large numbers of individuals and can be combined with direct DNA sequence studies to evaluate the relationship between different measures of variation.

1.2.2 Techniques for studying DNA variation

The biological and analytical properties of different molecular markers have been reviewed extensively (e.g., Avise 1994; Hillis *et al.*, 1996; Caetano-Anolles and Gresshoff 1997; Mitton 1994). A general agreement is that no genetic marker is best for all applications and each provides different insights and applications.

A range of molecular techniques is available to examine variation in nucleic acid sequences. Several of the methods described below are capable of detecting single nucleotide mutations. Highly variable regions of DNA can sometimes provide a unique "fingerprint" for each individual and access to such fine scale genetic variation is one of the best reasons for choosing to work with DNA markers. The physical aspects of DNA also offer several advantages over allozymes. DNA is found in nearly all cells of all organisms and can be recovered from both living and dead tissue. Furthermore, tissues can be easily stored under field conditions and in many cases only nanograms are needed for analysis, when amplified by PCR. The molecule is so stable that recognizable sequences can remain intact for thousands of years (e.g. Cano 1993). For most applications, however, fresh or recently preserved DNA is needed for analysis. It is also important to control carefully for contamination when the DNA

fragment of interest is amplified by PCR particularly when universal or non-specific primers are used.

There are many papers that have reviewed the applications of DNA techniques for answering various issues of population genetics (Karl and Avise 1993; Lessa and Applebaum 1993; Avise 1994; Skibinski 1994; Sole-Cava and Thorpe 1997). The types of variation usually considered are analysis of: restriction fragment length polymorphism (RFLPs), microsatellites, single-stranded conformational polymorphisms (SSCPs), intron length polymorphisms (ILPs) and sequencing. However, only SSCP analysis and sequencing were employed in the present study. Therefore a brief overview will be presented here to outline the basic characteristics of each technique. A more detailed account of SSCP and sequencing will be given in the following chapters.

Restriction fragment length polymorphism (RFLP) analysis is perhaps the technique predominantly used in early work (Karl and Avise 1993; Skibinski 1994; Sole-Cava and Thorpe 1997). DNA from an individual specimen is first extracted and purified. Purified DNA can be amplified by the polymerase chain reaction (PCR). In the first RFLP studies polymorphisms were detected by probing for nuclear DNA and in the case of mitochondrial DNA by the end-labelling of purified DNA. In more recent studies, the DNA is cut into restriction fragments by endonucleases, which only cut where there are specific DNA sequences recognized by the enzymes. The restriction fragments are then separated according to length by agarose gel electrophoresis, blotted to membranes (e.g. southern blotting) and can be probed with cloned radiolabelled DNA that binds to a single locus or the fragments might be stained

directly using a dye such as ethidium bromide. The distance between the locations cut by restriction enzymes (the restriction sites) varies between individuals and this can be detected by examining fragment length variation. This can be used to discriminate individuals genetically. It can also be used to measure allele frequencies and can also show the genetic relationship between individuals and species.

Microsatellites, also known as simple sequence repeats (SSRs), are polymorphic loci present in nuclear DNA that consist of repeating units of 1-6 base pairs in length. They are present in both coding and noncoding regions and are usually characterized by a high degree of length polymorphism. The origin of such polymorphism is still under debate though it appears most likely to be due to slippage events during DNA replication (Schlötterer and Tautz 1992). Microsatellites have high mutation rates and therefore may show high variation between individuals within a species. This makes them ideal for determining relationships between different populations of the same species (i.e. population genetics) or even between individuals. The relative ease of scoring in addition to the high polymorphism, represent the two major features that make microsatellites of particular interest for many genetic studies. The major drawback of microsatellites, however, is that they need to be isolated *de novo* from species that are being examined.

Lessa (1992) introduced intron-targeted PCR, in which a non-coding intron was amplified using primers designed from highly conserved exon sequences. This approach, called Exon-Primed Intron-Crossing (EPIC)-PCR, has been shown to yield substantial variability, mainly from intron length polymorphism and was successfully used in several population genetic surveys (Daguin and Borsa 1999, Hassan *et al.*,

2003). EPIC-PCR has several advantages in population genetics studies: (i) by using primers from heterologous genes, cloning and sequencing of the target can be avoided; (ii) cross-species amplification should be easier than when primers are designed in non-coding sequences because exon sequences are more conserved across species; (iii) for the same reason, within species, PCR artifacts such as null alleles are expected to be less frequent.

Single-strand conformational polymorphism (SSCP) analysis is one of the most widely used mutation scanning techniques because of its simplicity. The principle of this method is based on the fact that the electrophoretic mobility of nucleic acids in a non-denaturing gel is sensitive to both size and shape. Unlike double-stranded DNA, single-stranded DNA is flexible and will adopt a conformation determined by intramolecular interactions and base stacking that is uniquely dependent on sequence composition (Orita 1989). Further details of this technique will however be discussed in later chapters (Chapter 4 and Chapter 5).

DNA sequencing is undoubtedly one of the most important breakthroughs and one of the key techniques used in nearly all molecular biology and genetics laboratories. Currently most laboratories use automated Sanger sequencing techniques (Section 4.2.7); however, some laboratories are moving to new sequencing platforms like the Pyrosequencing-based platform or the massively parallel sequencing-by-synthesis approach.

The use of DNA markers in population genetics has provided considerably more flexibility and diversity than was previously available in electrophoretic surveys of

protein variation (Mitton 1994). Thus, it is becoming increasingly important for population biologists to be able to understand and evaluate molecular data and to know whether their own research questions could be addressed with molecular techniques. The assumptions that can be put forward using such molecular markers are that the characters in question are heritable, repeatable and independent (Dowling *et al.*, 1996).

1.2.3 Problems to which molecular techniques can be applied

Silva and Russo (2000) assessed the biological problems that are involved in population genetics using DNA technology. In general there are three main categories of biological problems. The first group comprises problems related to the analysis of variation in and between populations (population structure, kinship determination etc.). The second group of problems relates to the test of hypotheses within the debate of neutralism and selectionism (e.g. tests of neutrality, associative overdominance). The third group of problems is associated with more applied purposes, such as forensics, fisheries and conservation (Avise 1994, 1996; Cipriano and Palumbi 1999). In this thesis, however, problems belonging to the first group are investigated.

Genetic variation is generated by mutation and maintained and distributed by the processes of natural selection, migration and genetic drift. Genetic variation is usually lost by small populations through genetic drift and inbreeding (Falconer and Mackay 1996; Lacy 1997; Frankham 2002). Random genetic drift is, by definition, a stochastic process. It has three main conservation and evolutionary consequences, which include random allele frequency changes from one generation to the next, population divergence through the differentiation of even initially identical

populations and the fixation of alleles and resultant loss of genetic variation. These processes have larger impacts on small or subdivided populations than larger and continuous ones.

Genetic variation can be subdivided into four types: 1) variation within individuals related to the study of phenomena such as heteroplasmy (Fisher and Skibinski 1990; Hoech *et al.*, 1991; Zouros *et al.,* 1992, 1994a,b; Magoulas and Zouros 1993; Skibinski 1994a,b), multi-gene family evolution (Zhang and Nei 1996) and concerted evolution (Karnoven and Savolainen 1993; Elder and Turner 1994). 2) Variation within populations. This area deals with the effects of inbreeding and bottlenecks on genetic variation (Boulding *et al.*, 1993; Grewe *et al.*, 1993) as well as problems involving kinship determination such as nepotism (Pfennig and Reeve 1993), social structure (Morin *et al.*, 1994; Oldroyd *et al.*, 1994) and reproductive success (Bensche *et al.*, 1994; Lambert *et al.*, 1994; Wetton *et al.*, 1995). 3) Variation between populations. These studies focus on problems of population structuring such as stock definition in fisheries (Silberman and Walsh 1994; Hall *et al.*, 1995), bioinvasion (Echelle and Dowling, 1992; Geller *et al.*, 1994; Heath *et al.*, 1995) and gene flow (Zink and Dittmann 1993; Elliot *et al.*, 1994; Hurst and Skibinski 1995; Palumbi 1996a). Problems related to the taxonomic status of morphotypes (Vuorinen *et al.*, 1993; Rosel *et al.*, 1994) or ecotypes (Hanfstingl *et al.*, 1994; Cluster and Allard 1995) are also considered. 4) Genetic variation above the species level, including the effects of different life cycles on species isolation and differentiation (McMillan *et al.*, 1992), the study of hybridisation and hybrid zones (Edwards and Skibinski 1987; Moritz *et al.*, 1992; Zink *et al.*, 1994; Oldroyd *et al.*, 1995) and strain definition in unicellular organisms (Bell and Friedman 1994).

1.2.4 Application of DNA techniques in the study of Bryozoa

The phylum Bryozoa is a relatively little studied group. However, over the years DNA techniques have been employed by several researchers to answer a number of questions of interest. Of particular interest are the studies that were undertaken in order to elucidate taxonomic identities of various bryozoan species such as the study of Schwaninger (1999) who examined genetic relationships between two sympatric morphs of *Membranipora membranacea* one bearing spines and the other without, by using a combined approach of allozymes and mtDNA COI sequences. Results showed that the two morphs were genetically identical and that none of the mtDNA lineages corresponded with morphological characteristics. Therefore, the presence of spines, or not, was probably due to the presence of an external environmental factor such as the presence of predators. Another study that was involved with taxonomic identities of various bryozoan species is that of Hoare *et al.* (2001) who used cytochrome *c* oxidase I (COI) and 16S rDNA sequences and found distinct clades within the cosmopolitan species *Celleporella hyalina*, one in the U.K., one comprising Sweden and USA and a third from Chile. This provided evidence for cryptic speciation. Similar studies are those of Dick *et al.* (2003) that used genetic data in order to identify recent species of free-living bryozoans (Cupuladriidae) from both sides of the Isthmus of Panama and to examine their phylogenetic relationships, species richness and population structures. Cryptic speciation was also revealed from the study of Mackie and Keough (2001) that assessed levels of divergence in the mitochondrial cytochrome oxidase I gene from widespread populations of *Bugula dentata* Lamouroux. Their results showed an average pairwise distance of 21% (Kimura-2P corrected) between colonies. By assuming that COI mutation rates were comparable

with other organisms, they suggested that cladogenesis leading to these taxa took place in the Miocene. Moreover, morphological differences between specimens were consistent with regional taxonomic differences identified previously in the literature, whereas phylogenetic division between species was not diagnosed taxonomically. Finally, their examination of population level differences revealed a shallow phylogenetic structure, which is commensurable with a recent colonisation event by a small number of founders and genetic differences between populations were structured, suggesting small barriers to gene flow.

A molecular study that is concerned with phylogenetic relationships within the bryozoan phylum is that of Dick *et al.* (2000) who constructed a phylogeny based on partial 16S rDNA sequences. Results demonstrated that the bryozoan ctenostome species *Amathia lendigera, Flustrellidra hispida, Sundanella sibogae* and *Farella repens* are paraphyletic with both cheilostomes and cyclostomes, which is in agreement with the study of Todd (2000) based on fossil data. Furthermore, ascophoran bryozoans were found to be polyphyletic, which was also shown from a morphological study (Voigt 1991). The second study was carried out by Hao *et al.*, (2002) who sequenced the 18S rRNA gene of eight species of cheilostome bryozoans. They reconstructed phylogenetic trees using the neighbour-joining (NJ) and maximum-parsimony (MP) methods by combining these sequences with the sequences of other bryozans including entoprocts, ectoprocts, bachiopods and phoronids. The results showed that the entoprocts and the ectoprocts comprise two parallel branches in the molecular phylogenetic trees of bryozoans. In addition, *Membranipora grandicella* (which was sequenced by Hao *et al.* in their study) and *Membranipora* sp. as reported by Giribet *et al.* (2000) are far away from each other in

these trees. Moreover, their results supported the two-class morphological classification of Phylactolaemata and Gymnolaemata of ectoprots.

Of great interest is the study of Waeschenbach (2003). The phylogenetic relationships between species of the stoloniferan ctenostome bryozoan genus *Bowerbankia* were examined using DNA sequences for two mitochondrial genes, 12S rDNA and mtDNA COI. Results from that study show that the resultant phylogeny based on COI sequences was confounded by nuclear insertion mitochondrial sequences (Numts). 12S rDNA sequences with mutations in highly conserved regions, as determined by secondary structure folding, were also suspected to be Numts.

The present study is concerned with aspects of the population genetics of a particular marine bryozoan species using mtDNA. The results are discussed in Chapter 5.

1.3 On bryozoans

1.3.1 Bryozoan distribution and general structure

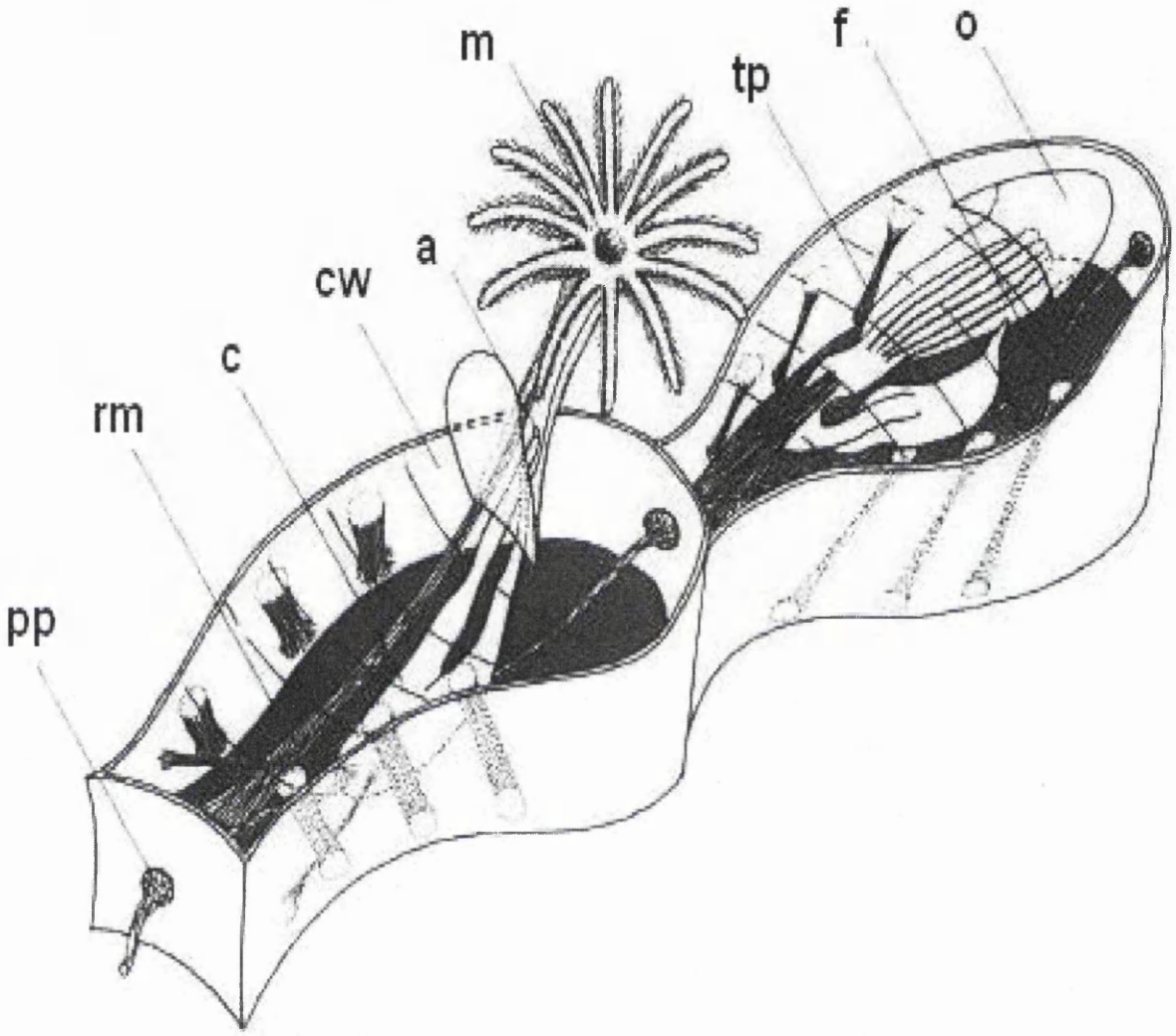
Bryozoa are aquatic colonial animals, which are abundant in modern marine environments and are important components of the fossil record. Despite the fact that there are more than 5600 living species worldwide (Todd 2000), with several times that number of fossil species, the Bryozoa remain largely unknown to most people. Bryozoans, or "moss animals," are aquatic organisms that live for most part in colonies of interconnected zooids. A few to many millions of these zooids may form one colony. Bryozoan colonies range from millimetres to metres in size, but the

individuals that make up the colonies are rarely larger than one millimetre. Colonies may be mistaken for hydroids, corals, or even seaweeds.

The British bryozoan fauna includes about 300 species. The world fauna is presently unknown. Possibly 1000 new species have been described worldwide in the second half of the 20th century and the rate of description of new species shows no sign of slowing down (Hayward and Ryland 1999). This is partly attributable to modern taxonomic precision, but also to the fact that bryozoan taxonomists have only recently discovered the astonishing diversity of the phylum in coral reef habitats, or in coarse ground continental shelf environments. It is probable that the true total number of living species of Bryozoa is more than twice the estimated 4000 (Hayward and Ryland 1999).

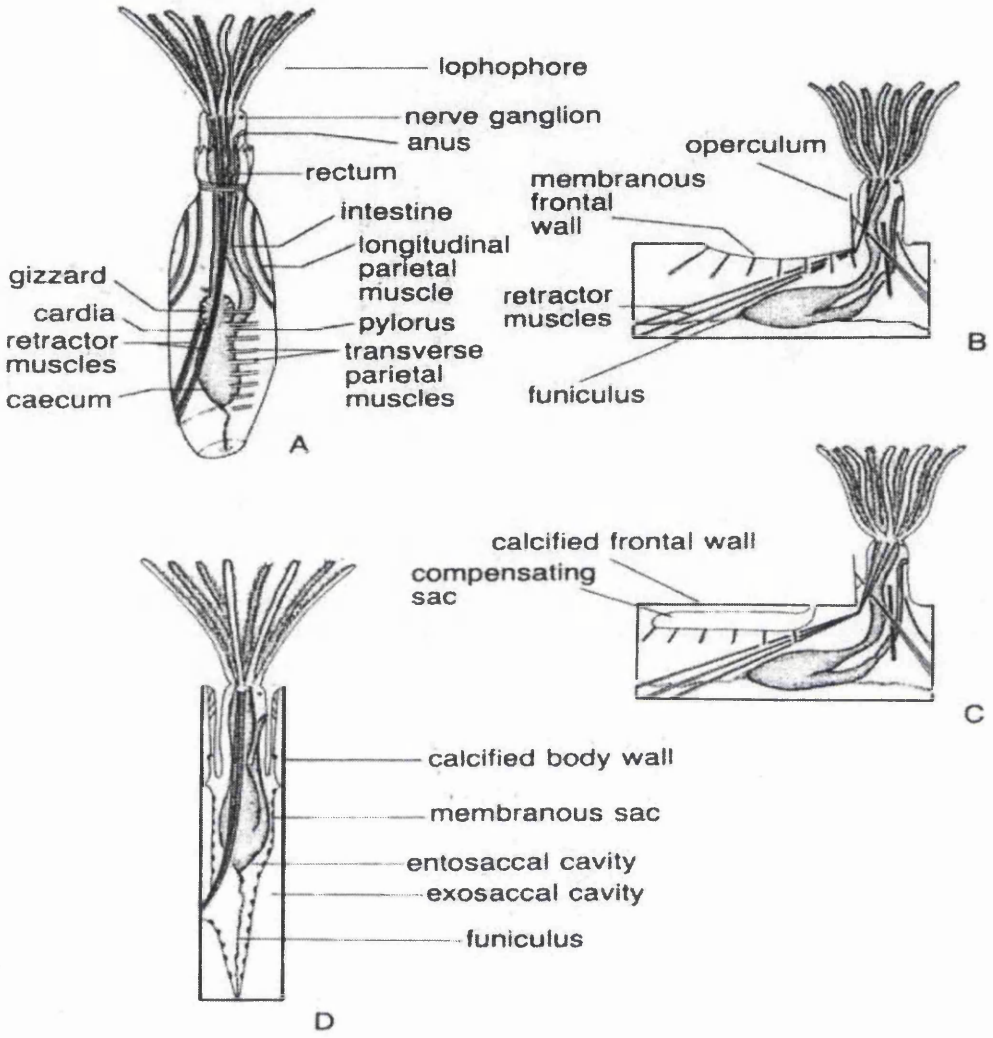
Bryozoans are modular organisms composed of many genetically identical connecting zooids each with its independent food gathering structure (lophophore), mouth, gut, muscles, nervous and reproductive systems (Figure 1.1). Despite their apparent autonomy, zooids share certain tissues and fluids which unify the colonies physiologically. In addition, even though zooids are genetically alike, zooid morphology and function can differ within colonies.

Figure 1.1 Generalised bryozoan autozooids in feeding (left) and retracted (right) positions based on hypothetical anascan cheilostome with a noncalcified frontal wall: (a) anus; (c) caecum; (cw) calcified wall; (f) funiculus; (m) mouth; (o) operculum; (pp) pore plate and rosette cells; (rm) retractor muscle; (tp) transverse parietal muscle. (Adapted from Ryland and Hayward 1977).



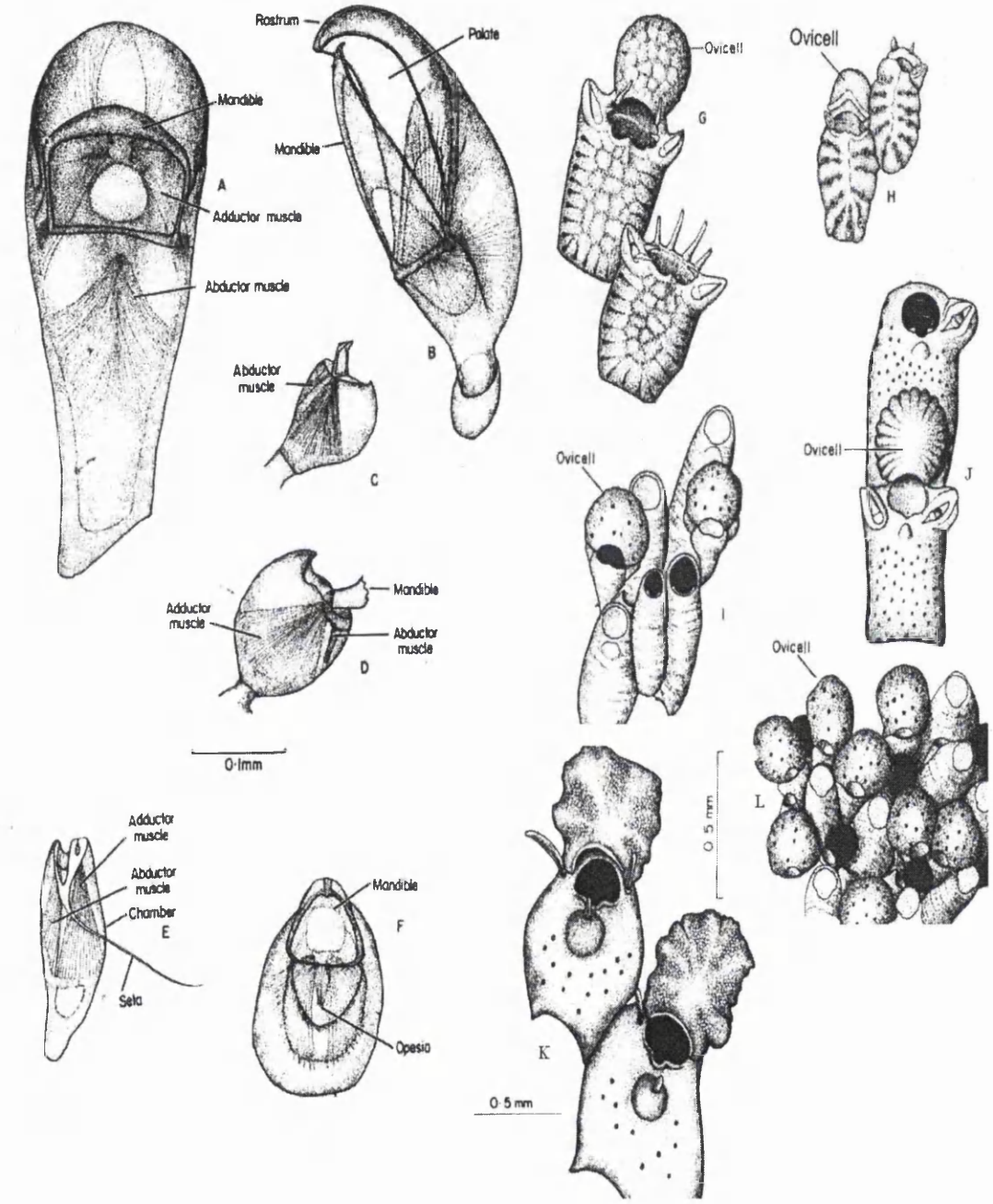
In most bryozoans, several different types of zooids coexist in a colony, each one specialised for a particular function. Feeding zooids are known as autozooids (Figure 1.2), whereas specialized nonfeeding zooids may be called heterozooids (Figure 1.3).

Figure 1.2 Autozooid structure in four groups of Bryozoa. (A) A stoloniferous ctenostome. (B) An anascan cheilostome. (C) An ascophoran cheilostome. (D) A cyclostome (Adapted from Ryland and Hayward 1985).



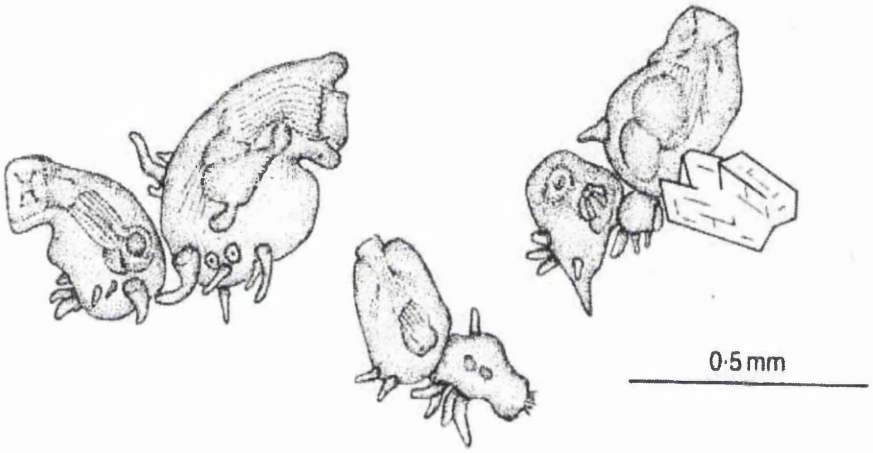
Heterozooids are dependent on functioning autozooids for nutrients. Heterozooids include forms specialised for producing and brooding eggs, or, more rarely, sperm. Avicularia are small heterozooids in which the zooid and operculum form a beak-like, snapping structure that deters small predators. Vibracula bear long setae, or bristles and are thought to function in cleaning the bryozoan colony, while kenozooids are small heterozooids that strengthen and support the colony as well as filling space.

Figure 1.3 Bryozoan heterozooids. (A) Avicularium of *Flustra foliacea*. (B) Avicularium of *Bugula avicularia*. (C) Avicularium of *Episomia bursaria*. (D) Avicularium of *Callopora lineata*. (D) Vibraculum of *Scrupocellaria scruposa*. (G) Ovicell in *Escharoides coccinea*. (H) Ovicell in *Cribrilina cryptooecium*. (I) Ovicell in *Hippothoa hyalina*. (J) Ovicell in *Schizoporella unicornis*. (K) Ovicell in *Phaeostachys spinifera*. (L) Ovicell in *Celleporella hyalina*. (Adapted from Ryland and Hayward 1977).



Bryozoan colonies thus show a range of integration of different zooid types. The most integrated colonies behave like individual organisms, for the zooids making up the colony are all specialized for certain functions and connected to each other. However, there is only a single apparent solitary species, *Monobryozoon ambulans*, which forms a colony of one autozooid and several rootlike heterozooids (Figure 1.4).

Figure 1.4 *Monobryozoon ambulans*. Part of a small population showing autozooids and autozooid buds in different stages of development. (Adapted from Ryland and Hayward 1985).



1.3.2 Classification of Bryozoa

The phylum Bryozoa is divided into three classes: the Stenolaemata, the Gymnnolaemata and the Phylactolaemata. Of these, the Phylactolaemata is wholly freshwater in distribution. The Stenolaemata are marine bryozoans with tubular zooids with strongly calcified walls. They are characterised by a lophophore which is protruded by the action of annular muscles. Most forms lack an operculum and there is limited polymorphism. This class includes several thousand species distributed among five orders: Cystoporata, Trepostomata, Cryptostomata, Fenestrata and Cyclostomata. However, living species are found only in the order Cyclostomata. The

Cyclostomata have a circular, terminal orifice, which is closed by muscular contraction. Cyclostomata lack an operculum (Hayward 1985). The Gymnolaemata are mostly marine bryozoans with cylindrical or flattened zooids. The body wall is calcified or uncalcified and the lophophore is protruded by action of muscles pulling on the frontal wall. Colonies are polymorphic with specialization of zooids. The Gymnolaemata is divided into the orders Ctenostomata and Cheilostomata. The Ctenostomata are characterised by membranous or gelatinous zooidal walls, a terminal orifice with no operculum and closed by a pleated collar in most. Heterozooids are absent, or only kenozooids are present. The Cheilostomata (whose characteristics will be illustrated in Chapter 2) were formerly separated into the suborders Anasca and Ascophora. The difference in the two suborders lies in the presence (in the Ascophora) or absence (in the Anasca) of a flexible sac (ascus), situated below the frontal shield. This is part of the hydrostatic system and assists the opening and closing of the operculum.

1.3.3 Bryozoan evolution

The Bryozoa is the only animal phylum with an extensive fossil record that does not appear in Cambrian or late Precambrian rocks. The oldest known fossil bryozoans, including representatives of both major marine groups, the Stenolaemata and Gymnolaemata, appear in the Early Ordovician. It is possible that the Bryozoa existed in the Cambrian but were soft-bodied or not preserved for some other reason. Perhaps they evolved from a phoronid-like ancestor at about this time.

The stenolaemate bryozoans quickly radiated in the early Palaeozoic and are very characteristic fossils of Palaeozoic rocks, sometimes making substantial contributions to the formation of reefs, calcareous shales and limestones. They included forms with robust skeletons, such as the trepostome *Hallopora*. Such forms were common in shallow-water habitats that today are dominated by corals. There were also forms with delicate, branching fanlike skeletons such as the fenestrates. With the exception of one order of stenolaemates, the Tubuliporata or Cyclostomata, all of these Palaeozoic bryozoan lineages were severely impacted in the Permian extinction. Cryptostomates disappeared at the end of the Permian (245 million years ago), while a few other lineages survived until the end of the Triassic, about 210 million years ago. Tubuliporate bryozoans have survived to this day and in fact underwent a remarkable radiation in the Cretaceous, but are no longer dominant today.

Uncalcified gymnolaemates are known as fossils from the Late Ordovician on carbonate substrates such as shells. Calcareous gymnolaemates did not appear in the oceans until the Cretaceous, during which time they diversified rapidly from a very few species in the early Cretaceous. By the end of the Cretaceous, there were over 100 genera of gymnolaemates. They continued to diversify in the Cenozoic. Today there are over 1000 genera, comprising the bulk of marine bryozoan diversity in today's seas.

1.3.4 Bryozoan reproduction and life histories

All bryozoans are hermaphroditic, meaning they produce both female and male gametes, or protandric hermaphrodites (i.e. change sex with age). In many

cheilostomes, ovaries and testes occur together in each autozooid but mature at different times, with protandry being the usual role. In others, an autozooid may first develop a testis, thus becoming an androzooid (Dyrynda and Ryland 1982). Following maturation and release of sperm, the polypide degenerates and is replaced and an ovary develops. The zooid thus becomes a gynozooid.

Bryozoans can reproduce both sexually and asexually. Asexual reproduction occurs by budding of new zooids as the colony grows and this is the main way by which a colony expands in size. If a piece of a bryozoan colony breaks off, the piece may continue to grow and will form a new colony. A colony formed this way is composed entirely of clones of the first zooid, which is called the ancestrula.

Sexual reproduction involves the release of sperm through the tips of the tentacles. Eggs come in two forms; they are either small and released into the water, where they undergo development, or they are larger and retained within the zooid, in ovicells in those forms that have them. Ovicells are solely found in cheilostomes. Eggs are fertilised by sperm that is brought in on the feeding current. In brooding species, the larvae are released after they hatch. These larvae can swim but do not feed. They swim towards the light at first then after a few hours they swim away from the light, down to the sea floor to look for a suitable substrate to settle and attach to. The ability to brood is thought to be the more advanced mode of reproduction (Taylor 1988). Most species of bryozoans practice some form of brood care such as this.

Species that do not brood their eggs have much smaller eggs than the eggs of those species which do brood their eggs by a ratio of about 1:10. The smaller eggs are also

much more numerous. After the eggs have been fertilised they become part of the plankton where they hatch into 'Cyphonautes' larvae which spend 1 to 2 months in the plankton feeding and growing until they too are large enough to descend to the substrate and found a new colony.

Cycles of growth and reproduction vary widely in bryozoans. Species such as *Celleporella hyalina* encrusting ephemeral substrata like *Laminaria saccharina* fronds are r-selected species and produce larvae continuously through spring and summer, giving rise to numerous short-lived generations, each of which grows, breeds and dies within a few months. On the other hand perennial species such as *Pentapora fascialis*, which may live more than 10 years and extensive sheet encrusting species such as *Schizoporella unicornis,* are k-selected species with limited annual reproductive periods. Between these two extremes there are numerous intermediates. For example, *Chartella papyracea* colonies live for about three years and pass through three cycles of colony growth, polypide recycling and sexual reproduction (Dyrynda and King 1982). In almost all temperate species growth and reproduction decline in the autumn. Colonies may die with the onset of winter, or polypides degenerate, leaving conspicuous brown bodies.

1.3.5 Bryozoan ecology

Bryozoans are ubiquitous in the marine benthic realm, occurring from the intertidal zone to the greatest abyssal depths. With a very few specialised exceptions the only requirement for their presence is a substratum to which an ancestrula can attach (Hayward and Ryland 1999). In aquatic habitats, bryozoans may be found on all types

of hard substrates: sand grains, rocks, shells, wood and blades of kelps and other algae may be heavily encrusted with bryozoans. Some bryozoan colonies, however, do not grow on solid substrates, but form colonies on sediment. While some species have been found at depths of 8200 metres, most bryozoans inhabit much shallower water. Sheltered rocky shores support a surprisingly large number of bryozoans, representing all three orders. Some are essentially subtidal, but others are primarily intertidal in distribution, or limited to very shallow sublittoral and lower intertidal habitats. They may also be extremely common beneath large stones on the lower shore.

Competition for space may be intense among encrusting, filter feeding animals and the diversity of particular communities will reflect the competitive abilities and growth and reproductive strategies of the species present (Hayward and Ryland 1999).

Bryozoan diet consists of small microorganisms, including diatoms and other unicellular algae that are obtained as fine particles filtered from the sea. There may be limitless quantities of food available to intertidal and shallow subtidal bryozoans and the fact that neighbouring colonies compete for food demonstrates that they do not rely on ambient water flow to supply it. Filter-feeding becomes less effective as ambient water flow increases (Okamura 1992) and the periods during which coastal species are able to feed may be quite short. Among encrusting communities competing for food resources those species which occupy the greatest area in the shortest time have an advantage and maintain their dominance in spite of intense spatial competition. Rapid growth, with an extensive broad peripheral multizooidal growing edge and ability to overgrow neighbouring colonies contribute to success in competitive interactions. Multispecies encrusting communities tend to consist of

competitive networks (Jackson 1979; Rubin 1982, 1985), in which the competitive abilities of the constituent species and colonies are affected by additional factors, such as the angle at which opposing colony edges intersect the thickness of the colony, whether the growth pattern is radial or directional and the regenerative capacity of each species. Factors such as interaction with other sessile organisms, predation and physical disturbance all contribute to the maintenance of the network (Hayward and Ryland 1999).

Predator-prey relationships in bryozoan communities are an interesting aspect of bryozoan ecology. Sea urchins, fish, crabs and some prosobranchs are all known to feed on bryozoans. They affect sessile community structure, creating patches of substratum for recolonisation and therefore contribute to species diversity. However, none of these predator effects has been quantified.

Because they are not mobile, bryozoans have developed mechanisms to deter potential predators. Harvell (1990, 1991) studied the effects of sea slug predation on kelp-encrusting colonies of *Membranipora* on the Washington coast and showed that *Membranipora* colonies develop surface spines following attack by *Doridella steinbergae* and that the production of spines could be induced in cultured colonies by the proximity of a tethered slug, suggesting that the bryozoan detected and recognised a potential predator through a chemosensory receptor.

Other studies have investigated the role of avicularia in bryozoan defensive strategies. Winston (1986, 1991) observed that avicularia appear to act in response to specific stimuli. They may be triggered to close by the passage of small syllid polychaetes and

tube-building amphipods. In addition they appear to be sensitive to chemical stimuli. Winston (1986) suggested that avicularia seen in many cheilostomes bryozoans may have evolved in response to particular predation pressure, perhaps imposed by small selective predators.

Bryozoans may also help protect themselves with chemicals. *Flustra foliacea, Chartella papyracea, Bugula neritina* and *Alcyonidium diaphanum* are typical examples (Christophersen 1985). The substances isolated, in some cases, appear to have potentially important pharmacological properties. For instance, bryostatin which was isolated from *Bugula neritina*, appears to have a potent anti-cancer activity (Zhang *et al.* 1996b). However, the significance of these compounds for the bryozoans themselves appears to be unknown. They may have an anti-fouling effect, or they may impart a toxic or harmful taste to the colony as a deterrent to predators.

1.4 Aims

The research in this thesis has two main objectives which have very important implications in Bryozoan studies. The first objective is to detect morphological variation in *Callopora* populations and to compare variation within colonies and populations. The second objective is to study population structure in these three *Callopora* species and to clarify phylogenetic relationships between them.

CHAPTER 2

SPECIES DESCRIPTION AND SAMPLING LOCALITIES

2.0 SPECIES DESCRIPTION AND SAMPLING LOCALITIES

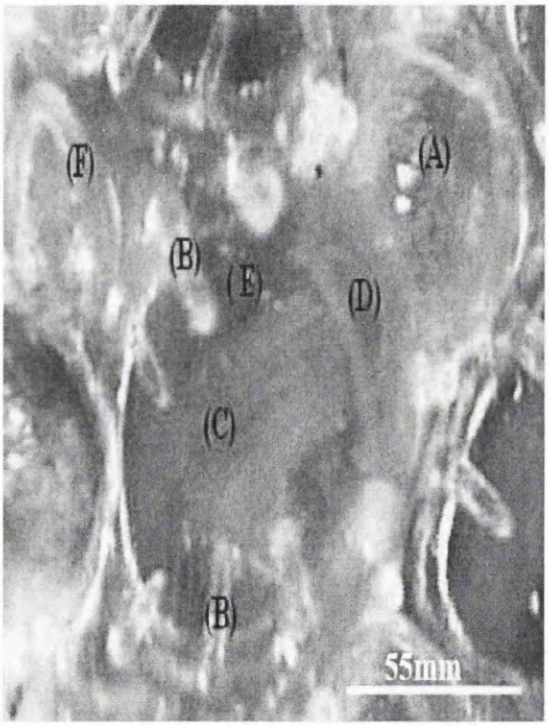
2.1 Introduction

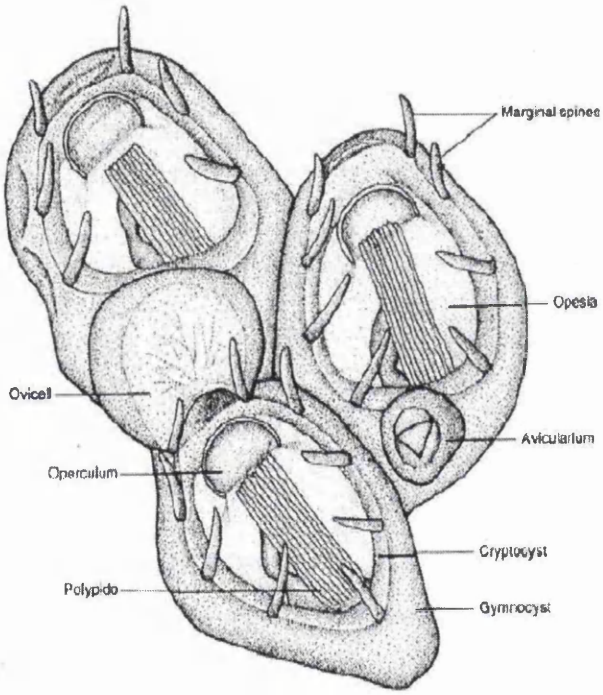
The aim of this Chapter is to provide an outline of the *Callopora* species that were examined in this thesis. Basic morphological characteristics, distribution and reproductive characters are also described. In addition this chapter includes information for the sampling localities that were selected in this study.

The genus *Callopora* was first described by Gray in 1848 and is one of the thirteen genera that make the family Calloporidae (Norman 1903). The genus *Callopora* is representative of a large number of encrusting genera and is often used in order to display the range of autozooid morphological characteristics in this family (Figure 2.1). Colonies form encrusting, unilaminar sheets, with new autozooids developing, through intrazooidal budding, from prominent chambers visible at the base of the vertical walls of peripheral autozooids. The vertical walls of the autozooids curve inwards and proximally are continuous with a smooth frontal gymnocyst, frequently bearing one or more avicularia. Much of the frontal surface is a transparent membrane, with the operculum set within it at the distal end, through which the polypide can be seen. The membrane is bordered by spines, which vary in number, set on a distinct ridge, the mural rim, marking the boundary between the gymnocystal calcification of the frontal and lateral walls and a narrow border of granular cryptocystal calcification underlying the membrane. The cryptocyst is generally reduced and defines a space, the opesia, which is a term used to describe the residual

area of uncalcified frontal surface. Prominent, hyperstomial ovicells are present, frequently with ridges or umbones. The ectooecium, a term used to describe the outer layer of the ooecial wall, is partly or completely membranous and closed by autozooidal operculum. Basal pore chambers are also present.

Figure 2.1 Autozooid structure in *Callopora lineata*; (A) Ovicell, (B) Spines, (C) Polypide, (D) Operculum, (E) Opesia, (F) Avicularium. (Diagrammatic representation of autozooid adapted from Ryland and Hayward 1999).





Five well defined species of *Callopora* are present in the British sea waters, two of which are also common in the Mediterranean. *Callopora minuta* (Harmelin 1973), which builds up uniserial colonies in cryptic coralligenous habitats is currently known only from the western Mediterranean. The West African species *Callopora depressa* has been found from the Straits of Gibraltar (Lopez de la Cuadra and Garcia Gomez 1993) and the northwest coast of Spain (Gil 1994). Another five additional species occur perhaps in the Arctic and subarctic northeast Atlantic region (Kluge 1962). In

this study, three *Callopora* species have been investigated, which are very common in the British sea area; *Callopora dumerilii*, *Callopora lineata* and *Callopora rylandi*.

2.2 Materials and methods

Specimens were observed alive using an Olympus dissection microscope SZ60, whenever possible, as colour, tentacle number as well as behaviour can be recorded. Fine paint brushes were routinely used for removing detritus as accurate identification requires that a portion of the specimen should be cleaned. Subsequently, colonies were examined again and their identity was confirmed based on the work of Hayward and Ryland (1998).

2.3 *Callopora dumerilii* (Audouin)

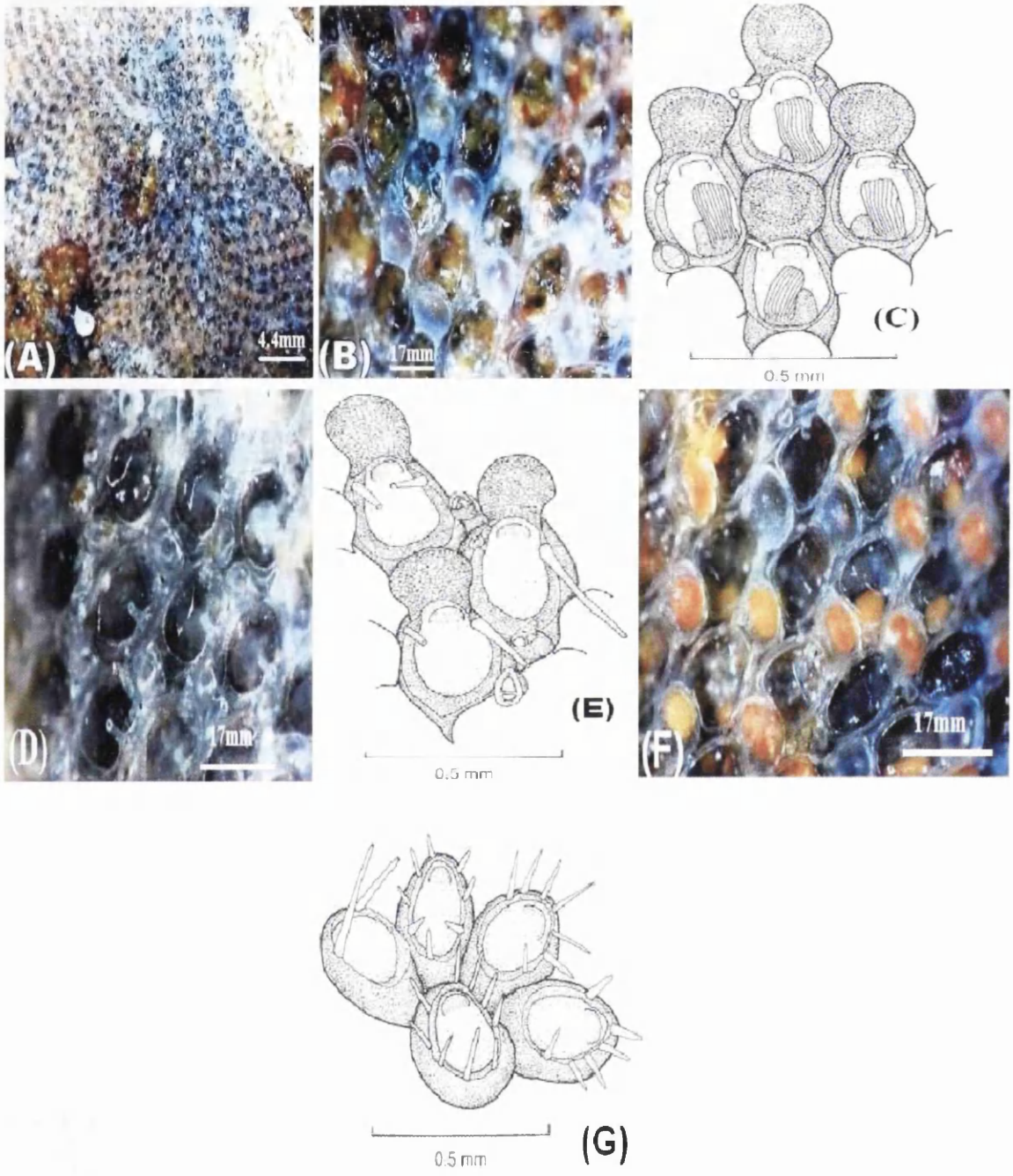
Callopora dumerilii is a cool temperate, shallow water species; although its lower bathymetric limits are unknown it has been reported from 300m. It is found on stones and shells but also on kelp holdfasts. Colonies form broad, irregular sheets (Figure 2.2A) composed of oval autozooids, which are sometimes broadened proximally to give pyriform outline (Figure 2.2B, 2.2C). Autozooids are very distinct, separated by deep grooves and are approximately 0.4-0.5 x 0.2-0.24mm in size. The gymnocyst in *Callopora dumerilii* is small, triangular in shape, smooth and vitreous whereas the cryptocyst is a narrow, crenellate shelf within the raised lateral walls. There are four, rarely six, spines at the distal end of the autozooid; the distal pair is small, pointed and erect, frequently missing especially in the presence of an ovicell. The proximal pair is

invariably present. One or both of these may be considerably lengthened and thickened, straight or curved, standing erect or draped across the frontal surface of the autozooid (Figure 2.2D, 2.2E). Avicularia in *Callopora dumerilii* are frequently absent, but when present are quite small, mounted on the gymnocyst, with a narrow, pointed rostrum directed proximally or distally (Figure 2.2B, 2.2C). Ovicells are globular in shape and very prominent. The ectooecium is almost entirely membranous with a small calcified portion which is restricted to a narrow peripheral band. The entooecium, however, is coarsely granular with a regularly reticulated surface. Eggs and embryos are orange in colour (Figure 2.2F).

Reproduction in *Callopora dumerilii* has been observed through most of the year, but mainly from June to November, with maximum settlement occurring during July and August.

The ancestrula is one-half to two-thirds the size of later autozooids, with ten peripheral spines (Figure 2.2G). However, succeeding autozooids display a gradual reduction in the number of spines until the usual number of four is reached.

Figure 2.2 (A) Overview of a *Callopora dumerilii* colony. (B),(C) Ovicellate zooids showing spine arrangement and presence of avicularia. (D),(E) Autozooids showing enlarged lateral spines. (F) Group of ovicellate zooids with embryos. (Diagrammatic representations adapted from Ryland and Hayward 1999).

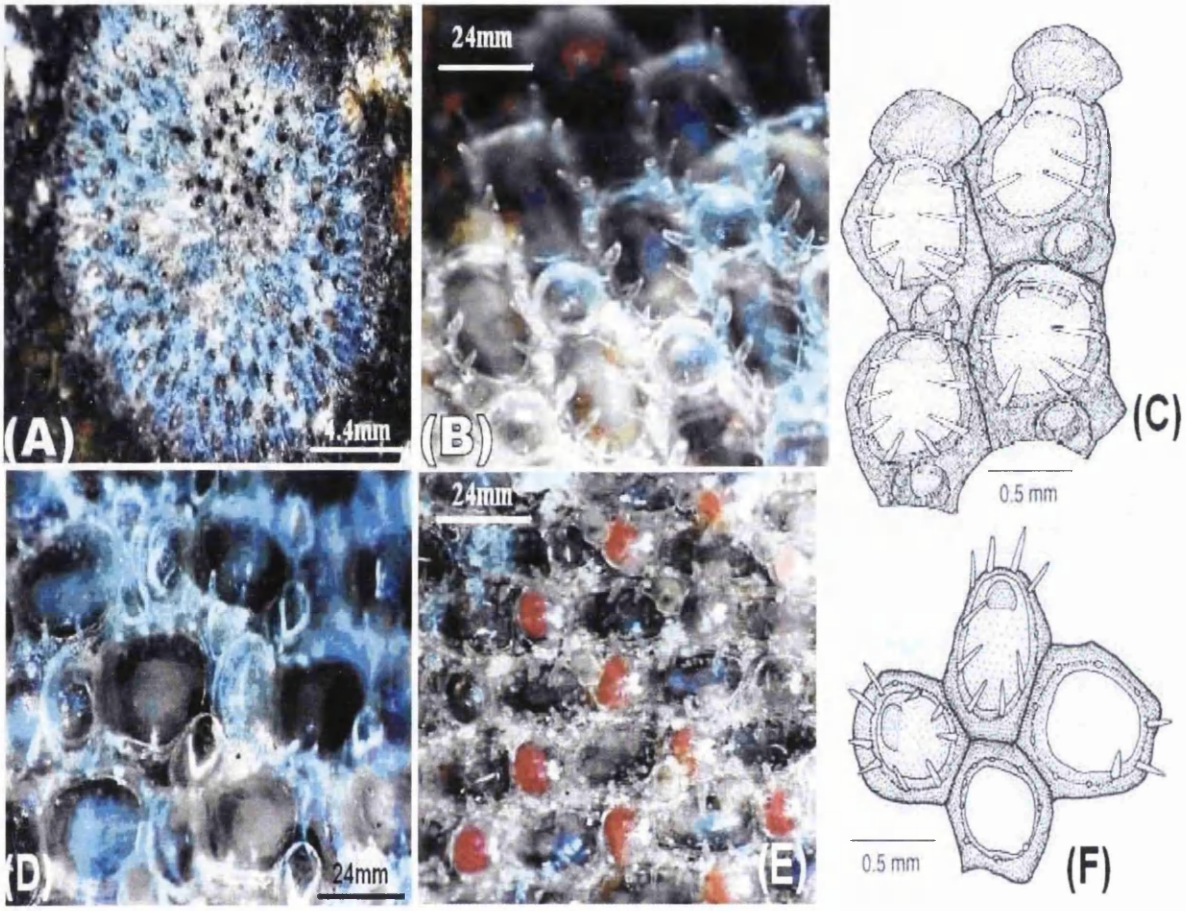


2.4 *Callopora lineata* (Linnaeus)

Callopora lineata is a distinctive member of rocky shore communities. It is found on a wide range of substrata, showing preference for shells, stones and the undersides of boulders. It is also found on the holdfasts of *Laminaria* species. It is mainly an intertidal species, ranging into the shallow sublittoral, but not usually below the limit of the kelp forests. Colonies of *Callopora lineata* form irregular, spreading sheets (Figure 2.3A). Autozooids vary in shape, from rectangular to irregularly polygonal and in size (0.4-0.54 x 0.2-0.3mm) and are separated by deep grooves. The gymnocyst is narrow, smooth and finely granular whereas the cryptocyst is reduced, more coarsely granular compared to the gymnocyst. Lateral walls in *Callopora lineata* are raised as a rim around the elongated oval opesia, bearing eight to eleven (exceptionally six to fourteen) straight, thick cylindrical spines (Figure 2.3B, 2.3C). The polypide bears thirteen to fourteen tentacles. The avicularium is borne on the gymnocyst, its cystid is squat and rounded with a triangular rostrum which is usually directed proximally and angled to the surface of the autozooid (Figure 2.3D). Ovicells are extremely prominent, globular in shape and finely granular in structure. The frontal surface has an extensive area of uncalcified ectooecium. Finally, several lateral pored and one distal pore chambers are present.

Embryos are red in colour (Figure 2.3E) present throughout much of the year mainly from June to November, with peak settlement from June to August. The ancestrula is similar to later autozooids (Figure 2.3F).

Figure 2.3 (A) Overview of a *Callopora lineata* colony. (B),(C) Ovicellate and non-ovicellate zooids showing spine arrangement. (D) Ovicellate zooids showing avicularia. (E) Group of ovicellate zooids with embryos. (F) Ancestrula and periancestrular zooids. (Diagrammatic representations adapted from Ryland and Hayward 1999).

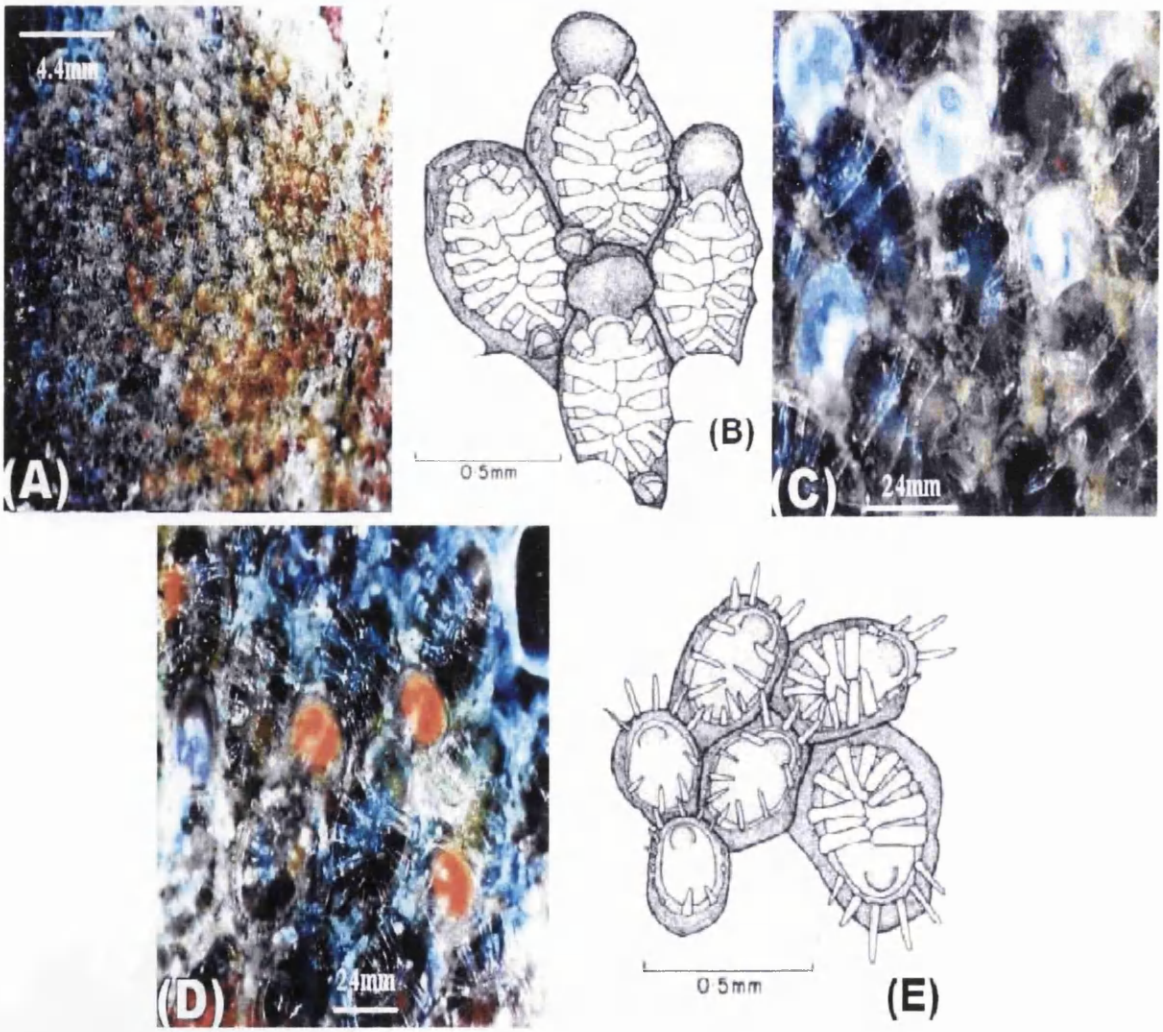


2.5 *Callopora rylandi* Bobin and Prenant

Callopora rylandi is found widespread in littoral environments on southern and western shores of the British Isles and Ireland. It is found on rocks, around *Himanthalia* buttons and in kelp holdfasts in the shallow sublittoral and on the lower shore. Colonies of *Callopora rylandi* are developed as small, rounded patches and may reach up to about 10 mm across (Figure 2.4A). Autozooids are oval in shape, very distinct, separated by shallow grooves and vary in shape and size (0.4-0.6 x 0.26-0.36mm). The gymnocyst is reduced to a narrow triangular region at the proximal extremity of the autozooid and is commonly obscured by the ovicell of the previous individual; the cryptocyst is evident as a narrow rim around the oval opesia. There are usually three to six lateral pairs of spines and a single proximal spine, which are broad and flattened, hollow, arching over the frontal membrane, eventually meeting in the midline, where they broaden or separate and then fuse (Figure 2.4B). Moreover, there are four to six erect cylindrical spines around the distal end of the autozooid (four in the presence of an ovicell). The avicularium in *Callopora rylandi* is small and borne on the gymnocyst with a rostrum pointed, acute to frontal plane and directed proximally or distally (Figure 2.4C). Ovicells are subglobular in shape and are flattened frontally. The ectooecium is membranous frontally, whereas the entooecium is finely granular. Embryos are orange in colour (Figure 2.4D); reproduction has been observed through most of the year, mainly from June to November, with maximum settlement occurring during August to October.

The ancestrula is about half the size of later autozooids, oval in shape, with negligible development of the gymnocyst and cryptocyst and ten erect, cylindrical spines regularly spaced around the frontal membrane (Figure 2.4E).

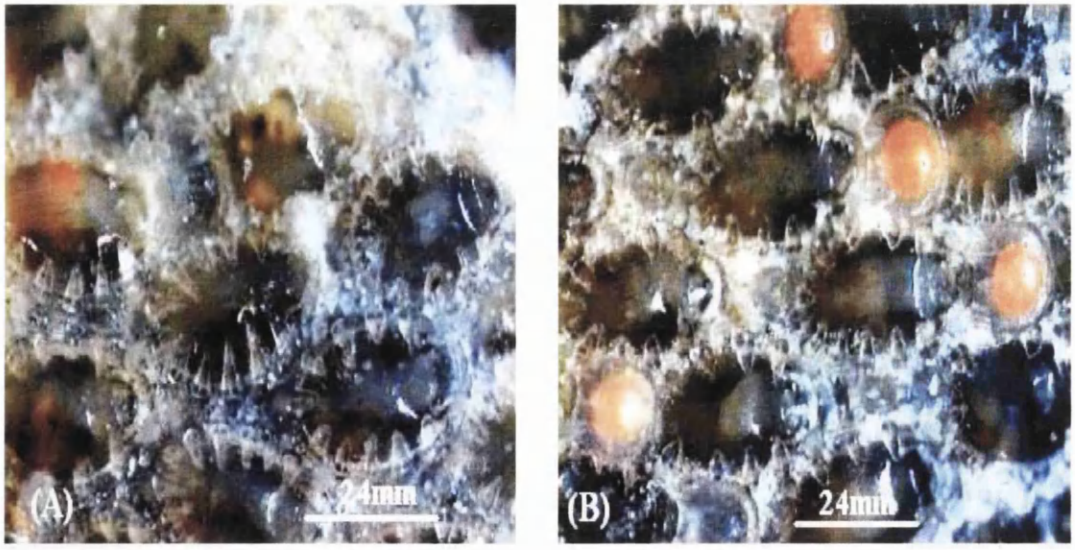
Figure 2.4 (A) Overview of *Callopora rylandi* colony. (B),(C) Ovicellate zooids showing spine arrangement and avicularia. (D) Group of ovicellate zooids with embryos. (E) Ancestrula and first zooids of a colony. (Diagrammatic representations adapted from Ryland and Hayward 1999).



2.6 A possible new species of *Callopora*

A possible new species of *Callopora* was first found by P.J. Hayward and A. Tsyganov at Lydstep Beach, Pembrokeshire and to date has not been found again. The overall colony morphology closely resembles that of *Callopora lineata*, with however, few notable differences. This form has 10 to 12 straight thick spines which are shorter than that of *Callopora lineata* (Figure 2.5A), the ovicell structure is more granular in compared to *Callopora lineata* and the embryo colour is orange (Figure 2.5B) whereas in *Callopora lineata* the embryo colour is red. Reproductive period could not be established, as the species has only been found once. However, at the time of collection (late March 2004), embryos were observed.

Figure 2.5: (A) Ovicellate zooids showing spine arrangement. (B) Ovicellate zooids with embryos.

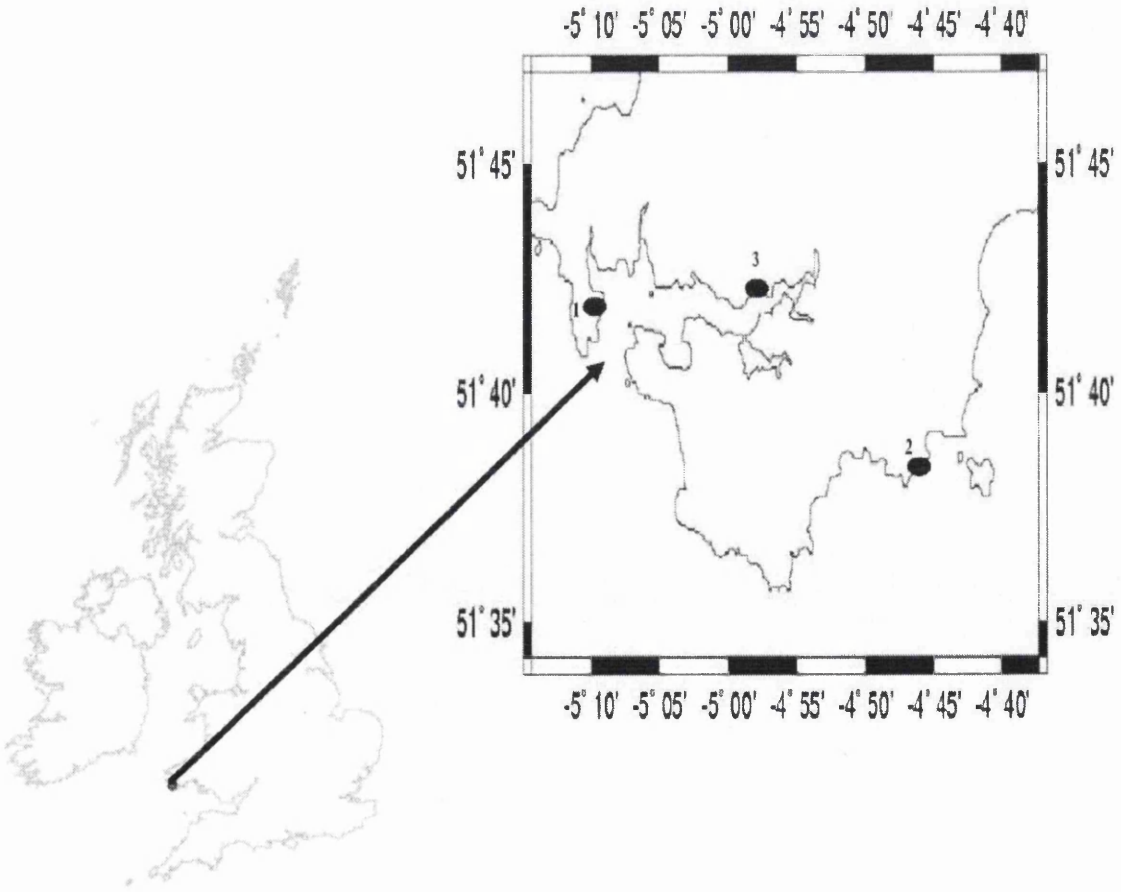


This new form occurred intertidally and was found growing on a stone in the same manner as *Callopora lineata*.

2.7 Study area

Colonies of three *Callopora* species were collected from three different locations around the South West Wales coast (Figure 2.6); Pembroke Ferry (Cleddau Bridge - Milford Haven), Watwick Bay (Milford Haven) and Lydstep Beach (Carmarthen Bay). The sites were selected as the species had been previously recorded there (Hayward and Ryland 1979). Sampling trips were planned to coincide with low water in order to allow coverage of the shore from the infralittoral through to the lower eulittoral, allowing the sampling to be as efficient as possible.

Figure 2.6 Map of South-West Wales showing the sampling localities; 1. Watwick Bay; 2. Lydstep Beach; 3. Pembroke Ferry (Cleddau Bridge). Maps extracted from "Coastline Extractor" available at http://www.rimmer.ngdc.noaa.gov/coast.



Milford Haven, situated near the mouth of the River Cleddau, at the extreme end of south west Wales, is a deep and rocky "drowned valley" (Nelson-Smith 1965) with a abundant marine flora and fauna. The climate is mild and oceanic. However, the south-west winds which prevail most of the time, are very strong and so the shores around the mouth are extremely exposed. The range of the tide, which can exceed 7m, increases to some extent towards the head of the estuary and weakens in the rivers. The freshwater volume which enters Milford Haven is relatively small compared with its tidal volume, thus high levels of salinity can be recorded even in the upper reaches. Mudbanks tend to be local and the waters contain little suspended material. The estuary is essentially unpolluted except by occasional oil spillages.

Carmarthen Bay is an extensive shallow bay. Throughout the bay, physical conditions vary considerably. Salinity varies from low salinity (at the estuaries) to fully marine, there are gradients in wave action from sheltered to exposed and strong tides sweep exposed headlands whilst other areas are sheltered from currents. There is a wide variety of seabed types, including mud, sand and rock, although the majority of the seabed is sandy (information obtained from: http://www.ccw.gov.uk/).

2.7.1 Watwick Bay (Millford Haven)

Watwick Bay (Figure 2.7) is a sandy bay with rocky areas to the north and south sides. The south side was used in this study. The rocky shore abruptly slopes and is composed of large boulders with numerous overhangs which contain a vast number of sessile colonial animals. All three *Callopora* species were found. *Callopora lineata* and *Callopora rylandi* were found in great abundance under flat rocks, whereas *Callopora dumerilii* were relatively scarce.

Figure 2.7 (A) Distant view of Watwick Bay. (B) Close-up of a sampling point.

2.7.2 Pembroke Ferry (Cleddau Bridge – Milford Haven)

Pembroke Ferry is an estuary with shores steadily sloping down to the base of the bridge pier (Figure 2.8). The sampling area of interest is reached by means of a rocky reef which protrudes into the channel under the bridge. The shore consists of small boulders which are overhanging at low water. Of the three *Callopora* species, only *Callopora dumerilii* was collected in great abundance, whereas *Callopora lineata* and *Callopora rylandi* were not found during the course of this study.

Figure 2.8 (A) Distant view of Cleddau Bridge. (B) Close-up of a sampling point.

2.7.3 Lydstep Beach (Carmarthen Bay)

Lydstep Beach (Figure 2.9) is a long rocky beach very close to the town of Tenby. Bryozoans were collected at low water tide, at the furthest possible point that could be accessed on foot. Small and large stones were well exposed at low tide. All three *Callopora* species were found in great abundance in this sampling site

Figure 2.9 (A) Distant view of Lydstep Beach. (B) Close-up of a sampling point.

CHAPTER 3

MORPHOLOGICAL VARIATION IN *CALLOPORA* POPULATIONS

3.0 MORPHOLOGICAL VARIATION IN *CALLOPORA* POPULATIONS

3.1 Aspects of morphometrics in Bryozoa

Over the years, understanding the intrinsic and extrinsic factors that control morphological variation within and among populations has been regarded as an essential prerequisite for most whole-organism studies. Documentation of phenotypic variation is essential for any group of organisms in order to develop sound species concepts and to provide for meaningful ecological and evolutionary inferences. Examples of phenotypic variation based on multiple quantitative morphological characters range from gradual to highly punctuated in well-defined, geographically widespread lineages with detailed fossil records. For example, Cheetham (1986a) used discriminant statistical analysis on multiple- character morphological data from closely spaced sequential populations of the Neogene bryozoan *Metrarabdotos* with the intention of comparing overall morphologies. Results showed that in nine comparisons of ancestor-descendant species pairs, all demonstrated within-species rates of morphological change that did not vary significantly from zero, hence accounting for none of the across-species differences. In all cases the ratio of within-species fluctuation to across-species difference was very low, thus the punctuated pattern could not be distinguished with certainty.

Anatomical characters have traditionally been used in bryozoan studies in order to describe taxonomic units both at the inter- and intraspecific level. Their use in population structure studies is made difficult by the fact that phenotypic variation in these characters is not only directly controlled by genetic factors, but is also

dependent on environmental modifications. The morphology of an organism is the end result of its genetic makeup and the effect of the immediate environment in which it has developed and lived. One of the most well documented responses to environmental changes is the temperature-size relation, based on the observations that large body size is attained in cooler environments. Of great importance is the study of Atkinson (1994) who reviewed 109 studies that had considered temperature mediated size responses. His study included examples from nine phyla both ectothermic and endothermic. He discovered that 84% of the studies reported body size to be larger at lower temperatures; 11% reported an opposite tendency but half of these results were unreliable as the organisms studied had been nurtured at unsuitable extreme temperatures.

Taxonomic analyses, based on morphological characters require understanding of the genetic and environmental components of morphological variation. This is particularly true for most fossil species. For example Jackson and Cheetham (1990) presented evidence from breeding experiments and protein electrophoresis that skeletal characters that are available in fossil material were sufficient to discriminate biological species of living cheilostome Bryozoa. From this, palaeontologists could therefore describe the tempo and infer the mode of evolutionary change at the species level in this group. In addition to this study, Cheetham *et al.* (1993) employed quantitative genetic analyses of skeletal morphology based on breeding data to explore the role of genetic change and natural selection in producing phenotypic divergence between two closely related species of living bryozoans. Their results showed that the hypothesis that morphological differences between the species are

caused by mutation and drift could not be rejected for reasonable rates of mutation maintained for as few as 10^3 to 10^4 generations.

3.2 Genetic and environmental variation in Bryozoa

Morphological variation can be studied efficiently in colonial organisms. Bryozoans are a study group particularly well suited for this purpose. From the slight differences observed in the multitude of zooids, which constitute a bryozoan colony, in addition to the presence of distinctions amongst colonies belonging to the same species, one could expect a range of zooid morphological characteristics. In cheilostome Bryozoa, for example, species are morphospecies based on skeletal characters of feeding zooids and, where present, of specialised polymorphs (Ryland and Hayward 1979) and despite their asexual origin, zooids within the same colony normally vary morphologically. Moreover, variation within colonies could be as great as that among zooids of different colonies and this variation provides the basis for a statistically rigorous approach to morphospecies discrimination that is applicable to both living and fossil cheilostomes (Cheetham 1986b).

The subject of morphological variation in Bryozoa is of great importance in many different areas of study. For the point of view of the taxonomist, measuring morphological variation within single colonies is important allowing assessment of morphological variation of individual colonies within a population (Schopf 1976). On the other hand, measuring morphological variation can help elucidate relationships between major groupings of bryozoans, since those groupings are largely based on morphological characters. This work has great impact in explaining biodiversity of

these animals as well as learning about the factors that are important in speciation processes. In addition, measuring morphological variation is of theoretical significance when one wants to compare this variation in terms of environmental versus genetic components. This type of work although rather difficult is much simpler than estimating aspects of behavioural traits.

The degree to which the variation in morphometric characteristics is influenced by environmental conditions is not known. However, compelling evidence for environmental effects on morphological characters of Bryozoa has been obtained in laboratory based experiments, where differences in temperature, oxygen concentration, salinity and diet induced morphological deviations (Okamura 1987; Hunter and Hughes 1994; Okamura 1985; O'Dea and Okamura 1999; Menon 1972; Morris *et al.*, 1976; Silen and Harmelin 1976). Genetic and environmental influences may depend on the morphological character in question as well as the species and environmental conditions. Moreover, Bryozoa are capable of adopting appropriate morphological responses to environmental changes reflecting genotypic and phenotypic changes (McKinney and Jackson 1989). Zooid size has been related not only to the colony size, (for example small zooids in young colonies (Occhipinti and Ambrogi 1987) but also to feeding resources, to growth rate (Okamura 1987; Hunter and Hughes 1994), salinity, oxygen concentration, extreme hydrodynamic conditions in aquatic environments (Okamura 1985; O'Dea and Okamura 1999) and to the variation of reproductive status by analysis of ovicell size (Jackson and Herrera-Cubilla 2000).

A variety of studies has indicated that the zooids in many cheilostome bryozoan species are influenced by variation in temperature. For example, laboratory based studies demonstrated that *Celleporella hyalina* (Hunter and Hughes 1994) *Membranipora membranacea* and *Conopeum reticulum* (Menon 1972) produced smaller zooids at higher temperatures. In addition, the genera *Haplopoma* and *Hippothoa* and the species *Haplopoma sciaphilum* displayed variation in zooid size along latitudinal and therefore temperature gradients (Morris 1976; Silen and Harmelin 1976). Of particular importance was the study of Okamura and Bishop (1988) that revealed changes in zooid size for a number of Recent and fossil groups in accordance with known temperature regimes. Their results provided proof that changes in zooid size within a species over geological time could be used to deduce relative changes in palaeotemperature. In accordance with this study was the study of O'Dea and Okamura (1999), who observed zooid size and colony growth of the estuarine bryozoan *Conopeum seurati.* Their data were analysed in combination with synchronous measurements of temperature, salinity and food availability and showed that the length, width and area of the zooid were highly dependent on temperature, whereas food availability and colony growth rate did not seem to have such an effect. Moreover, salinity and the interaction of temperature and salinity had a significant effect on zooid length and area, a result that implied that changes in zooid size could be attributed to oxygen limitation in warm waters. Similar findings were observed in colonies of *Pentapora fascialis* (Lombardi *et al.*, 2006). They showed that zooid length in colonies of *Pentapora fascialis* appeared more sensitive than zooid width and area to temperature fluctuations, with longest zooids being budded in areas that experienced low seasonal variation in temperature (3.2-7.5°C) and low mean annual temperature (~ 11°C). Zooid length displayed the strongest association with the range

of seasonal variation in temperature rather than with the mean annual temperature. Interestingly, a decrease in zooid size during warmer summer months in *Electra pilosa* suggested that a temperature-size effect occurs on a seasonal basis (Okamura 1987). It was speculated that this seasonal variation in size was due to temperature fluctuation and not food availability. However, the fact that neither temperature nor food availability were monitored made these results difficult to assess.

Experiments comparing both morphological and molecular data have been performed in order to establish if the morphological characters support the molecular data. Jackson and Cheetham (1990), for example, used this approach to explore the degree to which morphospecies correspond to biological species. They studied three distantly related genera of Panamanian cheilostomes; *Parasmittina*, which is morphologically complex, with nearly 40 quantifiable skeletal characters and *Steginoporella* and *Stylopoma*, which are less complex, each with approximately 20 characters. The range of morphometric complexity was chosen in such a way as to test whether ability to discriminate biologically significant taxa depends on the number of available characters. For this purpose horizontal starch gel electrophoresis was employed in order to study enzyme variation between different colonies in each of these three species. 402 colonies were collected in total, in three species each of *Steginoporella* and *Stylopoma* and two species of *Parasmittina* and each colony was assigned to a morphospecies by visual inspection. More than 30 enzymes were examined using conventional methods and staining. From these, only seven loci were resolved and scored. Nevertheless, all were polymorphic and varied within as well as between morphospecies. In addition, they found no diagnostic alleles between each of the pairs of morphospecies within all three genera. Moreover, genetic distances between

morphospecies were found to be consistently much higher than between populations of the same morphospecies. Heritability of morphospecies identity was studied by raising offspring of colonies from different populations in a common garden experiment. Offspring colonies were grown from embryos derived from known maternal colonies but of unknown paternity. Two generations of offspring, F_1 and F_2, were raised to test for maternal effects on development, such as could result from maturation of embryos within maternal colonies in their natural environment. In *Parasmittina* and *Steginoporella*, all offspring colonies were virtually identical to their parents ($P>0.99$) despite having been raised in unnatural environment. Slightly different results were obtained in *Stylopoma*. In both species, the probability of assignment of several F_2 colonies to their parental morphospecies dropped below 0.99, apparently reflecting sensitivity to the unnatural conditions of the experiment. In summary evidence from their experiments showed that morphospecific identity of cheilostomes is heritable and that morphospecies are genetically distinct with no indication of morphologically cryptic species.

In the same way, Porter *et al.* (2001a) used a combination of comparative morphological and molecular genetic techniques to resolve the problem of the taxonomic identity of the common subtidal ctenostomate bryozoan *Alcyonidium diaphanum*. Colony morphology and genetic characters of the three commonly occurring morphotypes were examined. Results from this study showed that just a single genetic species was recognised based on the 12s rRNA and COI mitochondrial DNA genes.

Similar work, where statistical methods have been employed in order to elucidate the identity of bryozoan species, is very common in the literature. Lidgard and Buckley (1994) used three multivariate types of analysis, such as principal component analysis, cluster analysis and discriminant analysis, to investigate phenotypic variation and the possibility of morphologically cryptic species in *Adeonellopsis yarraensis* in populations from Australia and New Zealand. Their analysis showed that at least five subgroups, which were previously recognised as *Adeonellopsis yarraensis*, are actually separate species. This raised a number of questions as to whether a number of cryptic species may exist within currently accepted yet poorly defined species boundaries predominantly among taxa that are morphologically variable and geographically widespread. However, Levinton (1991) questioned as to whether skeletal morphology alone is sufficient in order to distinguish species. His point of view was that skeletal morphology either is insufficient alone to separate closely related forms or may be too variable to confer species status on members of the same population.

3.3 Aims

In the present study, morphometric analyses were conducted in order to study morphological variation in different populations each of three *Callopora* species and to partition the morphological variation within and between sites and colonies for each species. In this way patterns of variation could be compared in these related species.

3.4 Materials and methods

3.4.1 Fieldwork

Colonies of *Callopora rylandi*, *Callopora lineata* and *Callopora dumerilii* were collected from three different sites around South West Wales (Chapter 2). Sampling collections were carried out monthly, but extensive sampling was performed between May and October as these species are known to reach the peak of their reproduction during these months (Hayward and Ryland 1998). The sampling dates and locations were chosen to coincide with low tide. For this purpose the Admiralty tidal tables (Great Britain. Hydrographic Dept) as well as the National Tidal and Sea Level Facility were used to confirm the time of low tide.

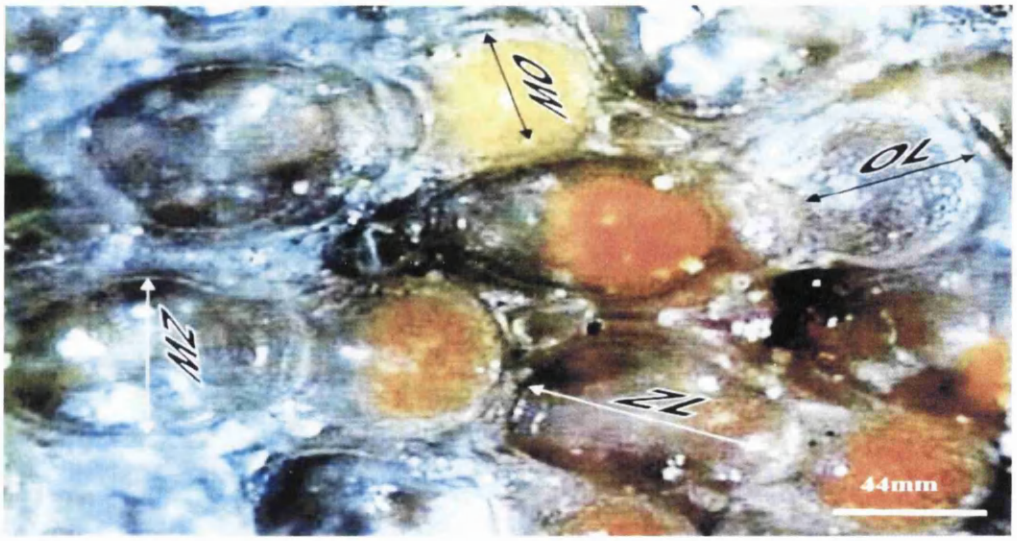
Epiphytic bryozoans are conspicuous and easily found, whereas hard substratum species, such as those in this study, required the use of a x10 hand lens to recognise the bryozoan colony of interest. Upon collection, encrusted stones were carefully packed in damp seaweed to avoid crushing their sessile fauna and cautiously transferred into thermo insulated containers containing fresh sea water. In all instances care was taken to minimise damage to intertidal habitats and collecting was strictly minimal. Any large stones that were collected in the search for specimens were returned to their original position.

3.4.2 Laboratory analysis of zooids

Upon return to the lab, samples were stored in large containers according to sampling locality in a constant temperature room at ambient temperature. The containers were connected to an aeration system in order to extend the life of the colonies for further analysis, primarily larval extraction as well as measurement of the zooids.

As mentioned in preceding section (Section 2.2) specimens were observed alive, whenever possible and fine paint brushes were used for removing detritus as accurate identification required that a portion of the specimen should be cleaned. Subsequently, colonies were examined again, their identity was confirmed based on the work of Hayward and Ryland (1998) and morphological measurements consequently were taken. Morphological measurements that were reliably measurable at the available resolution were used. These were zooid length (ZL); zooid width (ZW); ovicell length (OL) and ovicell width (OW) (Figure 3.1).

Figure 3.1 Zooids of *Callopora dumerilii* showing the morphological characters selected.



Zooids were examined using a colour video camera (Media Cybernetics) which was attached to a stereomicroscope (Wild M420). Digital calibration of the microscope was performed using a specific video capture utility (Image-Pro Plus Version 4.1.09) and measurements were taken under standard magnification (x32) making sure that no more than two zooids filled a screen. The positions from which zooids were selected for measurement from a colony were chosen according to the protocol by Hageman *et al.*, (2002). Based on this, 20 zooids were selected from each colony distributed over three 120 degree sectors: one sector running from 12 o'clock to 4 o'clock = "Right", one from 4 to 8 = "Bottom" and one from 8 to 12 = "Left". The objective was to select different parts of the colony in order for the widest variation within the colony to be measured. Measurements were arranged in spreadsheets according to site ID as well as colony ID. Further analysis of the data was carried out in SPSS.

3.4.3 Statistical methods

Zooid length, zooid width, ovicell length and ovicell width (Figure 3.1) were measured in colonies collected from three sites in total (Table 3.1). For each site, a different number of colonies was sampled for each species. For *Callopora lineata*, 21 colonies were collected in total at Lydstep Beach and 13 colonies at Watwick Bay. No colonies were collected from Pembroke Ferry. For *Callopora rylandi*, 10 colonies were collected from Lydstep Beach and 17 colonies from Watwick Bay. No colonies were collected from Pembroke Ferry. For *Callopora dumerilii*, 16 colonies were collected from Lydstep Beach, 4 colonies from Watwick Bay and 18 colonies from Pembroke Ferry (Table 3.1).

Table 3.1 Number of colonies collected for each species from each site.

	Callopora rylandi	*Callopora lineata*	*Callopora dumerilii*
Lydstep	10	21	16
Watwick Bay	17	13	4
Pembroke Ferry	-	-	18

The morphological measurements were analysed between sites and colonies using a Nested ANOVA approach. A principal component analysis was also performed on the dataset, as it is known to be a very effective statistical method in providing morphologically interpretable results without requiring unlikely assumptions about the distribution of the data (Morrison 1967). With morphometric data, the first component generally expresses aspects of general size and among the remaining components one or more expresses shape. The data can then be summarised with respect to size and shape vectors to compare zooids of different morphology (Cheetham 1973).

3.5 Results

3.5.1 Tests of Normality

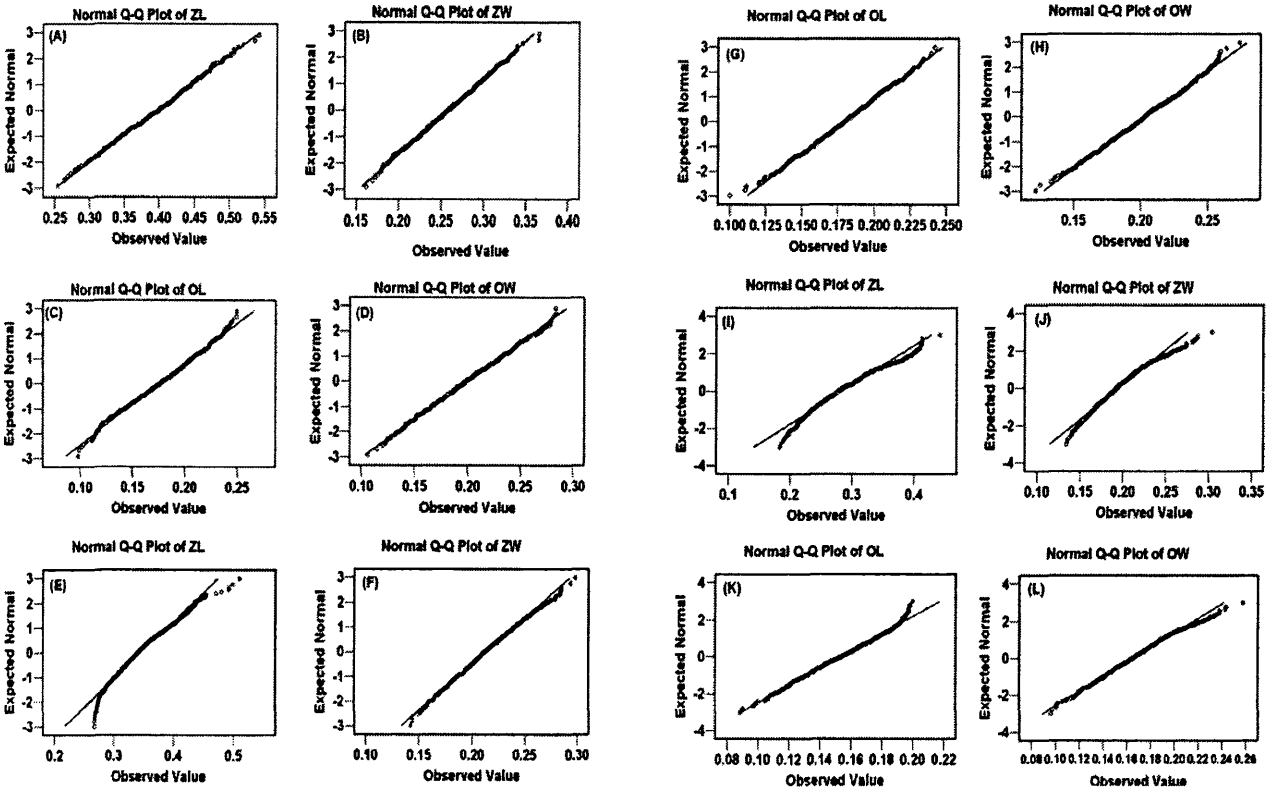
Prior to any analysis, all four measurements zooid length, zooid width, ovicell length, ovicell width were tested for normality using the Kolmogorov-Smirnov test (Table 3.2).

Table 3.2 Kolmogorov-Smirnov test of normality for three species of *Callopora* and four morphological characters.

	Species name	Statistic	df	P
ZL	*Callopora rylandi*	0.032	540	0.632
	Callopora lineata	0.054	680	0.039
	Callopora dumerilii	0.058	760	0.012
ZW	*Callopora rylandi*	0.016	540	0.999
	Callopora lineata	0.028	680	0.677
	Callopora dumerilii	0.053	760	0.029
OL	*Callopora rylandi*	0.030	540	0.710
	Callopora lineata	0.022	680	0.909
	Callopora rylandi	0.023	760	0.826
OW	*Callopora rylandi*	0.023	540	0.928
	Callopora lineata	0.026	680	0.763
	Callopora dumerilii	0.024	760	0.762

Nine of the values are non-significant suggesting no significant departure from normality. Three of the values are significant *a priori*. However, they are non-significant after Bonferroni correction taking account that there are twelve values in the table. The distribution of the variables was also examined with Q-Q plots (Figure 3.2).

Figure 3.2 Normal Q-Q plot distributions: (A) ZL *Callopora rylandi*; (B) ZW *Callopora rylandi*; (C) OL *Callopora rylandi*; (D) OW *Callopora rylandi*; (E) ZL *Callopora lineata*; (F) ZW *Callopora lineata*; (G) OL *Callopora lineata*; (H) OW *Callopora lineata*; (I) ZL *Callopora dumerilii*; (J) ZW *Callopora dumerilii*; (K) OL *Callopora dumerilii*; (L) OW *Callopora dumerilii*.

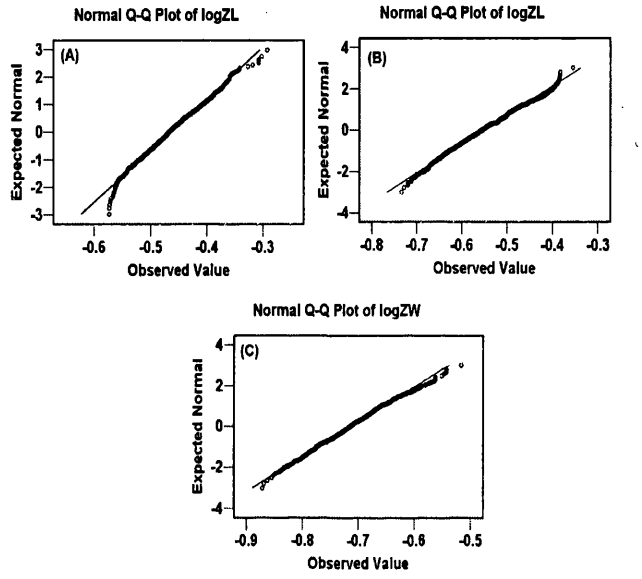


In general the points fall on straight lines in these plots consistent with normality. The biggest deviations, in graphs (E) and (I) and (J), correspond with the *a priori* significant values observed in Table 3.2. To try to correct for this, a $\log_{10}$ transformation (Sokal and Rohlf 1995) was carried out on these three sets of measurements. The Kolmogorov-Smirnov statistics were non-significant after transformation (Table 3.3) and the corresponding Q-Q plots (Figure 3.3) showed improved fit to a straight line.

Table 3.3 Kolmogorov-Smirnov test for normality for $\log_{10}$ transformed variables.

	Species name	Statistic	df	P
logZL	*Callopora lineata*	0.029	680	0.618
	Callopora dumerilii	0.025	760	0.737
logZW	*Callopora dumerilii*	0.026	760	0.703

Figure 3.3 Normal Q-Q plots for $\log_{10}$ transformed variables; (A) ZL for *Callopora lineata;* (B) ZL for *Callopora dumerilii*; (C) ZW for *Callopora dumerilii.*



Because of the improved fit to normality after transformation for these three datasets it was decided to use the transformed values for ANOVA. For the other nine datasets the untransformed values were used.

3.5.2 ANOVA for measured zooid characters

3.5.2.1 Zooid Length (ZL)

Prior to any analysis the values for ZL where checked for homogeneity of variance using the Levene test (Table 3.4).

Table 3.4 Levene test for homogeneity of variance.

	Species name	F	df1	df2	P
SITE	*Callopora rylandi*	17.998	1	538	0.000
"	*Callopora lineata*	7.680	1	678	0.006
"	*Callopora dumerilii*	10.079	2	757	0.000
COLONY	*Callopora rylandi*	1.521	26	513	0.05
"	*Callopora lineata*	1.519	33	646	0.033
"	*Callopora dumerilii*	4.189	37	722	0.000

Significant departures from homogeneity of variance were observed when comparing sites and when comparing colonies for each of the three species. Analysis of variance is generally quite robust to deviations from normality and the power to detect deviations is high here because of the high error degrees of freedom (df2). Thus it was decided to pursue ANOVA but also to carry out the non-parametric Kruskal-Wallis test when comparing sites. This test can be used in place of a one-way ANOVA when there are more than two groups to compare. As a rule of thumb, Kruskal-Wallis test was performed if the P value in the Levene test is less that 0.05.

The results of a Nested ANOVA for all three species for ZL are shown in Table 3.5. The mean square values are tested in a hierarchical fashion. 'Between Colonies within Site' is tested against the error. Site is then tested against 'Between Colonies within Site' to determine whether there is any significant variation between sites on top of

that due to differences between colonies. The component Colony tests for significant differences between colonies disregarding sites.

Table 3.5 Nested ANOVA results for all species for ZL variable.

Species Name	Source	SS	df	MS	F	P
Callopora rylandi	Colony	0.418	26	0.016	9.691	0.000
Callopora lineata	"	0.672	33	0.020	10.423	0.000
Callopora dumerilii	"	1.749	37	0.047	15.977	0.000
Callopora rylandi	Site	0.001	1	0.001	0.051	0.823
Callopora lineata	"	0.019	1	0.019	0.976	0.326
Callopora dumerilii	"	0.482	2	0.241	6.651	0.001
Callopora rylandi	Between	0.417	25	0.017	10.059	0.000
Callopora lineata	Colonies	0.652	32	0.020	10.431	0.000
Callopora dumerilii	Within Site	1.237	35	0.036	12.238	0.000
Callopora rylandi	Error	0.852	513	0.002		
Callopora lineata	"	1.262	646	0.002		
Callopora dumerilii	"	2.136	722	0.003		

With the exceptions, *Callopora rylandi* and *Callopora lineata* for Site, there are significant differences both between site and between colonies. The mean values corresponding to the ANOVA results are given in Figure 3.4. The small differences between means and overlap of confidence intervals for *Callopora rylandi* and *Callopora lineata* fits with the observed non-significant results for Site in these species. By contrast the significant results for Site for *Callopora dumerilii* fit well with the large differences between means. Because there were three sites for *Callopora dumerilii* and thus three possible comparisons between sites, post hoc comparisons using the Bonferroni procedure were carried out (Table 3.6). All three comparisons were highly significant following this procedure.

Figure 3.4 Mean Plots for the ANOVA results with 95% confidence intervals for ZL in *Callopora rylandi* for sites (A) and colonies (B) (colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick); in *Callopora lineata* (C and D) (colonies 1-21 are from Lydstep and 22-34 are from Watwick) and in *Callopora dumerilii* (E and F) (colonies 1-18 are from Pembroke Ferry, 19-35 are from Lydstep and 36-39 are from Watwick).

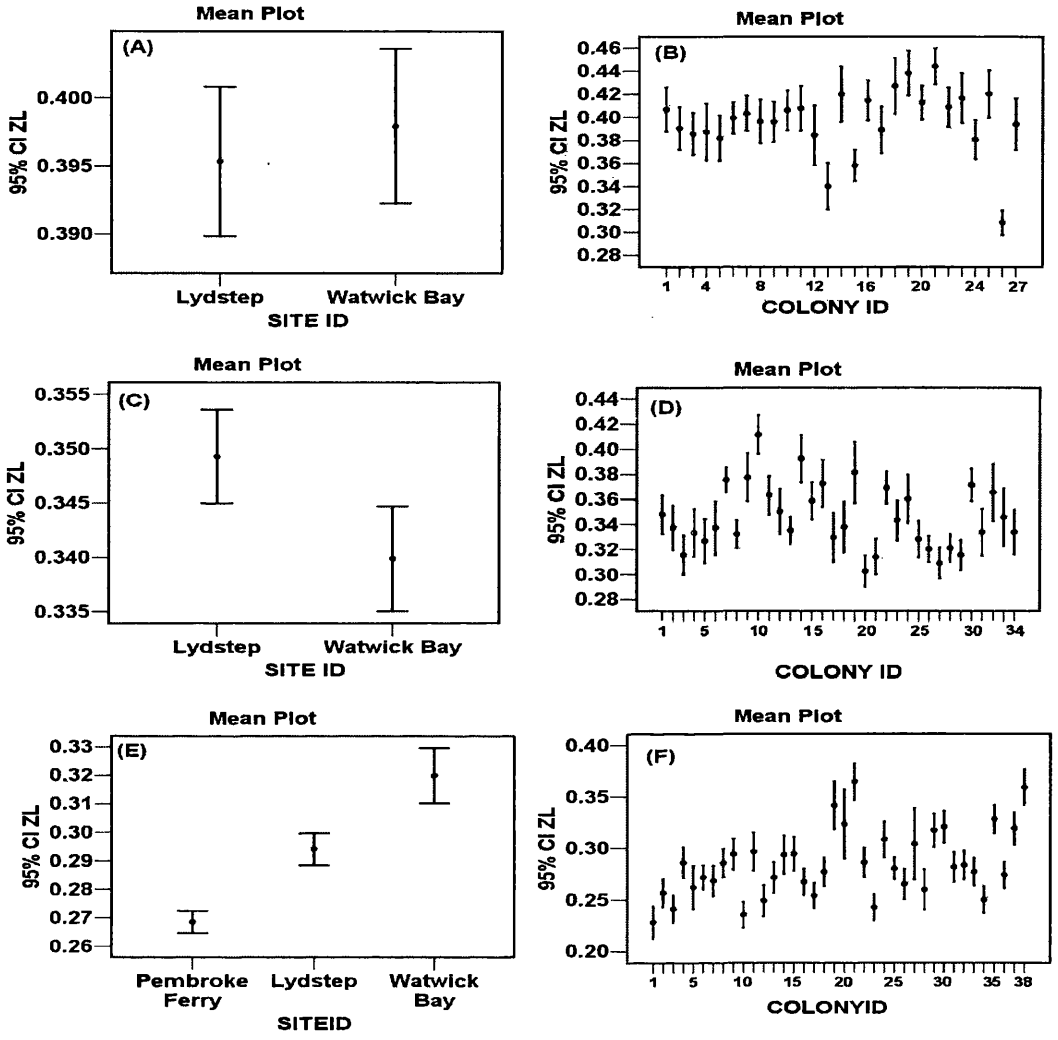


Table 3.6 Post hoc test using Bonferroni procedure for differences between sites for *Callopora dumerilii*.

Comparison		Mean Difference between sites	STD Error	P
Pembroke Ferry	Lydstep	-0.037	0.005	0.000
Lydstep	Watwick Bay	-0.039	0.008	0.000
Watwick Bay	Pembroke Ferry	0.076	0.008	0.000

The plots of colony means in Figure 3.4 show a scatter of values. However, it is clear that in many cases when comparing two colonies that the 95% confidence intervals do not overlap. Further analysis was done on the colony means by carrying out the Student-Newman-Keuls (SNK) test. This is a post hoc test which sorts means into groups which are homogeneous subsets. Within a subset means are not significantly different from one another. Subsets that do not share members can be regarded as distinct significantly different groups of colonies. The results for *Callopora rylandi* are shown in Table 3.7. Most of the groups overlap and share colonies. However, group 1 is distinct from the other groups; group 2 is distinct from groups 4-7 and group 3 is distinct from group 7.

Table 3.7 Student-Newman-Keuls (SNK) post hoc test for *Callopora rylandi.* Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7
26	0.308						
13		0.340					
15		0.358	0.358				
24			0.381	0.381			
5			0.382	0.382	0.382		
12			0.384	0.384	0.384		
3			0.386	0.386	0.386		
4			0.387	0.387	0.387		
17			0.389	0.389	0.389		
2			0.390	0.390	0.390		
27			0.394	0.394	0.394	0.394	
9			0.396	0.396	0.396	0.396	
8			0.396	0.396	0.396	0.396	
6			0.399	0.399	0.399	0.399	
7				0.403	0.403	0.403	0.403
10				0.406	0.406	0.406	0.406
1				0.407	0.407	0.407	0.407
11				0.408	0.408	0.408	0.408
22				0.409	0.409	0.409	0.409
20				0.413	0.413	0.413	0.413
16				0.415	0.415	0.415	0.415
23				0.417	0.417	0.417	0.417
14				0.420	0.420	0.420	0.420
25				0.420	0.420	0.420	0.420
18					0.427	0.427	0.427
19						0.438	0.438
21							0.444
Sig.	1.000	0.163	0.066	0.215	0.067	0.053	0.082

The SNK test was also carried out in a similar way for *Callopora lineata* (Table 3.8) and *Callopora dumerilii* (Table 3.9). For *Callopora lineata* examples of distinct differences are group 1 being distinct from groups 8-10, groups 2-3 being distinct from groups 9-10 and groups 4-8 being distinct from group 10.

Table 3.8 Student-Newman-Keuls (SNK) post hoc test for *Callopora lineata*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-21 are from Lydstep and colonies 22-34 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10
20	-0.52									
27	-0.512	-0.512								
21	-0.505	-0.505	-0.505							
3	-0.503	-0.503	-0.503							
29	-0.502	-0.502	-0.502							
26	-0.496	-0.496	-0.496	-0.496						
28	-0.495	-0.495	-0.495	-0.495	-0.495					
5	-0.488	-0.488	-0.488	-0.488	-0.488	-0.488				
25	-0.486	-0.486	-0.486	-0.486	-0.486	-0.486				
17	-0.486	-0.486	-0.486	-0.486	-0.486	-0.486				
4	-0.48	-0.48	-0.48	-0.48	-0.48	-0.48	-0.48			
8	-0.48	-0.48	-0.48	-0.48	-0.48	-0.48	-0.48			
31	-0.479	-0.479	-0.479	-0.479	-0.479	-0.479	-0.479			
34	-0.479	-0.479	-0.479	-0.479	-0.479	-0.479	-0.479			
13	-0.476	-0.476	-0.476	-0.476	-0.476	-0.476	-0.476			
6	-0.475	-0.475	-0.475	-0.475	-0.475	-0.475	-0.475			
18	-0.475	-0.475	-0.475	-0.475	-0.475	-0.475	-0.475			
2	-0.474	-0.474	-0.474	-0.474	-0.474	-0.474	-0.474			
23		-0.466	-0.466	-0.466	-0.466	-0.466	-0.466	-0.466		
33		-0.465	-0.465	-0.465	-0.465	-0.465	-0.465	-0.465		
1			-0.46	-0.46	-0.46	-0.46	-0.46	-0.46		
12			-0.458	-0.458	-0.458	-0.458	-0.458	-0.458		
15				-0.447	-0.447	-0.447	-0.447	-0.447	-0.447	
24					-0.446	-0.446	-0.446	-0.446	-0.446	
11						-0.441	-0.441	-0.441	-0.441	
32						-0.44	-0.44	-0.44	-0.44	
22							-0.434	-0.434	-0.434	
16							-0.431	-0.431	-0.431	
30							-0.431	-0.431	-0.431	
7								-0.426	-0.426	
9								-0.425	-0.425	
19								-0.422	-0.422	
14									-0.408	-0.408
10										-0.387
Sig.	0.095	0.085	0.089	0.052	0.055	0.067	0.057	0.1	0.178	0.121

For *Callopora dumerilii*, SNK post hoc comparisons showed significant differences amongst group 1 versus groups 7-15, group 2 versus groups 9-15, group 3 versus groups 10-15, group 4 versus groups 11-15, group 5 versus groups 12-15, group 6

versus groups 12-15, groups 7-8 versus groups 13-15, groups 9-11 versus groups 14-15 and group 12 versus group15 (Table 3.9).

Table 3.9 Student-Newman-Keuls (SNK) post hoc test for *Callopora dumerilii.* Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-18 are from Pembroke Ferry, colonies 19-35 are from Lydstep and colonies 36-39 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	-0.646														
10	-0.631	-0.631													
3	-0.621	-0.621	-0.621												
24	-0.618	-0.618	-0.618	-0.618											
12	-0.607	-0.607	-0.607	-0.607	-0.607										
35	-0.604	-0.604	-0.604	-0.604	-0.604	-0.604									
18	-0.597	-0.597	-0.597	-0.597	-0.597	-0.597									
2		-0.593	-0.593	-0.593	-0.593	-0.593	-0.593								
29		-0.591	-0.591	-0.591	-0.591	-0.591	-0.591	-0.591							
5		-0.587	-0.587	-0.587	-0.587	-0.587	-0.587	-0.587							
27		-0.579	-0.579	-0.579	-0.579	-0.579	-0.579	-0.579							
17		-0.575	-0.575	-0.575	-0.575	-0.575	-0.575	-0.575							
7			-0.574	-0.574	-0.574	-0.574	-0.574	-0.574	-0.574						
13			-0.569	-0.569	-0.569	-0.569	-0.569	-0.569	-0.569						
6			-0.568	-0.568	-0.568	-0.568	-0.568	-0.568	-0.568						
37			-0.564	-0.564	-0.564	-0.564	-0.564	-0.564	-0.564						
19				-0.56	-0.56	-0.56	-0.56	-0.56	-0.56	-0.56					
34				-0.56	-0.56	-0.56	-0.56	-0.56	-0.56	-0.56					
26					-0.554	-0.554	-0.554	-0.554	-0.554	-0.554	-0.554				
32					-0.553	-0.553	-0.553	-0.553	-0.553	-0.553	-0.553				
33					-0.55	-0.55	-0.55	-0.55	-0.55	-0.55	-0.55				
8						-0.546	-0.546	-0.546	-0.546	-0.546	-0.546				
4						-0.546	-0.546	-0.546	-0.546	-0.546	-0.546				
23						-0.546	-0.546	-0.546	-0.546	-0.546	-0.546				
14							-0.536	-0.536	-0.536	-0.536	-0.536	-0.536			
9							-0.534	-0.534	-0.534	-0.534	-0.534	-0.534			
16							-0.534	-0.534	-0.534	-0.534	-0.534	-0.534			
28								-0.529	-0.529	-0.529	-0.529	-0.529			
25									-0.514	-0.514	-0.514	-0.514	-0.514		
30										-0.502	-0.502	-0.502	-0.502		
21										-0.501	-0.501	-0.501	-0.501		
38											-0.498	-0.498	-0.498		
31											-0.496	-0.496	-0.496		
36												-0.486	-0.486	-0.486	
20													-0.472	-0.472	-0.472
39														-0.447	-0.447
22															-0.441
Sig.	0.077	0.051	0.068	0.056	0.076	0.072	0.053	0.051	0.054	0.053	0.071	0.125	0.167	0.064	0.171

An interesting feature that emerged from the SNK post hoc test was that certain groups for each of the *Callopora* species contained colonies that were specific to a particular site. For example, *Callopora rylandi* group 2 in Table 3.7 includes colonies 13 and 15 that were from Lydstep and group 4 includes colonies 1-10 that were from Lydstep and colonies 11, 12, 14, 16, 17, 20, 22-25 that were from Watwick. Similar groupings were found for the *Callopora lineata* as well as *Callopora dumerilii.*

The non-parametric Kruskal-Wallis test was performed by comparing colonies within each site separately for each species. The results are shown in Table 3.10.

Table 3.10 Kruskal-Wallis test for all three sites for all three species.

Species name	Source	Chi-Square	df	P
Callopora rylandi	Lydstep	8.397	9	0.495
Callopora lineata	"	152.944	20	0.000
Callopora dumerilii	"	124.227	15	0.000
Callopora rylandi	Watwick Bay	134.624	16	0.000
Callopora lineata	"	80.451	12	0.000
Callopora dumerilii	"	37.486	3	0.000
Callopora dumerilii	Pembroke Ferry	106.600	17	0.000

Significant differences were observed for all sites where the species were sampled, with one exception, for *Callopora rylandi* from Lydstep. The exceptional result fits in with the observed colony means in Figure 3.4B, which are quite closely similar to each in value. In general the results of Kruskal-Wallis test are consistent with the ANOVA results in showing significant differences between colonies.

3.5.2.2 Zooid Width (ZW)

Similarly, prior to analysis the scores for ZW variable were checked for homogeneity of variance using the Levene test (Table 3.11).

Table 3.11 Levene test for homogeneity of variance.

	Species name	F	df1	df2	P
SITE	*Callopora rylandi*	14.334	1	538	0.000
"	*Callopora lineata*	2.483	1	678	0.116
"	*Callopora dumerilii*	2.909	2	757	0.055
COLONY	*Callopora rylandi*	1.591	26	513	0.000
"	*Callopora lineata*	2.235	33	646	0.000
"	*Callopora dumerilii*	1.567	37	722	0.018

With the exceptions *Callopora lineata* and *Callopora dumerilii* for Site, significant departures from homogeneity of variance were observed when comparing sites and when comparing colonies for each of the three species. Similarly ANOVA was conducted and the non-parametric Kruskal-Wallis test for Site for those that have P value less than 0.05 in the Levene test. The results of a Nested ANOVA for all three species for ZW are shown in Table 3.12.

Table 3.12 Nested ANOVA results for all species for ZW variable.

Species Name	Source	SS	df	MS	F	P
Callopora rylandi	Colony	0.219	26	0.008	9.907	0.000
Callopora lineata	"	0.183	33	0.006	11.782	0.000
Callopora dumerilii	"	0.566	37	0.015	5.451	0.000
Callopora rylandi	Site	0.001	1	0.001	0.150	0.702
Callopora lineata	"	0.003	1	0.003	0.477	0.490
Callopora dumerilii	"	0.089	2	0.045	3.272	0.001
Callopora rylandi	Between	0.218	25	0.009	10.616	0.000
Callopora lineata	Colonies	0.179	32	0.006	11.972	0.000
Callopora dumerilii	Within Site	0.477	35	0.014	4.854	0.000
Callopora rylandi	Error	0.437	513	0.001		
Callopora lineata	"	0.303	646	0.001		
Callopora dumerilii	"	2.026	722	0.003		

With two exceptions, *Callopora rylandi* and *Callopora lineata* for Site, there are significant differences both between sites and between colonies. The mean values corresponding to the ANOVA results are given in Figure 3.5. The small differences between means and overlap of confidence intervals for *Callopora rylandi* and *Callopora lineata* fits with the observed non-significant results for Site this species. By contrast the significant results for *Callopora dumerilii* for Site fit well with the large differences between means. Because there were three sites for *Callopora dumerilii* and thus three possible comparisons between sites, post hoc comparisons using the Bonferroni procedure were carried out (Table 3.13). Two comparisons were highly significant following this procedure. However, the comparison between Lydstep and Watwick Bay was not significant, which fits in with the observed overlap of 95% confidence intervals in Figure 3.5E.

Figure 3.5 Mean Plots for the ANOVA results with 95% confidence intervals for ZW in *Callopora rylandi* for sites (A) and colonies (B) (colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick); in *Callopora lineata* (C and D) (colonies 1-21 are from Lydstep and 22-34 are from Watwick) and in *Callopora dumerilii* (E and F) (colonies 1-18 are from Pembroke Ferry, 19-35 are from Lydstep and 36-39 are from Watwick).

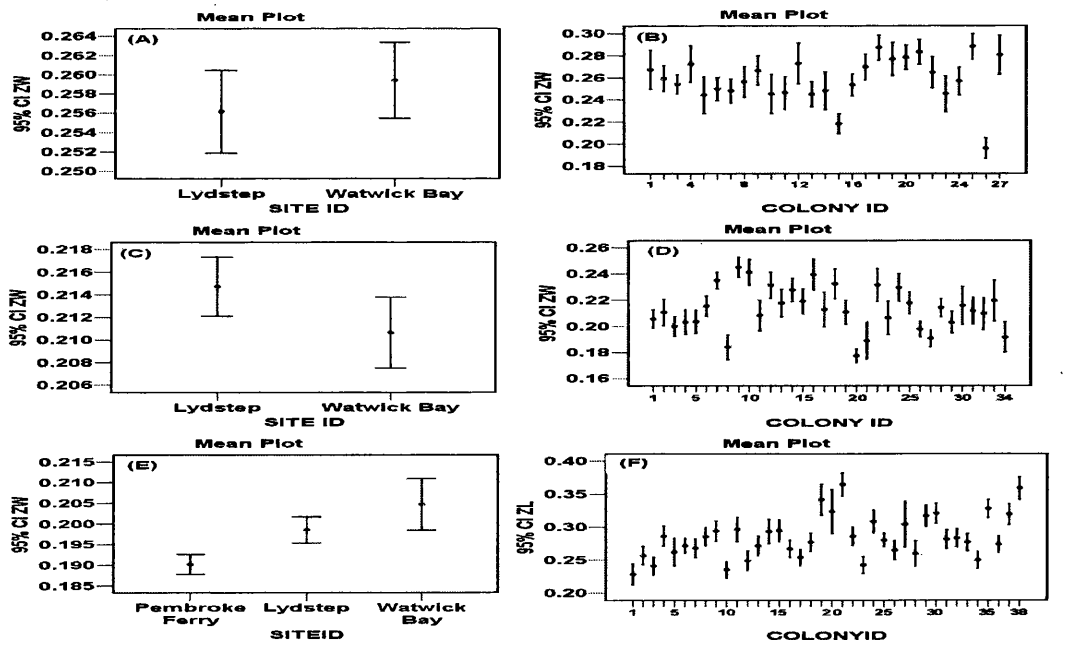


Table 3.13 Post hoc test using Bonferroni procedure for differences between sites for *Callopora dumerilii.*

Comparison		Mean Difference between sites	STD Error	P
Pembroke Ferry	Lydstep	-0.017	0.005	0.000
Lydstep	Watwick Bay	-0.014	0.007	0.166
Watwick Bay	Pembroke Ferry	0.031	0.007	0.000

As with ZL, the plots of colony means in Figure 3.5 show a scatter of values. However, it is clear that in many cases when comparing two colonies that the 95% confidence intervals do not overlap. Further analysis was done on the colony means by carrying out the Student-Newman-Keuls (SNK) test. The results for *Callopora rylandi* are shown in Table 3.14.

Table 3.14 Student-Newman-Keuls (SNK) post hoc test for *Callopora rylandi.* Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8
26	0.196							
15		0.218						
5			0.244					
13			0.245					
23			0.245					
10			0.245					
11			0.246					
7			0.248	0.248				
14			0.248	0.248				
6			0.25	0.25	0.25			
16			0.253	0.253	0.253	0.253		
3			0.254	0.254	0.254	0.254		
8			0.256	0.256	0.256	0.256	0.256	
24			0.257	0.257	0.257	0.257	0.257	
2			0.259	0.259	0.259	0.259	0.259	0.259
22			0.265	0.265	0.265	0.265	0.265	0.265
9			0.266	0.266	0.266	0.266	0.266	0.266
1			0.267	0.267	0.267	0.267	0.267	0.267
17			0.27	0.27	0.27	0.27	0.27	0.27
4			0.272	0.272	0.272	0.272	0.272	0.272
12			0.273	0.273	0.273	0.273	0.273	0.273
19			0.277	0.277	0.277	0.277	0.277	0.277
20				0.278	0.278	0.278	0.278	0.278
27					0.28	0.28	0.28	0.28
21						0.283	0.283	0.283
18							0.287	0.287
25								0.288
Sig.	1	1	0.052	0.081	0.061	0.082	0.051	0.09

Most of the groups overlap and share colonies. However group 1 is distinct from the other groups and group 2 is also distinct from groups 3-8.

In the same manner, the SNK test was also carried out in a similar way for *Callopora lineata* (Table 3.15) and *Callopora dumerilii* (Table 3.16). For *Callopora lineata* group 1 is distinct from groups 5-12, group 2 is distinct from groups 7-12, groups 3-4 are distinct from groups 11-12 and group 5 is distinct from group 12.

Table 3.15 Student-Newman-Keuls (SNK) post hoc test for *Callopora lineata*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-21 are from Lydstep and colonies 22-34 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10	11	12
20	0.177											
8	0.184	0.184										
21	0.189	0.189	0.189									
27	0.191	0.191	0.191	0.191								
34	0.192	0.192	0.192	0.192								
26		0.198	0.198	0.198	0.198							
3		0.2	0.2	0.2	0.2							
29		0.203	0.203	0.203	0.203							
4		0.203	0.203	0.203	0.203							
5		0.204	0.204	0.204	0.204							
1		0.205	0.205	0.205	0.205	0.205						
23			0.206	0.206	0.206	0.206	0.206					
11			0.208	0.208	0.208	0.208	0.208	0.208				
32			0.209	0.209	0.209	0.209	0.209	0.209	0.209			
2			0.211	0.211	0.211	0.211	0.211	0.211	0.211			
19			0.211	0.211	0.211	0.211	0.211	0.211	0.211			
31			0.212	0.212	0.212	0.212	0.212	0.212	0.212	0.212		
17				0.213	0.213	0.213	0.213	0.213	0.213	0.213		
28				0.214	0.214	0.214	0.214	0.214	0.214	0.214		
6					0.215	0.215	0.215	0.215	0.215	0.215		
30					0.216	0.216	0.216	0.216	0.216	0.216		
25					0.218	0.218	0.218	0.218	0.218	0.218	0.218	
13					0.218	0.218	0.218	0.218	0.218	0.218	0.218	
15					0.219	0.219	0.219	0.219	0.219	0.219	0.219	
33					0.219	0.219	0.219	0.219	0.219	0.219	0.219	
14						0.228	0.228	0.228	0.228	0.228	0.228	0.228
24							0.229	0.229	0.229	0.229	0.229	0.229
12								0.231	0.231	0.231	0.231	0.231
22								0.231	0.231	0.231	0.231	0.231
18									0.232	0.232	0.232	0.232
7										0.235	0.235	0.235
16											0.239	0.239
10												0.241
9												0.245
Sig.	0.232	0.057	0.064	0.056	0.158	0.09	0.064	0.068	0.075	0.05	0.056	0.225

For *Callopora dumerilii* group 1 is distinct from group 4.

Table 3.16 Student-Newman-Keuls (SNK) post hoc test for *Callopora dumerilii*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-18 are from Pembroke Ferry, colonies 19-35 are from Lydstep and colonies 36-39 are from Watwick Bay.

COLONY ID	1	2	3	4
12	-0.751			
26	-0.75			
33	-0.743			
18	-0.743			
6	-0.738			
1	-0.735			
7	-0.735			
27	-0.735			
4	-0.733			
8	-0.733			
32	-0.726	-0.726		
14	-0.725	-0.725		
17	-0.724	-0.724		
23	-0.722	-0.722		
29	-0.72	-0.72		
2	-0.719	-0.719		
25	-0.718	-0.718		
34	-0.718	-0.718		
9	-0.717	-0.717		
3	-0.716	-0.716		
24	-0.716	-0.716		
5	-0.716	-0.716		
13	-0.714	-0.714		
37	-0.714	-0.714		
10	-0.714	-0.714		
11	-0.713	-0.713		
36	-0.711	-0.711		
31	-0.708	-0.708		
35	-0.707	-0.707		
28	-0.704	-0.704	-0.704	
16	-0.703	-0.703	-0.703	
19	-0.703	-0.703	-0.703	
30	-0.695	-0.695	-0.695	
39	-0.692	-0.692	-0.692	
21		-0.67	-0.67	
20		-0.668	-0.668	
38			-0.653	
22				-0.605
Sig.	0.141	0.097	0.052	1

The non-parametric Kruskal-Wallis test was performed by comparing colonies within each site separately for *Callopora rylandi*. It was not performed for the other two species, as the P value in the Levene test was higher than 0.05. The results are shown in Table 3.17.

Table 3.17 Kruskal-Wallis test for *Callopora rylandi.*

Species name	Source	Chi-Square	df	P
Callopora rylandi	Lydstep	60.537	9	0.000
Callopora rylandi	Watwick Bay	111.549	16	0.000

Significant differences were observed for all sites where *Callopora rylandi* were sampled. This result fits in with the observed colony means in Figure 3.5B, which are quite closely similar to each other in value. In general the results of the Kruskal-Wallis tests are consistent with the ANOVA results in showing significant differences between colonies.

3.5.2.3 Ovicell Length (OL)

Values for OL were checked for homogeneity of variance using the Levene test (Table 3.18).

Table 3.18 Levene test for homogeneity of variance.

	Species name	F	df1	df2	P
SITE	*Callopora rylandi*	0.421	1	538	0.517
"	*Callopora lineata*	4.472	1	678	0.034
"	*Callopora dumerilii*	15.729	2	757	0.000
COLONY	*Callopora rylandi*	1.647	26	513	0.024
"	*Callopora lineata*	2.673	33	646	0.000
"	*Callopora dumerilii*	2.299	37	722	0.000

With one exception, *Callopora rylandi* for Site, significant departures from homogeneity of variance were observed when comparing sites and when comparing colonies for each of the three species. As for the zooid characters ANOVA was conducted as well as the non-parametric Kruskal-Wallis test for those sites in which the P value in the three species was less than 0.05. The results of a Nested ANOVA for all three species for OL are shown in Table 3.19.

Table 3.19 Nested ANOVA results for all species for OL variable.

Species Name	Source	SS	df	MS	F	P
Callopora rylandi	Colony	0.199	26	0.008	12.433	0.000
Callopora lineata	"	0.109	33	0.003	8.977	0.000
Callopora dumerilii	"	0.105	37	0.003	8.511	0.000
Callopora rylandi	Site	0.052	1	0.052	8.911	0.006
Callopora lineata	"	0.010	1	0.010	3.250	0.072
Callopora dumerilii	"	0.006	2	0.003	1.112	0.329
Callopora rylandi	Between	0.146	25	0.006	9.532	0.000
Callopora lineata	Colonies	0.098	32	0.003	8.405	0.000
Callopora dumerilii	Within Site	0.099	35	0.003	8.460	0.000
Callopora rylandi	Error	0.315	513	0.001		
Callopora lineata	"	0.237	646	0.0001		
Callopora dumerilii	"	0.241	722	0.0003		

With the exceptions of *Callopora lineata* and *Callopora dumerilii* for Site, there are significant differences both between site and between colonies. The mean values corresponding to the ANOVA results are given in Figure 3.6. The significant result for Site for *Callopora rylandi* fit well with the large differences between means. Because there were three sites for *Callopora dumerilii* and thus three possible comparisons between sites, post hoc comparisons using the Bonferroni procedure were carried out (Table 3.20). Following this procedure, only two comparisons were highly significant; that of Lydstep with Watwick Bay and that of Watwick Bay with Pembroke Ferry.

Figure 3.6 Mean Plots for the ANOVA results with 95% confidence intervals for OL in *Callopora rylandi* for sites (A) and colonies (B) (colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick); in *Callopora lineata* (C and D) (colonies 1-21 are from Lydstep and 22-34 are from Watwick) and in *Callopora dumerilii* (E and F) (colonies 1-18 are from Pembroke Ferry, 19-35 are from Lydstep and 36-39 are from Watwick).

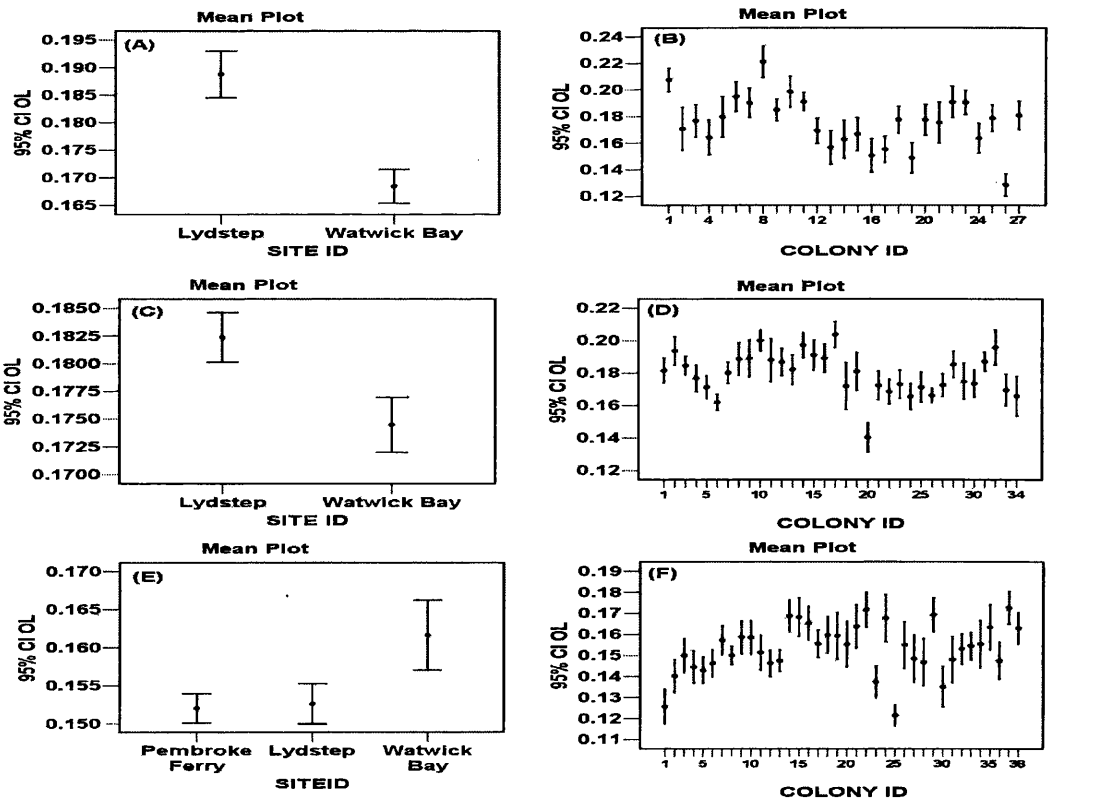


Table 3.20 Post hoc test using Bonferroni procedure for differences between sites for *Callopora dumerilii*.

Comparison		Mean Difference between sites	STD Error	P
Pembroke Ferry	Lydstep	-0.001	0.002	1.000
Lydstep	Watwick Bay	-0.009	0.003	0.002
Watwick Bay	Pembroke Ferry	0.010	0.003	0.001

Further analysis was done on the colony means by carrying out the SNK test. The results for *Callopora rylandi* are shown in Table 3.21.

Table 3.21 Student-Newman-Keuls (SNK) post hoc test for *Callopora rylandi*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10
26	0.129									
19		0.149								
16		0.151	0.151							
17		0.155	0.155	0.155						
13		0.157	0.157	0.157						
14		0.163	0.163	0.163	0.163					
24		0.164	0.164	0.164	0.164					
4		0.164	0.164	0.164	0.164	0.164				
15		0.167	0.167	0.167	0.167	0.167				
12		0.169	0.169	0.169	0.169	0.169	0.169			
2		0.171	0.171	0.171	0.171	0.171	0.171			
21			0.175	0.175	0.175	0.175	0.175	0.175		
3				0.177	0.177	0.177	0.177	0.177		
20				0.177	0.177	0.177	0.177	0.177		
18				0.178	0.178	0.178	0.178	0.178		
25				0.179	0.179	0.179	0.179	0.179		
5				0.18	0.18	0.18	0.18	0.18		
27				0.181	0.181	0.181	0.181	0.181		
9					0.185	0.185	0.185	0.185	0.185	
7						0.19	0.19	0.19	0.19	
23						0.19	0.19	0.19	0.19	
22						0.191	0.191	0.191	0.191	
11						0.191	0.191	0.191	0.191	
6							0.195	0.195	0.195	
10								0.198	0.198	
1									0.207	0.207
8										0.221
Sig.	1	0.14	0.056	0.076	0.22	0.052	0.072	0.152	0.078	0.083

Most of the groups overlap and share colonies. However group 1 is distinct from the other groups, group 2 is distinct from groups 8-10, groups 3-4 from groups 9-10 and groups 5-8 from group 10.

In the same manner, the SNK test was also carried out *Callopora lineata* (Table 3.22) and *Callopora dumerilii* (Table 3.23).

Table 3.22 Student-Newman-Keuls (SNK) post hoc test for *Callopora lineata*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-21 are from Lydstep and colonies 22-34 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10	11
20	0.14										
6		0.162									
24		0.165	0.165								
34		0.166	0.166								
26		0.166	0.166	0.166							
22		0.169	0.169	0.169	0.169						
33		0.17	0.17	0.17	0.17	0.17					
5		0.171	0.171	0.171	0.171	0.171					
25		0.171	0.171	0.171	0.171	0.171					
18		0.172	0.172	0.172	0.172	0.172	0.172				
21		0.172	0.172	0.172	0.172	0.172	0.172				
27		0.172	0.172	0.172	0.172	0.172	0.172				
23		0.173	0.173	0.173	0.173	0.173	0.173				
30		0.174	0.174	0.174	0.174	0.174	0.174				
29		0.175	0.175	0.175	0.175	0.175	0.175	0.175			
4		0.177	0.177	0.177	0.177	0.177	0.177	0.177	0.177		
7		0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	
19		0.181	0.181	0.181	0.181	0.181	0.181	0.181	0.181	0.181	
1		0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.182	
13		0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.182	0.182	
3			0.185	0.185	0.185	0.185	0.185	0.185	0.185	0.185	0.185
28			0.185	0.185	0.185	0.185	0.185	0.185	0.185	0.185	0.185
12			0.187	0.187	0.187	0.187	0.187	0.187	0.187	0.187	0.187
31			0.187	0.187	0.187	0.187	0.187	0.187	0.187	0.187	0.187
11				0.188	0.188	0.188	0.188	0.188	0.188	0.188	0.188
8					0.189	0.189	0.189	0.189	0.189	0.189	0.189
9					0.189	0.189	0.189	0.189	0.189	0.189	0.189
16					0.189	0.189	0.189	0.189	0.189	0.189	0.189
15						0.191	0.191	0.191	0.191	0.191	0.191
2							0.194	0.194	0.194	0.194	0.194
32								0.196	0.196	0.196	0.196
14									0.197	0.197	0.197
10										0.2	0.2
17											0.204
Sig.	1	0.09	0.057	0.05	0.102	0.07	0.054	0.053	0.078	0.093	0.097

For *Callopora lineata* group 1 is distinct from the other groups and group 2 is distinct from group 11.

Table 3.23 Student-Newman-Keuls (SNK) post hoc test for *Callopora dumerilii*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-18 are from Pembroke Ferry, colonies 19-35 are from Lydstep and colonies 36-39 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10	11	12	13
26	0.121												
1	0.126	0.126											
31		0.135	0.135										
24		0.137	0.137	0.137									
2		0.14	0.14	0.14	0.14								
5			0.143	0.143	0.143	0.143							
4			0.145	0.145	0.145	0.145	0.145						
12			0.146	0.146	0.146	0.146	0.146						
6			0.146	0.146	0.146	0.146	0.146						
29			0.147	0.147	0.147	0.147	0.147	0.147					
37			0.147	0.147	0.147	0.147	0.147	0.147	0.147				
13			0.147	0.147	0.147	0.147	0.147	0.147	0.147				
32			0.148	0.148	0.148	0.148	0.148	0.148	0.148	0.148			
28			0.148	0.148	0.148	0.148	0.148	0.148	0.148	0.148	0.148		
3			0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15		
8			0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15		
11			0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151	
33			0.153	0.153	0.153	0.153	0.153	0.153	0.153	0.153	0.153	0.153	0.153
34			0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155
27			0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155
21			0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155
35			0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155	0.155
18			0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156	0.156
7				0.157	0.157	0.157	0.157	0.157	0.157	0.157	0.157	0.157	0.157
10					0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159
9					0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159
20					0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159
19					0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
39						0.163	0.163	0.163	0.163	0.163	0.163	0.163	0.163
36						0.163	0.163	0.163	0.163	0.163	0.163	0.163	0.163
22						0.164	0.164	0.164	0.164	0.164	0.164	0.164	0.164
17							0.165	0.165	0.165	0.165	0.165	0.165	0.165
25								0.168	0.168	0.168	0.168	0.168	0.168
16									0.168	0.168	0.168	0.168	0.168
14										0.169	0.169	0.169	0.169
30											0.169	0.169	0.169
23												0.172	0.172
38													0.173
Sig.	0.467	0.059	0.058	0.082	0.112	0.064	0.07	0.055	0.064	0.055	0.054	0.062	0.092

For *Callopora dumerilii* group 1 is distinct from groups 3-13 and group 2 from groups 6-13.

The non-parametric Kruskal-Wallis test was performed by comparing colonies within each site separately for *Callopora dumerilii* and *Callopora lineata*. The results are shown in Table 3.24.

Table 3.24 Kruskal-Wallis test for *Callopora dumerilii* and *Callopora lineata.*

Species name	Source	Chi-Square	df	P
Callopora dumerilii	Lydstep	92.756	15	0.000
Callopora lineata	"	135.978	20	0.000
Callopora dumerilii	Watwick Bay	151.106	3	0.002
Callopora lineata	"	48.621	12	0.000
Callopora dumerilii	Pembroke Ferry	109.578	17	0.000

Significant differences were observed for all sites where the species were sampled. In general the results of the Kruskal-Wallis tests are consistent with the ANOVA results in showing significant differences between colonies.

3.5.2.4 Ovicell Width (OW)

Finally, values for OW were checked for homogeneity of variance using the Levene test (Table 3.25).

Table 3.25 Levene test for homogeneity of variance.

	Species name	F	df1	df2	P
SITE	*Callopora rylandi*	0.129	1	538	0.719
"	*Callopora lineata*	0.829	1	678	0.363
"	*Callopora dumerilii*	23.142	2	757	0.044
COLONY	*Callopora rylandi*	2.461	26	513	0.000
"	*Callopora lineata*	1.246	33	646	0.165
"	*Callopora dumerilii*	2.323	37	722	0.000

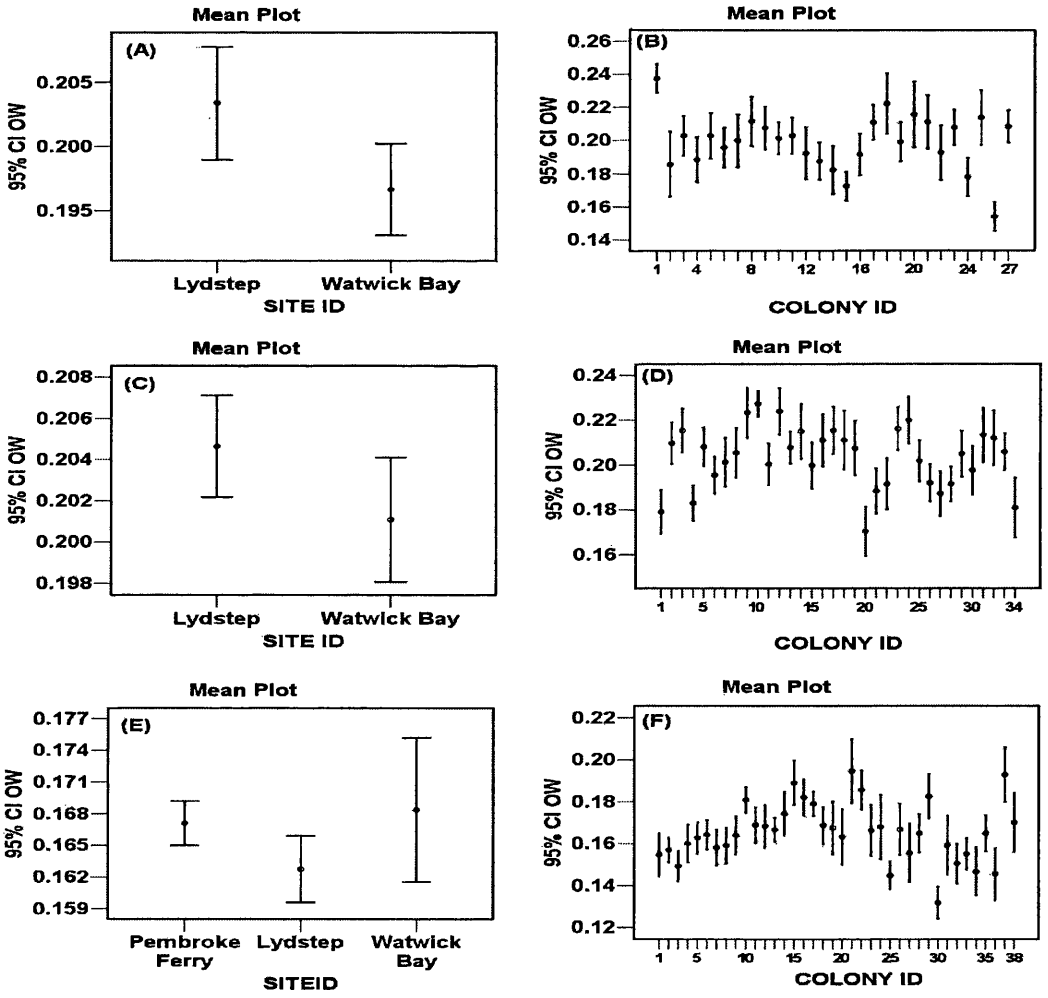
Similarly, with the exceptions of *Callopora rylandi* and *Callopora lineata*, significant departures from homogeneity of variance were observed when comparing sites and when comparing colonies for each of the three species. In the same manner, it was decided to pursue ANOVA but also to carry out the non-parametric Kruskal-Wallis test for those sites that had P values less than 0.05. The results of a Nested ANOVA for all three species for OW are shown in Table 3.26.

Table 3.26 Nested ANOVA results for all species for OW variable.

Species Name	Source	SS	df	MS	F	P
Callopora rylandi	Colony	0.146	26	0.006	6.654	0.000
Callopora lineata	"	0.126	33	0.004	7.892	0.000
Callopora dumerilii	"	0.141	37	0.004	7.107	0.000
Callopora rylandi	Site	0.006	1	0.006	1.014	0.324
Callopora lineata	"	0.002	1	0.002	0.529	0.467
Callopora dumerilii	"	0.004	2	0.002	0.510	0.601
Callopora rylandi	Between	0.140	25	0.006	6.655	0.000
Callopora lineata	Colonies	0.123	32	0.004	8.001	0.000
Callopora dumerilii	Within	0.137	35	0.004	8.159	0.000
	Site					
Callopora rylandi	Error	0.432	513	0.001		
Callopora lineata	"	0.311	646	0.001		
Callopora dumerilii	"	0.347	722	0.001		

It appears that for ovicell width, there are no significant differences between sites for any of the three species for Site, whereas there are significant differences for all three species between colonies. The mean values corresponding to the ANOVA results are given in Figure 3.7. Post hoc comparisons for *Callopora dumerilii* using the Bonferroni procedure were not carried out as the ANOVA results for this species was not significant.

Figure 3.7 Mean Plots for the ANOVA results with 95% confidence intervals for OW in *Callopora rylandi* for sites (A) and colonies (B) (colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick); in *Callopora lineata* (C and D) (colonies 1-21 are from Lydstep and 22-34 are from Watwick) and in *Callopora dumerilii* (E and F) (colonies 1-18 are from Pembroke Ferry, 19-35 are from Lydstep and 36-39 are from Watwick).



Further analysis was done on the colony means by carrying out the Student-Newman-Keuls (SNK) test. The results for *Callopora rylandi* are shown in Table 3.27. Most of the groups overlap and share colonies. However group 1 is distinct from the other groups and groups 2-3 are distinct from group 7.

Table 3.27 Student-Newman-Keuls (SNK) post hoc test for *Callopora rylandi.* Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7
26	0.154						
15		0.172					
24		0.178	0.178				
14		0.182	0.182	0.182			
2		0.186	0.186	0.186	0.186		
13		0.188	0.188	0.188	0.188		
4		0.188	0.188	0.188	0.188		
16		0.192	0.192	0.192	0.192	0.192	
12		0.192	0.192	0.192	0.192	0.192	
22		0.193	0.193	0.193	0.193	0.193	
6		0.196	0.196	0.196	0.196	0.196	
19		0.199	0.199	0.199	0.199	0.199	
7		0.2	0.2	0.2	0.2	0.2	
10		0.201	0.201	0.201	0.201	0.201	
3		0.203	0.203	0.203	0.203	0.203	
11		0.203	0.203	0.203	0.203	0.203	
5		0.203	0.203	0.203	0.203	0.203	
9			0.208	0.208	0.208	0.208	
23			0.208	0.208	0.208	0.208	
27			0.208	0.208	0.208	0.208	
17				0.211	0.211	0.211	0.211
21				0.211	0.211	0.211	0.211
8				0.212	0.212	0.212	0.212
25				0.214	0.214	0.214	0.214
20					0.216	0.216	0.216
18						0.222	0.222
1							0.237
Sig.	1	0.072	0.086	0.079	0.127	0.092	0.06

In the same manner, the SNK test was also carried out in a similar way for *Callopora lineata* (Table 3.28) and *Callopora dumerilii* (Table 3.29).

Table 3.28 Student-Newman-Keuls (SNK) post hoc test for *Callopora lineata*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-21 are from Lydstep and colonies 22-34 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8
20	0.17							
1	0.179	0.179						
34	0.181	0.181						
4	0.183	0.183	0.183					
27	0.187	0.187	0.187	0.187				
21	0.188	0.188	0.188	0.188				
28	0.191	0.191	0.191	0.191	0.191			
22	0.191	0.191	0.191	0.191	0.191			
26	0.192	0.192	0.192	0.192	0.192			
6		0.195	0.195	0.195	0.195	0.195		
30		0.198	0.198	0.198	0.198	0.198		
15		0.2	0.2	0.2	0.2	0.2	0.2	
11		0.2	0.2	0.2	0.2	0.2	0.2	
7		0.201	0.201	0.201	0.201	0.201	0.201	
25		0.202	0.202	0.202	0.202	0.202	0.202	
29			0.205	0.205	0.205	0.205	0.205	0.205
8			0.205	0.205	0.205	0.205	0.205	0.205
33			0.206	0.206	0.206	0.206	0.206	0.206
19				0.207	0.207	0.207	0.207	0.207
13				0.208	0.208	0.208	0.208	0.208
5				0.208	0.208	0.208	0.208	0.208
2				0.21	0.21	0.21	0.21	0.21
18				0.211	0.211	0.211	0.211	0.211
16				0.211	0.211	0.211	0.211	0.211
32				0.212	0.212	0.212	0.212	0.212
31					0.213	0.213	0.213	0.213
14					0.215	0.215	0.215	0.215
3					0.215	0.215	0.215	0.215
17					0.215	0.215	0.215	0.215
23					0.216	0.216	0.216	0.216
24						0.22	0.22	0.22
9							0.223	0.223
12							0.224	0.224
10								0.227
Sig.	0.05	0.066	0.069	0.051	0.069	0.062	0.079	0.126

For *Callopora lineata* group 1 is distinct from groups 6-8 and group 2 is distinct from group 8 (Table 3.28), whereas, for *Callopora dumerilii* group 1 is distinct from groups 4-10 and group 2 from group 10 (Table 3.29).

Table 3.29 Student-Newman-Keuls (SNK) post hoc test for *Callopora dumerilii*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-18 are from Pembroke Ferry, colonies 19-35 are from Lydstep and colonies 36-39 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10
31	0.132									
26	0.145	0.145								
37	0.146	0.146								
35	0.147	0.147								
3	0.149	0.149	0.149							
33	0.151	0.151	0.151							
1		0.155	0.155	0.155						
34		0.155	0.155	0.155						
28		0.156	0.156	0.156	0.156					
2		0.157	0.157	0.157	0.157	0.157				
7		0.158	0.158	0.158	0.158	0.158	0.158			
8		0.159	0.159	0.159	0.159	0.159	0.159			
32		0.159	0.159	0.159	0.159	0.159	0.159			
4		0.16	0.16	0.16	0.16	0.16	0.16			
5		0.163	0.163	0.163	0.163	0.163	0.163	0.163		
21		0.163	0.163	0.163	0.163	0.163	0.163	0.163		
9		0.164	0.164	0.164	0.164	0.164	0.164	0.164		
6		0.164	0.164	0.164	0.164	0.164	0.164	0.164		
29		0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.165	
36		0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.165	
24		0.166	0.166	0.166	0.166	0.166	0.166	0.166	0.166	
13		0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	
27		0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	
20		0.168	0.168	0.168	0.168	0.168	0.168	0.168	0.168	
25		0.168	0.168	0.168	0.168	0.168	0.168	0.168	0.168	
12		0.168	0.168	0.168	0.168	0.168	0.168	0.168	0.168	
19		0.169	0.169	0.169	0.169	0.169	0.169	0.169	0.169	
11		0.169	0.169	0.169	0.169	0.169	0.169	0.169	0.169	
39		0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	
14			0.174	0.174	0.174	0.174	0.174	0.174	0.174	0.174
18				0.179	0.179	0.179	0.179	0.179	0.179	0.179
10					0.181	0.181	0.181	0.181	0.181	0.181
17						0.182	0.182	0.182	0.182	0.182
30							0.183	0.183	0.183	0.183
23								0.186	0.186	0.186
16									0.189	0.189
38										0.193
22										0.195
Sig.	0.077	0.065	0.062	0.083	0.05	0.053	0.074	0.119	0.054	0.089

The non-parametric Kruskal-Wallis test was performed by comparing colonies within each site separately for *Callopora dumerilii*. The results are shown in Table 3.30.

Table 3.30 Kruskal-Wallis test for *Callopora dumerilii.*

Species name	Source	Chi-Square	df	P
Callopora dumerilii	Lydstep	92.756	15	0.000
Callopora dumerilii	Watwick Bay	15.106	3	0.002
Callopora dumerilii	Pembroke Ferry	109.578	17	0.000

Significant differences were observed for all sites where *Callopora dumerilii* were sampled and this result of the Kruskal-Wallis test is consistent with the ANOVA results in showing significant differences between colonies.

3.5.3 Correlations between morphological variables

The correlations between the four morphological variables were calculated for each species. Examination of the correlation matrix reveals significant values (Table 3.31).

Table 3.31 Pearson correlation coefficients (r) for all morphological characters; Double asterisks denote significance at the 0.01 level (2-tailed). Values in parentheses are the coefficients of determination (r^2).

		ZL	ZW	OL	OW
Callopora rylandi	ZL	1			
Callopora lineata	"				
Callopora dumerilii	"				
Callopora rylandi	ZW	0.374** (0.14)	1		
Callopora lineata	"	0.238** (0.057)			
Callopora dumerilii	"	0.413** (0.171)			
Callopora rylandi	OL	0.196**(0.04)	0.098**(0.01)	1	
Callopora lineata	"	0.103**(0.011)	0.126**(0.016)		
Callopora dumerilii	"	0.190**(0.036)	0.081**(0.007)		
Callopora rylandi	OW	0.175**(0.03)	0.313**(0.01)	0.404**(0.160)	1
Callopora lineata	"	0.075**(0.006)	0.335**(0.112)	0.300**(0.090)	
Callopora dumerilii	"	0.147**(0.022)	0.194**(0.038)	0.218**(0.048)	
		ZL	ZW	OL	OW

The correlation coefficients are positive. This means that high scores on ZL, for example are paired with relatively high scores with ZW, OL and OW and vice versa. In other words, if a zooid scored high on ZL variable, we would predict that the zooid would have scored relatively high on the rest of the variables.

The coefficient of determination (r^2) is a measure of how much of the variability in one variable can be explained by variation in the other. For instance, for the variables

ZW and ZL for *Callopora rylandi*, r^2 =0.14. We would therefore say that about 14% of the variability in zooid width is attributable to differences in zooid length or vice versa. It should be noted that even though the correlation coefficients are statistically significant, their values and the r^2 values are not high. To explore further the sources of variation and correlations between the morphological measurements a multivariate analysis was carried out.

3.5.4 Multivariate exploratory methods

3.5.4.1 Tests of adequacy

Prior to any analysis the data were tested to see whether they were suitable for principal component analysis. Two tests were carried out; the Kaiser-Meyer-Olkin measure of sampling adequacy and the Bartlett's test of Sphericity (Table 3.32).

Table 3.32 Kaiser-Meyer-Olkin Measure of sampling adequacy and Bartlett's Test of Sphericity.

	Kaiser-Meyer-Olkin Measure of Sampling Adequacy	Bartlett's Test of Sphericity		
		χ^2	df	P
Callopora rylandi	0.553	248.416	6	0.000
Callopora lineata	0.557	188.649	6	0.000
Callopora dumerilii	0.567	247.702	6	0.000

The Kaiser-Meyer-Olkin statistic ranges from 0 to 1. For any value over 0.5 it is considered acceptable to proceed with principal component analysis. Values below this would mean that principal component analysis will not be able to account for much of the variability in the data and so is not worth undertaking. Similarly the Bartlett's test of Sphericity output is accepted for further principal component analysis, as the test statistic is significant.

3.5.4.2 Principal component analysis

Since both conditions for factor analysis were fulfilled, a principal component analysis was then carried out. Principal component analysis was performed for all morphological measurements of zooids, including zooid length (ZL), zooid width (ZW), ovicell length (OL) and ovicell width (OW). Data from all sites were used.

Principal Component Analysis produced four principal components or factors. However, only those factors with eigenvalues greater than or very close to 1.0 were considered for the analysis. The eigenvalue is analogous to the variance and is the proportion of the total variation that is explained by the factor. This criterion of using eigenvalues of 1.0 or greater was proposed by Kaiser and is probably the one most widely used. Using this criterion, 2 factors were retained. These two factors explain a total of 70% of variation in *Callopora rylandi*, 65% of variation in *Callopora lineata* and 67% of variation in *Callopora dumerilii* (Table 3.33).

Table 3.33 Principal components and their corresponding eigenvalues.

		Initial Eigenvalues		
Variable		Eigenvalue	% of Variance Explained	Cummulative % explained
PC1	*C. rylandi*	1.784	44.595	44.595
	C. lineata	1.609	40.232	40.232
	C. dumerilii	1.66	41.496	41.496
PC2	*C. rylandi*	1.01	25.243	69.838
	C. lineata	0.998	24.698	64.93
	C. dumerilii	1.011	25.276	66.773
PC3	*C. rylandi*	0.723	18.078	87.916
	C. lineata	0.828	20.695	85.625
	C. dumerilii	0.774	19.36	86.133
PC4	*C. rylandi*	0.484	12.084	100
	C. lineata	0.575	14.375	100
	C. dumerilii	0.555	13.867	100

Factor loadings, which by definition are the correlations between the original variables and the factors and the key to understanding the nature of a particular factor, were examined without orthogonal rotation (Table 3.34). Orthogonal rotation is often used for factors in order to improve the separation of each variable in the factors. Whilst this method is non-compulsory for the analysis, it sometimes greatly improves the way data could be analysed and visualised. Squared factor loadings indicate what percentage of the variance in an original variable was explained by a factor.

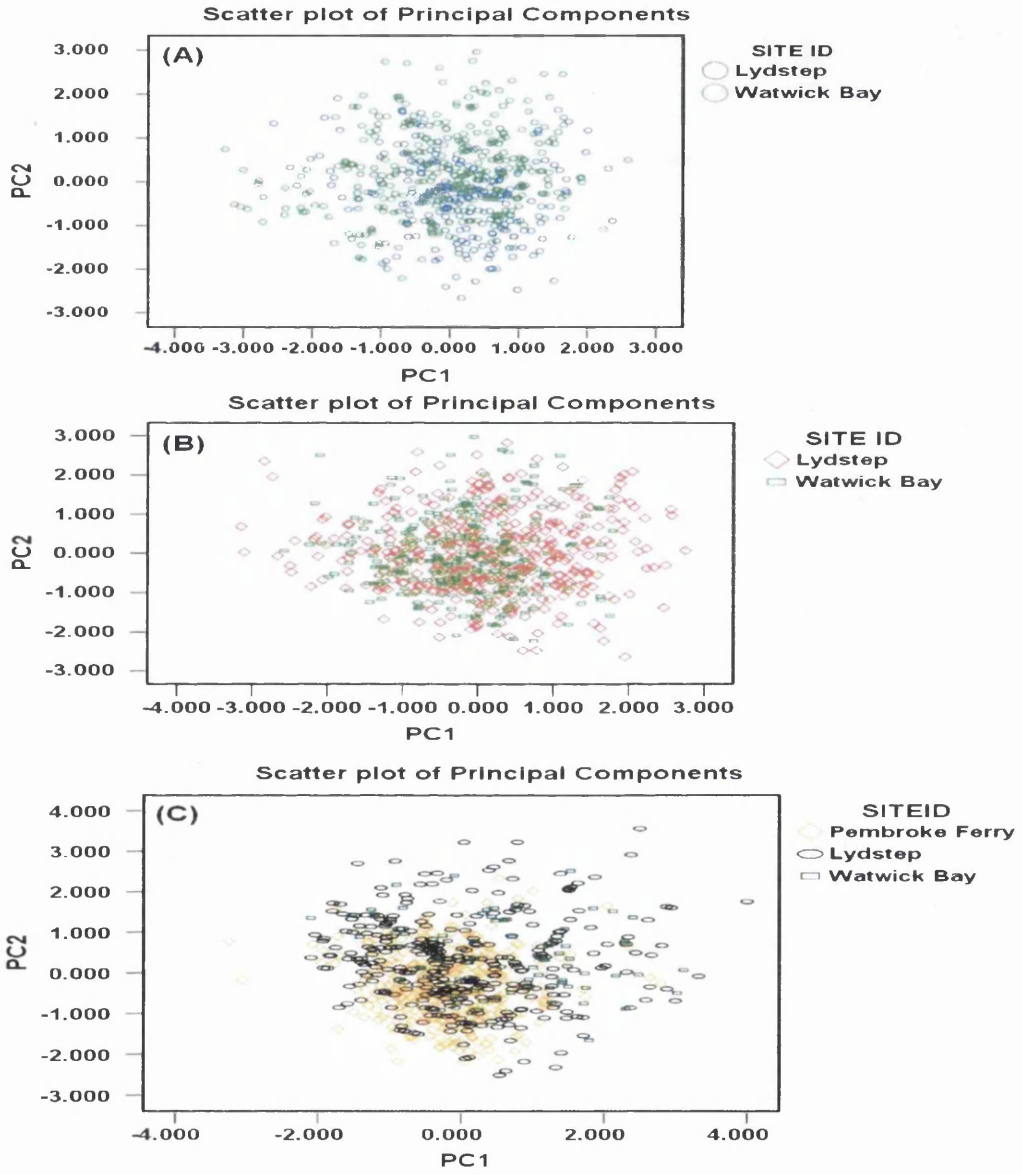
Table 3.34 Factor loadings for all variables for all three species.

	Variables	Factor loading 1	Factor loading 2
Callopora rylandi	ZL	0.640	0.486
Callopora lineata	"	0.468	0.738
Callopora dumerilii	"	0.524	0.641
Callopora rylandi	ZW	0.674	0.508
Callopora lineata	"	0.709	0.284
Callopora dumerilii	"	0.591	0.476
Callopora rylandi	OL	0.621	-0.613
Callopora lineata	"	0.588	-0.488
Callopora dumerilii	"	0.728	-0.387
Callopora rylandi	OW	0.731	-0.373
Callopora lineata	"	0.737	-0.352
Callopora dumerilii	"	0.711	-0.472

The first Factor can be interpreted as defining aspects of overall size of the zooid, whereas the second factor defines aspects of shape. In *Callopora rylandi* for Factor 1 as variables ZL and ZW increase, so do the variables OL and OW. However, for Factor 2 when ZL and ZW increase, then OL and OW show a relative decrease, because the loadings are different in sign and when ZL and ZW decrease then OL and OW show a relative increase. The same pattern was observed for *Callopora lineata* and *Callopora dumerilii*.

The two factor values are plotted against each other and categorized by site in Figure 3.8)

Figure 3.8 Scatter plots of principal components. The scores are colour coded for each site. (A) *Callopora rylandi* scatter plot; (B) *Callopora lineata* scatter plot; (C) *Callopora dumerilii* scatter plot.



There was a noticeable separation in all sites. The unequal number of plotted symbols however, is due to the fact that sites were represented by unequal number of colonies.

3.5.4.3 Analysis of variance of Factors

3.5.4.3.1 Analysis of variance for Factor 1

The analysis of Factor 1 represents an analysis of overall size of zooids and ovicells. Prior to analysis the values for Factor 1 were checked for homogeneity of variance using the Levene test (Table 3.35).

Table 3.35 Levene test for homogeneity of variance.

	Species name	F	df1	df2	P
SITE ID	*Callopora rylandi*	8.864	1	538	0.003
	Callopora lineata	11.187	1	678	0.009
	Callopora dumerilii	26.168	2	757	0.000
COLONY ID	*Callopora rylandi*	2.134	26	513	0.001
	Callopora lineata	2.266	33	646	0.000
	Callopora dumerilii	3.222	37	722	0.000

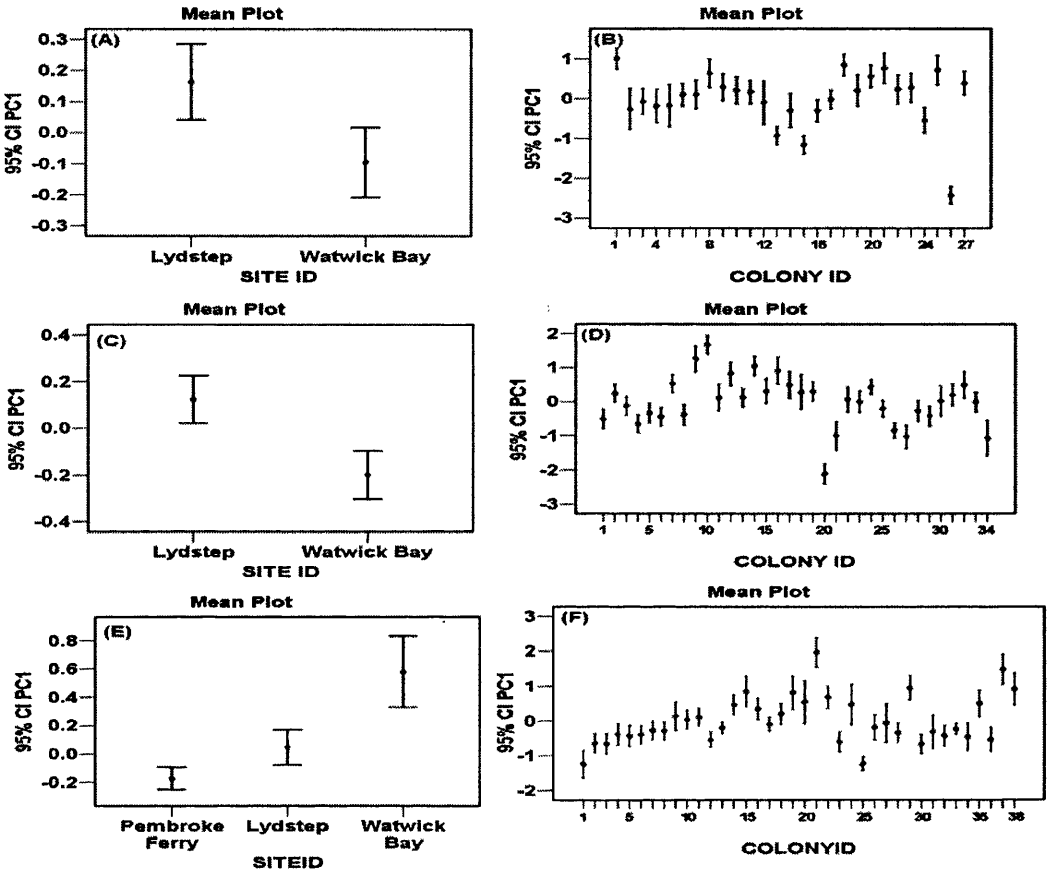
Significant departures from homogeneity of variance were observed when comparing sites and when comparing colonies for each of the three species. As in previous analyses non-parametric Kruskal-Wallis test for those sites with P value less than 0.05 was used as well as ANOVA. The results of a Nested ANOVA for all three species for Factor 1 are shown in Table 3.36.

Table 3.36 Nested ANOVA results for all species for Factor 1.

Species Name	Source	SS	df	MS	F	P
Callopora rylandi	Colony	254.533	26	9.789	17.655	0.000
Callopora lineata	"	361.377	33	10.951	22.272	0.000
Callopora dumerilii	"	348.510	37	9.419	16.567	0.000
Callopora rylandi	Site	37.909	1	37.909	4.375	0.037
Callopora lineata	"	16.737	1	16.737	1.554	0.213
Callopora dumerilii	"	38.261	2	19.130	2.158	0.116
Callopora rylandi	Between	216.624	25	8.665	15.626	0.000
Callopora lineata	Colonies	344.639	32	10.769	21.904	0.000
Callopora dumerilii	Within site	310.249	35	8.864	15.591	0.000
Callopora rylandi	Error	284.467	513	0.555		
Callopora lineata	"	317.623	646	0.491		
Callopora dumerilii	"	410.489	722	0.569		

With the exceptions of *Callopora lineata* and *Callopora dumerilii* for Site, there are significant differences both between sites and between colonies. The mean values corresponding to the ANOVA results are given in Figure 3.9. Post hoc comparisons for *Callopora dumerilii* were not performed as the ANOVA gave not significant results when comparing sites.

Figure 3.9 Mean Plots for the ANOVA results with 95% confidence intervals for Factor 1 in all three species; (A), (B) *Callopora rylandi* mean plots (colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick); (C), (D) *Callopora lineata* mean plots (colonies 1-21 are from Lydstep and 22-34 are from Watwick); (E), (F) *Callopora dumerilii* mean plots (colonies 1-18 are from Pembroke Ferry, 19-35 are from Lydstep and 36-39 are from Watwick).



Further analysis was done on the colony means by carrying out the Student-Newman-Keuls (SNK) test. The results for *Callopora rylandi* are shown in Table 3.37 Most of the groups overlap and share colonies. However group 1 is distinct from the other groups, group 2 is distinct from groups 4-11, group 3 is distinct from groups 5-11 and group 4 is distinct from group 11.

Table 3.37 Student-Newman-Keuls (SNK) post hoc test for *Callopora rylandi.* Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10	11
26	-2.427										
15		-1.159									
13		-0.929	-0.929								
24			-0.541	-0.541							
16				-0.303	-0.303						
14				-0.297	-0.297						
2				-0.265	-0.265						
4				-0.185	-0.185	-0.185					
5				-0.173	-0.173	-0.173					
12				-0.096	-0.096	-0.096	-0.096				
3				-0.074	-0.074	-0.074	-0.074	-0.074			
17				-0.022	-0.022	-0.022	-0.022	-0.022	-0.022		
6				0.094	0.094	0.094	0.094	0.094	0.094	0.094	
7				0.105	0.105	0.105	0.105	0.105	0.105	0.105	
11				0.166	0.166	0.166	0.166	0.166	0.166	0.166	
19				0.198	0.198	0.198	0.198	0.198	0.198	0.198	
10				0.209	0.209	0.209	0.209	0.209	0.209	0.209	
22				0.24	0.24	0.24	0.24	0.24	0.24	0.24	
23					0.276	0.276	0.276	0.276	0.276	0.276	0.276
9					0.287	0.287	0.287	0.287	0.287	0.287	0.287
27					0.389	0.389	0.389	0.389	0.389	0.389	0.389
20						0.557	0.557	0.557	0.557	0.557	0.557
8							0.634	0.634	0.634	0.634	0.634
25								0.71	0.71	0.71	0.71
21									0.761	0.761	0.761
18										0.843	0.843
1											1.002
Sig.	1	0.327	0.1	0.066	0.218	0.106	0.109	0.056	0.058	0.087	0.055

The SNK test was also carried out in a similar way for *Callopora lineata* (Table 3.38) and *Callopora dumerilii* (Table 3.39). For *Callopora lineata*, group 1 is distinct from groups 2-14, groups 2-3 are distinct from groups 8-14, groups 4-5 are distinct from groups 10-14, groups 6-7 are distinct from groups 12-14, groups 8-9 are distinct from groups 13-14 and groups 10-12 are distinct from group 14.

Table 3.38 Student-Newman-Keuls (SNK) post hoc test for *Callopora lineata*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-21 are from Lydstep and colonies 22-34 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
20	-2.102													
34		-1.058												
27		-1.019												
21		-0.997	-0.997											
26		-0.842	-0.842	-0.842										
4		-0.651	-0.651	-0.651	-0.651									
1		-0.512	-0.512	-0.512	-0.512	-0.512								
6		-0.446	-0.446	-0.446	-0.446	-0.446	-0.446							
29		-0.413	-0.413	-0.413	-0.413	-0.413	-0.413							
8		-0.383	-0.383	-0.383	-0.383	-0.383	-0.383							
5			-0.327	-0.327	-0.327	-0.327	-0.327							
28				-0.272	-0.272	-0.272	-0.272	-0.272						
25				-0.203	-0.203	-0.203	-0.203	-0.203	-0.203					
3					-0.119	-0.119	-0.119	-0.119	-0.119					
33					-0.009	-0.009	-0.009	-0.009	-0.009					
23					0.001	0.001	0.001	0.001	0.001					
30					0.02	0.02	0.02	0.02	0.02					
22					0.069	0.069	0.069	0.069	0.069					
11						0.122	0.122	0.122	0.122	0.122				
13						0.127	0.127	0.127	0.127	0.127				
31						0.199	0.199	0.199	0.199	0.199	0.199			
2						0.248	0.248	0.248	0.248	0.248	0.248			
18							0.282	0.282	0.282	0.282	0.282			
19							0.302	0.302	0.302	0.302	0.302			
15							0.308	0.308	0.308	0.308	0.308			
24								0.44	0.44	0.44	0.44	0.44		
17								0.494	0.494	0.494	0.494	0.494		
32								0.495	0.495	0.495	0.495	0.495		
7									0.533	0.533	0.533	0.533		
12										0.82	0.82	0.82	0.82	
16											0.913	0.913	0.913	
14												1.053	1.053	
9													1.255	1.255
10														1.672
Sig.	1	0.061	0.053	0.095	0.064	0.053	0.069	0.052	0.08	0.074	0.052	0.085	0.204	0.06

For *Callopora dumerilii* group 1 is distinct from groups 3-11 and groups 2-10 are distinct from group 11 (Table 3.39).

Table 3.39 Student-Newman-Keuls (SNK) post hoc test for *Callopora dumerilii*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-18 are from Pembroke Ferry, colonies 19-35 are from Lydstep and colonies 36-39 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10	11
1	-1.244										
26	-1.239										
31	-0.677	-0.677									
3	-0.655	-0.655									
2	-0.641	-0.641									
24	-0.609	-0.609									
12	-0.555	-0.555									
37	-0.543	-0.543									
35		-0.468	-0.468								
33		-0.442	-0.442								
5		-0.427	-0.427								
6		-0.395	-0.395								
4		-0.388	-0.388								
29		-0.355	-0.355	-0.355							
32		-0.32	-0.32	-0.32	-0.32						
8		-0.285	-0.285	-0.285	-0.285						
7		-0.276	-0.276	-0.276	-0.276						
34		-0.237	-0.237	-0.237	-0.237	-0.237					
13		-0.206	-0.206	-0.206	-0.206	-0.206					
27		-0.191	-0.191	-0.191	-0.191	-0.191					
18		-0.101	-0.101	-0.101	-0.101	-0.101	-0.101				
28		-0.071	-0.071	-0.071	-0.071	-0.071	-0.071				
10		0.031	0.031	0.031	0.031	0.031	0.031				
11		0.106	0.106	0.106	0.106	0.106	0.106	0.106			
9		0.131	0.131	0.131	0.131	0.131	0.131	0.131			
19		0.189	0.189	0.189	0.189	0.189	0.189	0.189	0.189		
17			0.337	0.337	0.337	0.337	0.337	0.337	0.337		
14				0.455	0.455	0.455	0.455	0.455	0.455		
25				0.46	0.46	0.46	0.46	0.46	0.46		
36					0.492	0.492	0.492	0.492	0.492		
21						0.538	0.538	0.538	0.538		
23							0.673	0.673	0.673		
20								0.804	0.804		
16								0.84	0.84		
39									0.905		
30									0.934		
38										1.468	
22											1.962
Sig.	0.066	0.053	0.08	0.053	0.056	0.071	0.055	0.078	0.068	1	1

The non-parametric Kruskal-Wallis test was performed by comparing colonies within each site separately for each species. The results are shown in Table 3.40.

Table 3.40 Kruskal-Wallis test for all three sites for all three species.

Species name	Source	Chi-Square	df	P
Callopora rylandi	Lydstep	41.635	9	0.000
Callopora lineata	"	106.671	20	0.000
Callopora dumerilii	"	143.134	15	0.000
Callopora rylandi	Watwick Bay	171.948	16	0.000
Callopora lineata	"	57.399	12	0.000
Callopora dumerilii	"	34.487	3	0.000
Callopora dumerilii	Pembroke Ferry	125.233	17	0.000

Significant differences were observed for all sites where the species were sampled. This result provides support to the ANOVA results in showing significant differences between colonies.

3.5.4.3.2 Analysis of variance for Factor 2

The analysis of Factor 2 represents an analysis of overall shape reflecting the relative size of zooids and ovicells. Scores for the Factor 2 were checked for homogeneity of variance using the Levene test for homogeneity of variance (Table 3.41).

Table 3.41 Levene test for homogeneity of variance.

	Species name	F	df1	df2	P
SITE ID	*Callopora rylandi*	1.395	1	538	0.238
	Callopora lineata	0.037	1	678	0.847
	Callopora dumerilii	9.678	2	757	0.000
COLONY ID	*Callopora rylandi*	1.627	26	513	0.027
	Callopora lineata	2.635	33	646	0.000
	Callopora dumerilii	1.349	37	722	0.083

Significant departures from homogeneity of variance were observed when comparing only sites in *Callopora dumerilii* and when comparing colonies for *Callopora rylandi* and *Callopora lineata.* ANOVA was conducted as well as the non-parametric Kruskal-Wallis test. The results of a Nested ANOVA for all three species for Factor 2 are shown in Table 3.42.

Table 3.42 Nested ANOVA results for all species for Factor 2.

Species Name	Source	SS	df	MS	F	P
Callopora rylandi	Colony	144.016	26	5.539	7.194	0.000
Callopora lineata	"	162.409	33	4.921	6.154	0.000
Callopora dumerilii	"	246.158	37	6.653	9.366	0.000
Callopora rylandi	Site	37.909	1	37.909	8.932	0.003
Callopora lineata	"	0.044	1	0.044	0.009	0.926
Callopora dumerilii	"	68.992	2	34.496	6.815	0.001
Callopora rylandi	Between	106.107	25	4.244	5.512	0.000
Callopora lineata	Colonies	162.365	32	5.073	6.345	0.000
Callopora dumerilii	Within site	177.166	35	5.062	7.126	0.000
Callopora rylandi	Error	394.984	513	0.769		
Callopora lineata	"	516.591	646	0.709		
Callopora dumerilii	"	512.841	722	0.710		

With the exceptions of *Callopora lineata* for Site, there are significant differences both between sites and between colonies. The mean values corresponding to the ANOVA results are given in Figure 3.10. The similarities between the two sites for *Callopora lineata* is clear but the other species show larger differences between sites. Because there were three sites for *Callopora dumerilii* and thus three possible comparisons between sites, post hoc comparisons using the Bonferroni procedure were carried out (Table 3.43) and significant differences appeared between Pembroke Ferry and Lydstep and between Watwick Bay and Pembroke Ferry.

Figure 3.10 Mean Plots for the ANOVA results with 95% confidence intervals for Factor 2 in all three species; (A), (B) *Callopora rylandi* mean plots (colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick); (C), (D) *Callopora lineata* mean plots (colonies 1-21 are from Lydstep and 22-34 are from Watwick); (E), (F) *Callopora dumerilii* mean plots (colonies 1-18 are from Pembroke Ferry, 19-35 are from Lydstep and 36-39 are from Watwick).

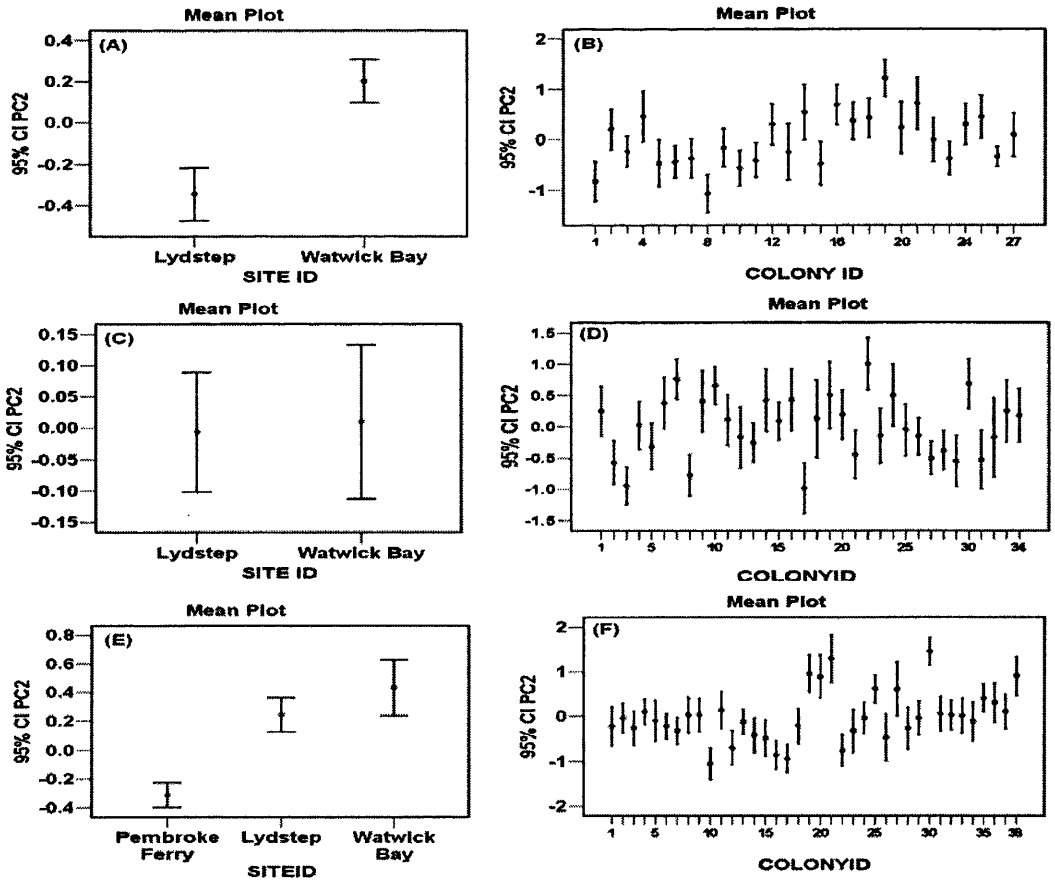


Table 3.43 Post hoc test using Bonferroni procedure for differences between sites for *Callopora dumerilii.*

Comparison		Mean Difference between sites	STD Error	P
Pembroke Ferry	Lydstep	-0.556	0.073	0.000
Lydstep	Watwick Bay	-0.188	0.119	0.347
Watwick Bay	Pembroke Ferry	0.744	0.118	0.000

Further analysis was done on the colony means by carrying out the Student-Newman-Keuls (SNK) test. The results for *Callopora rylandi* are shown in Table 3.44. Most of the groups overlap and share colonies. However groups 1-3 are distinct from group 7.

Table 3.44 Student-Newman-Keuls (SNK) post hoc test for *Callopora rylandi.* Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-10 are from Lydstep and colonies 11-27 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7
8	-1.067						
1	-0.825	-0.825					
10	-0.565	-0.565	-0.565				
15	-0.477	-0.477	-0.477	-0.477			
5	-0.466	-0.466	-0.466	-0.466			
6	-0.435	-0.435	-0.435	-0.435	-0.435		
11	-0.407	-0.407	-0.407	-0.407	-0.407		
23	-0.374	-0.374	-0.374	-0.374	-0.374		
7	-0.369	-0.369	-0.369	-0.369	-0.369		
26	-0.331	-0.331	-0.331	-0.331	-0.331		
13	-0.245	-0.245	-0.245	-0.245	-0.245	-0.245	
3	-0.234	-0.234	-0.234	-0.234	-0.234	-0.234	
9	-0.162	-0.162	-0.162	-0.162	-0.162	-0.162	
22		-0.008	-0.008	-0.008	-0.008	-0.008	
27		0.092	0.092	0.092	0.092	0.092	
2			0.206	0.206	0.206	0.206	
20			0.231	0.231	0.231	0.231	
12			0.299	0.299	0.299	0.299	
24			0.301	0.301	0.301	0.301	
17			0.363	0.363	0.363	0.363	
18				0.424	0.424	0.424	0.424
25				0.443	0.443	0.443	0.443
4				0.461	0.461	0.461	0.461
14					0.536	0.536	0.536
16						0.688	0.688
21						0.709	0.709
19							1.212
Sig.	0.061	0.061	0.082	0.088	0.056	0.051	0.07

The SNK test was also carried out in a similar way for *Callopora lineata* (Table 3.45) and *Callopora dumerilii* (Table 3.46). For *Callopora lineata*, group 1 is distinct from group 8.

Table 3.45 Student-Newman-Keuls (SNK) post hoc test for *Callopora lineata*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-21 are from Lydstep and colonies 22-34 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8
17	-0.985							
3	-0.945	-0.945						
8	-0.776	-0.776	-0.776					
2	-0.57	-0.57	-0.57	-0.57				
29	-0.55	-0.55	-0.55	-0.55				
31	-0.527	-0.527	-0.527	-0.527	-0.527			
27	-0.499	-0.499	-0.499	-0.499	-0.499			
21	-0.443	-0.443	-0.443	-0.443	-0.443			
28	-0.379	-0.379	-0.379	-0.379	-0.379			
5	-0.317	-0.317	-0.317	-0.317	-0.317	-0.317		
13	-0.256	-0.256	-0.256	-0.256	-0.256	-0.256	-0.256	
32	-0.175	-0.175	-0.175	-0.175	-0.175	-0.175	-0.175	
12	-0.172	-0.172	-0.172	-0.172	-0.172	-0.172	-0.172	
26	-0.156	-0.156	-0.156	-0.156	-0.156	-0.156	-0.156	
23	-0.15	-0.15	-0.15	-0.15	-0.15	-0.15	-0.15	
25	-0.054	-0.054	-0.054	-0.054	-0.054	-0.054	-0.054	
4		0.024	0.024	0.024	0.024	0.024	0.024	0.024
15			0.087	0.087	0.087	0.087	0.087	0.087
11			0.109	0.109	0.109	0.109	0.109	0.109
18			0.124	0.124	0.124	0.124	0.124	0.124
34			0.181	0.181	0.181	0.181	0.181	0.181
20			0.191	0.191	0.191	0.191	0.191	0.191
33				0.251	0.251	0.251	0.251	0.251
1				0.251	0.251	0.251	0.251	0.251
6				0.377	0.377	0.377	0.377	0.377
9				0.405	0.405	0.405	0.405	0.405
14				0.416	0.416	0.416	0.416	0.416
16				0.425	0.425	0.425	0.425	0.425
24					0.499	0.499	0.499	0.499
19					0.502	0.502	0.502	0.502
10						0.657	0.657	0.657
30						0.685	0.685	0.685
7							0.762	0.762
22								1.007
Sig.	0.078	0.052	0.077	0.082	0.056	0.066	0.055	0.055

For *Callopora dumerilii* group 1 is distinct from groups 7-10, groups 2-4 are distinct from groups 8-10 and group 5 is distinct from groups 9-10.

Table 3.46 Student-Newman-Keuls (SNK) post hoc test for *Callopora dumerilii*. Group numbers are indicated in the first row with colony membership and means below. The value (Sig.) at the foot of each column gives the P value for the null hypothesis that group means are homogeneous. Colonies 1-19 are from Pembroke Ferry, colonies 20-35 are from Lydstep and colonies 36-39 are from Watwick Bay.

COLONY ID	1	2	3	4	5	6	7	8	9	10
10	-1.054									
18	-0.932	-0.932								
17	-0.854	-0.854	-0.854							
23	-0.759	-0.759	-0.759	-0.759						
12	-0.699	-0.699	-0.699	-0.699						
16	-0.477	-0.477	-0.477	-0.477	-0.477					
27	-0.471	-0.471	-0.471	-0.471	-0.471					
14	-0.424	-0.424	-0.424	-0.424	-0.424					
7	-0.323	-0.323	-0.323	-0.323	-0.323	-0.323				
24	-0.322	-0.322	-0.322	-0.322	-0.322	-0.322				
29	-0.27	-0.27	-0.27	-0.27	-0.27	-0.27				
3	-0.257	-0.257	-0.257	-0.257	-0.257	-0.257				
6	-0.221	-0.221	-0.221	-0.221	-0.221	-0.221				
1	-0.22	-0.22	-0.22	-0.22	-0.22	-0.22				
19	-0.211	-0.211	-0.211	-0.211	-0.211	-0.211				
13		-0.123	-0.123	-0.123	-0.123	-0.123				
35		-0.112	-0.112	-0.112	-0.112	-0.112				
5		-0.095	-0.095	-0.095	-0.095	-0.095				
30		-0.041	-0.041	-0.041	-0.041	-0.041				
25		-0.04	-0.04	-0.04	-0.04	-0.04				
2		-0.037	-0.037	-0.037	-0.037	-0.037				
34		0.015	0.015	0.015	0.015	0.015	0.015			
33		0.025	0.025	0.025	0.025	0.025	0.025			
8		0.03	0.03	0.03	0.03	0.03	0.03			
9		0.031	0.031	0.031	0.031	0.031	0.031			
32			0.051	0.051	0.051	0.051	0.051			
4			0.105	0.105	0.105	0.105	0.105			
38			0.108	0.108	0.108	0.108	0.108			
11				0.14	0.14	0.14	0.14			
37					0.305	0.305	0.305	0.305		
36					0.407	0.407	0.407	0.407		
28						0.611	0.611	0.611	0.611	
26						0.616	0.616	0.616	0.616	
21							0.887	0.887	0.887	0.887
39							0.906	0.906	0.906	0.906
20								0.958	0.958	0.958
22									1.293	1.293
31										1.456
Sig.	0.102	0.057	0.066	0.133	0.154	0.08	0.053	0.18	0.109	0.206

The non-parametric Kruskal-Wallis test was performed by comparing colonies within each site separately for *Callopora dumerilii*. The results are shown in Table 3.47.

Table 3.47 Kruskal-Wallis test for *Callopora dumerilii*.

Species name	Source	Chi-Square	df	P
Callopora dumerilii	Lydstep	105.989	15	0.000
Callopora dumerilii	Watwick Bay	8.084	3	0.044
Callopora dumerilii	Pembroke Ferry	60.676	17	0.000

Significant differences were observed for all sites where *Callopora dumerilii* were sampled. This result is consistent with the ANOVA results in showing significant differences between colonies.

3.6 Discussion

The aim of this chapter was to analyse morphological variation for three *Callopora* populations. To do this, variation was partitioned in each species by separate nested ANOVAs. The levels of variation in each ANOVA were: among sites from which colonies were collected (Sites) and among colonies within sites (Colonies). In addition, a principal component analysis was performed in order to compare individuals of different morphology with respect to size and shape. The present morphometric investigation involving the use of zooid length and width and ovicell length and width showed that between colonies within sites there was a great deal of variation, as indicated by the nested ANOVA, regardless of the species. Analyses conducted between sites, however, showed that there were significant differences for certain morphological characters but not for others, depending on the species.

The morphological characters zooid length and zooid width were significantly different between different sites for *Callopora lineata* and *Callopora dumerilii*, but not for *Callopora rylandi*. However, major differences for these two morphological variables appeared in all three species in between colony within site comparisons. When comparing the ovicell length variable between different sites, noteworthy differences appeared only for *Callopora rylandi*, whereas considerable differences appeared in all three sites for between colonies within site comparisons. On the other hand, non-significant differences appeared for all three species when comparing ovicell width between different sites whereas highly significant differences appeared for between colony within site comparisons. Thus the general conclusion that might be drawn from these analyses is that while colonies show significant differences, sites

tend not to be different. There may however be some differences in this pattern between species.

Examination of the correlation matrix revealed highly significant correlation between the morphological characters. The positive correlation coefficients indicated that an increase in one morphological variable tends to be associated with an increase in the other and vice versa.

When reviewing the results of principal component analysis together with the results from nested ANOVA, the following pattern was revealed. For Factor 1, which defines aspects of the overall size of the zooid, there were significant differences between sites, as well as between colonies within sites for *Callopora rylandi*. This finding was further supported by the results of the non-parametric Kruskal-Wallis test that was performed for different sites. This helps to clarify the contrasting results for between sites for *Callopora rylandi* that were significant for ovicell but not zooid measurements. On the other hand, for *Callopora dumerilii* and *Callopora lineata*, it appeared that there were no significant differences between different sites whereas there were noteworthy differences between different colonies within sites. However, the results of the non-parametric tests showed that there were significant differences between different sites. For Factor 2, which defines aspects of the shape of the organism, there were significant differences between sites as well as between colonies within sites for both *Callopora rylandi* and *Callopora dumerilii*. These findings were supported by the results of the Kruskal-Wallis test. For *Callopora lineata* it emerged that there were no significant differences between sites, but there were important differences between colonies within sites.

The results from these analyses are summarised in table 3.48.

Table 3.48 A summary table of the nested ANOVA analyses for the four morphological measurements ZL, ZW, OL and OW, as well as Factor 1 and Factor 2; (+) sign indicates significant differences and (-) sign not significant differences.

	Variables	Between Sites	Between Colonies Within Site
Callopora rylandi	ZL	-	+
Callopora lineata	"	-	+
Callopora dumerilii	"	+	+
Callopora rylandi	ZW	-	+
Callopora lineata	"	-	+
Callopora dumerilii	"	+	+
Callopora rylandi	OL	+	+
Callopora lineata	"	-	+
Callopora dumerilii	"	-	+
Callopora rylandi	OW	-	+
Callopora lineata	"	-	+
Callopora dumerilii	"	-	+
Callopora rylandi	Factor 1	+	+
Callopora lineata	"	-	+
Callopora dumerilii	"	-	+
Callopora rylandi	Factor 2	+	+
Callopora lineata	"	-	+
Callopora dumerilii	"	+	+

An interesting point that can be made from Table 3.48 is that in *Callopora rylandi*, for example, there are significant differences when comparing size (Factor 1) and shape (Factor 2), between sites, but when comparing individual measurements only OL shows significant differences. This perhaps signifies the need of comparing overall size and shape rather than individual measurements.

For bryozoans, Schopf (1976) suggested that morphological variation within individual colonies, also referred to as individual variation, is due to developmental and micro environmental differences, such as budding of zooids in different parts of the colony at different times under different conditions, since the genotype of a colony

is identical throughout. These differences, under the general heading "temporal micro-environmental differences" contrast with the "spatial micro-environmental differences" such as growth of different parts of the colony that are affected by substratum or other local heterogeneities (Hageman *et al.*, 1999). On the other hand, morphological variation within a population is due to developmental and micro environmental differences, as in the case of individual variation, but also due to genotypic variability in different colonies as well as any environmental differences between colonies in the region that were sampled (Boardman *et al.,* 1970). In these observations one can add morphological variation between different populations which is attributed to all the above factors, plus heterogeneity of the physical environment. However, as analysis of these factors was not an aim in this study, a theoretical approach to explain these results will ensue. The fact that the three sites under investigation in this study were visited and collections made during the summer months could perhaps correlate with previous research work that has investigated morphological variation under certain environmental conditions in various bryozoan species in laboratory based experiments.

Several controlling factors for within-colony variation have been identified (Boardman *et al.*, 1970). These factors, which are ontogeny, astogeny, polymorphism and microenvironment, could be recognised based on their resultant morphological expression and may also represent possible sources of error if not taken into consideration (Key 1987). Concepts of astogenetic variation were not recognised uniformly particularly as they relate to ontogenetic, extrazooidal and polymorphic variation (Pachut *et al.*, 1991). Astogenetic variation occurs mainly in the primary zone of astogenetic change, but may also occur in succeeding zones. Ontogenetic

variation arises due to changes from the growth histories of individual zooids and related extrazooidal material. Ontogenetic variation is very common in bryozoan colonies, since young zooids are generally formed at the distal end of the colonies, whereas older zooids are at the proximal end (Hageman 1995). Zooid polymorphism adds to within colony variation through the repeated occurrence of a small number of different zooid morphs especially in the zone of astogenetic repetition (Key 1987). The distribution of those morphs in the colony may be either uniform or systematic, contributing little to intracolonial variation, or they may be in discrete patches and highly variable (Hageman 1995). This polymorph allocation could be tied to associated zooidal ontogeny, colony astogeny or induced by microenvironmental changes. The final extragenetic constituent of within-colony variation is microenvironmental differentiation. This includes changes in the organism's environment that affect zooid morphology in one or more regions of the colony. Microenvironmental changes include all those aspects that cannot be attributed to astogeny, ontogeny and zooid polymorphism. Examples of microenvironmental changes include recovery from predation, water turbulence or any other environmental perturbation. Microenvironmental factors are mostly important in colonies found in less stable environments (Key 1987).

A possible source of variation can also be attributed to human error while taking morphological measurements. In the present study all morphological characters were taken to 0.001 μm precision; however the actual accuracy of the measurements should be expected to be no more than 0.01 μm, as this was the true resolution of the software used with the camera. In literature there are examples that support the above statement. Schopf (1976) found that errors in accuracy that are caused by operator

variation in making measurements, together with lack of precision, resulted in significant differences in measurements as large as 0.0046mm. This is of particular importance for morphological measurements such as orifice length and width, as well as opesia width measurements, which are on the order of 0.10, 0.13 and 0.23 mm respectively, as the error could be on the order of 2 to 3% of the reported values and may reach 4 to 5% in extreme cases.

In the present study, a few extreme morphological measurements were found, mainly zooid length and zooid width variables. These extreme zooids, which are often referred to as enigmatic monster zooids (Hageman *et al.,* 1999) could be due to zooid arrangement in a colony as well as to the type of substratum in which the colony was located. In some cases, zooids next to ovicells appeared to be altered in their dimensions. This was particularly true for small colonies that were constricted at the edges of stones. In other colonies of the same species from the same locality, such zooids showed no change in their dimensions due to the adjacent ovicells, therefore, the presence or absence of ovicell did not influence zooid dimensions and morphological variation hence was not a physical result of ovicell occurrence. Colonies collected from flat stones appeared to have zooids with regular shape and size, whereas stones with uneven surfaces were likely to have zooids with miscellaneous shapes and sizes.

Zooid sizes in cheilostomes vary considerably among environments. Zooid size is correlated with diet, growth rate and ecological dominance among species and may increase within species with decreasing temperature. Other potential environmental differences could be due to changes in salinity, temperature, light intensity or

duration, sediment accumulation, substrate availability and obstacles (Pachut *et al.*, 1991). In addition, predatory activity, competition for space and self crowding can also affect morphology (Boardman *et al.*, 1983). Ovicell size is linked with clutch size, larval size and larval and post-larval survival which are correlated with ecological attributes associated with zooid size (Jackson and Herrera-Cubilla 1996). However, the knowledge as to what influences the ovicell size (and shape) in cheilostomes is relatively limited. Only thing that is known is that most species of encrusting ascophorines that grow large and dominate substrate have large ovicells, whereas most species that do not grow large have small ovicells. The latter consists of colonising species which are typical of ephemeral substrata or subject to high physical disturbances (such as wave actions). It could also be attributed from effects of grazing from predators (Bishop *et al.*, 1989), or to low food availability as it happens for bryozoans living in caves (Harmelin *et al.*, 1986).

According to many authors, the amount of food and sea temperature are the main factors influencing zooid size. For example, Dzik (1975) suggested that zooid size is directly proportional to the amount of food that was available to "parent" zooids. In the same manner, Sebens (1979) put forward that change in size in colonial suspension feeders are due to the food level availability. Essentially, these two studies considered that less food resulted in more zooids per unit surface area because the possibility of catching food was higher. This would, therefore, yield colonies with smaller zooids. In accordance with these studies was the study of Hunter and Hughes (1994) who proposed that Bryozoa may use temperature in order to predict productivity and therefore achieve the optimum zooid size for the amount of food available. Changes in zooid size could also be attributed to deviations in the energy

that was allocated by individual Bryozoa colonies in order to form new zooids. Characteristic was the study of Jebram (1977) who monitored colony growth rate in laboratory based experiments and suggested that as the rate increased in warm water the zooid size decreased. In addition he added that as gamete production occurred during warmer months, changes in the energy requirements of the colony may result in smaller zooids as well. Similar was the study of Lombardi *et al.* (2006) that explored zooid size variation and growth patterns in the bryozoan *Pentapora fascialis* at different seawater temperatures and demonstrated that zooid length showed greatest sensitivity to temperature fluctuations compared to zooid width and area.

These studies, however, have been challenged by the study of O'Dea and Okamura (1999) that put forward two very important limitations to the above hypotheses. One was that productivity does not parallel temperature in the natural environment and the second that an autozooid will carry on feeding, not only after it has been budded but also after the environment and availability of food changed. To justify their arguments, they looked at the zooid size and colony growth rate of the bryozoan species *Conopeum seurati* in relation to temperature, salinity and food availability at the same time. Their results complied with the principle that zooid size could be used as an indicator of temperature fluctuations while growth rates were dependent on food availability as well as on the combined effect of food availability and temperature. It has also been demonstrated that rising oxygen requirements at high temperatures have an important effect on changes in body size (Atkinson 1994). Since both temperature and salinity have an effect on zooid morphology, it also has a crucial effect on oxygen solubility in water; therefore oxygen limitation may have an effect in zooid size (O'Dea and Okamura 1999).

CHAPTER 4

MOLECULAR TECHNIQUES, PRIMER DESIGN AND TESTING

4.0 MOLECULAR TECHNIQUES, PRIMER DESIGN AND TESTING

4.1 Introduction

The advent of molecular techniques has had a major effect on the way in which questions concerning population genetics can be approached. Since 1966, when Lewontin and Hubby published their classic paper, allozyme electrophoresis has become very widely used in almost all groups of animals and plants and has been applied to almost every sort of problem in population genetics and its branches (Murphy *et al.*, 1996). The development of recombinant DNA techniques in the 1970s, however, gave the ability to look directly at DNA sequences and since then a rapidly increasing number of studies employ genetic data in analysing spatial and temporal patterns of population structure.

The aim of this chapter is to illustrate the techniques that were used in this study in order to obtain the sequence data needed to answer the questions addressed in this thesis. This chapter is divided in two parts. In the first part (Section 4.2) there is emphasis on the background of each technique. In the second part (Section 4.3) the methods are described.

4.2 Background to techniques used

4.2.1 DNA extraction methods

The most favourable method for extracting DNA from a bryozoan species depends on several factors, for example, on the amount of tissue that is available. For many cheilostomes, the principal source of DNA extraction is from larvae. For other bryozoan species, for example those in the genus *Alcyonidium*, DNA extraction can be performed from whole tissue using the method of Fisher and Skibinski (1990). Extraction methods are also selected on the basis of whether mitochondrial (Porter *et al.*, 2001) or nuclear DNA is needed (Waeschenbach 2003).

4.2.2 Polymerase Chain Reaction (PCR)

4.2.2.1 Introduction

The polymerase chain reaction (PCR) (Saiki *et al.*, 1985) is perhaps the most important laboratory technique of molecular biology. Possibly one of the most powerful laboratory techniques ever discovered, PCR is both very sensitive and specific with a great degree of flexibility. With PCR it is possible to specifically use a particular DNA sequence and to amplify this sequence to extremely high copy numbers.

The significance and consequences of the PCR method is similar to the invention of DNA sequencing, the determination of the base sequence by the chain determination process, according to the method of F. Sanger (Sanger *et al.*, 1977). Within a few years, the PCR method had lead to a variety of new techniques which had not been possible previously.

The main contributory factors to the significance of PCR derive principally from its ability to duplicate a DNA fragment indefinitely and to accurately detect very small amounts of a specific DNA target. Because of this, PCR has become an essential instrument in medical, forensic and environmental diagnostics. The high sensitivity and specificity of PCR often enables bacteria or viruses to be detected directly, thus avoiding all problems connected with the cultivation of these organisms. For the first time, non-cultivatable organisms can now be recorded, a process that was extremely limited using the previous methods, such as those involving DNA hybridization.

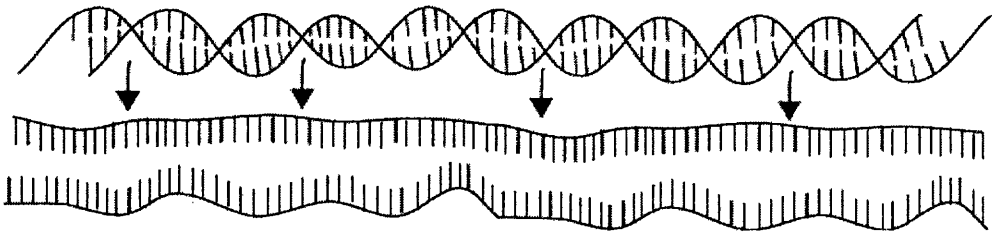
As a method for *in vitro* amplification of DNA, PCR is based on a few simple parameters. Three different reactions combine in a cycle which is repeated 20- 40 times. In theory, the number of copies of the target fragment is doubled after each cycle.

4.2.2.2 Polymerase Chain Reaction: The steps

The basics of the PCR cycling process are described next.

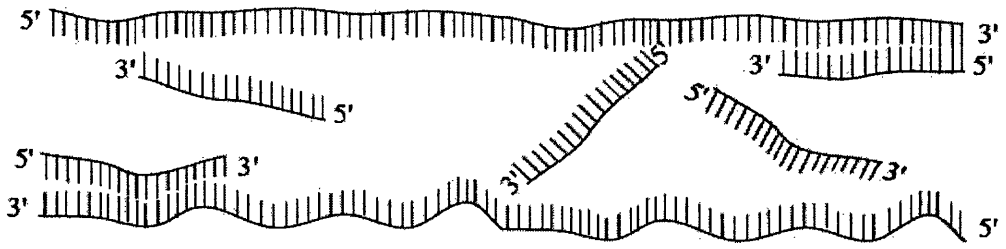
1. DNA Denaturation (Figure 4.1A): The double-strand DNA molecules of the template DNA, which is the target for amplification, are heated until they melt i.e. separated into single strands. This requires temperatures of between 92°C and 96°C.

Figure 4.1A The denaturation step of PCR (Adapted from: http://users.ugent.be/~avierstr/principles/pcr.html).



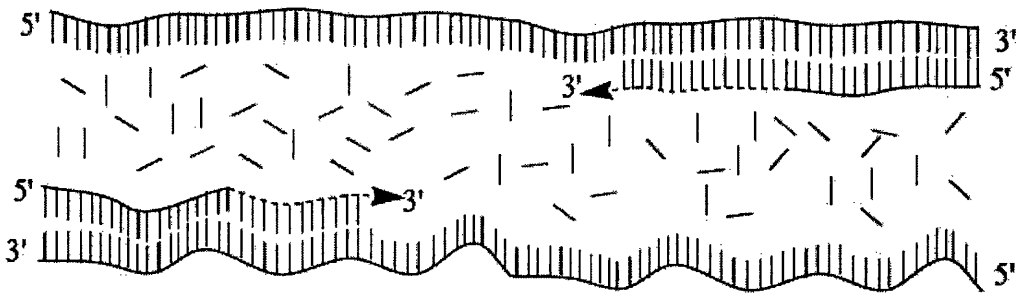
2. Hybridization or annealing of the primer (Figure 4.1B): In this phase, the temperature is lowered to around 45 to 65°C so that oligonucleotide primers added to the reaction can bind to complementary sequences at specific sites in the template DNA. This is the most critical phase, because if primers bind correctly to only the target positions in the template, then there is a good probability that the expected synthesis product will result. It should be noted that primer concentration should be much higher compared to template concentration as depletion of primers would render an unsuccessful or incomplete amplification.

Figure 4.1B The Annealing or hybridisation step of PCR showing primers and target DNA.



3. Extension (Figure 4.1C): This phase allows the enzyme to synthesise new DNA using the target DNA segment as template. The DNA polymerase (e.g. *Taq* polymerase from the *Thermus aquaticus* bacterium) works well at about 72°C and this is the temperature usually chosen for the extension reactions. *Taq* polymerase is, however, not inactivated by the high temperature in the denaturation step. The enzyme is active at lower temperatures, however and this is important for the success of most amplifications. *Taq* polymerase synthesises the complementary strand using free nucleotides, which are also added to the reaction. The primer molecules then become part of the new DNA strands. The end products of the reaction are double-stranded DNA molecules.

Figure 4.1C The extension step of PCR. Dotted line shows extension in the 3′ direction.

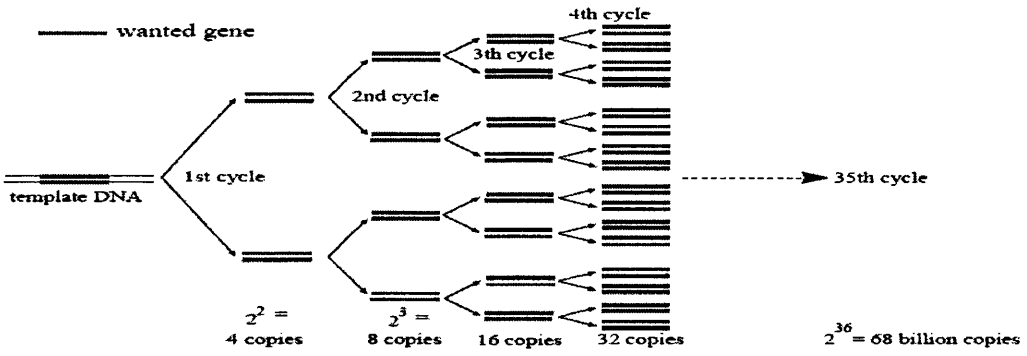


These three steps constitute one cycle of PCR. The number of PCR cycles will depend on the expected yield of the PCR product. Assuming that there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, three cycles will result in 8 copies and so on. In an optimal reaction, less than 10 template molecules can be amplified in less than 40 cycles to give a product that is easily detectable on a gel stained with ethidium bromide. Most PCR reactions should, therefore, include only 25 to 35 cycles. As cycle number increases, nonspecific products can accumulate. These are caused by the primers binding to other positions on the template.

4. Final Extension: Usually, after the last cycle, the reaction tubes are held at 72°C for 5–15 minutes to promote completion of partial extension products and annealing of single-stranded complementary products.

Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. The whole PCR amplification process is summarised in Figure 4.2.

Figure 4.2 The exponential amplification of the gene in PCR (Modified from http://users.ugent.be/~avierstr/principles/pcr.html).



4.2.2.3 PCR optimisation

Depending on the success of the PCR amplification it is necessary to optimise the conditions of the reaction. As there is no single set of conditions that is optimal for all PCR reactions, each PCR requires specific optimisation for the template/primer pairs chosen. This section deals with various factors that may affect the specificity, fidelity and efficiency of PCR. It is important to perform control reactions in parallel with the test samples in order to indicate whether any specificity or contamination problems exist. Two controls are essential; a reaction containing no DNA and one containing no primers.

High specificity in PCR is favoured by:

1. Optimal concentration of Magnesium chloride, buffers, *Taq* DNA polymerase, dNTPs and primers.

The concentration of magnesium ions (Mg^{2+}) is a crucial factor affecting the performance of *Taq* DNA polymerase. Reaction components, including template DNA, dNTPs and proteins, can affect the amount of free magnesium. In the absence of enough free magnesium, *Taq* DNA polymerase is inactive. Conversely, excess free magnesium reduces enzyme fidelity (Eckert and Kunkel 1990) and may increase the level of non-specific amplification (Williams *et al.*, 1989, Ellsworth *et al.*, 1993). For these reasons, it is important to empirically determine the optimal magnesium concentration for each reaction.

Similarly, choosing the right buffer for a particular DNA polymerase and specific application plays a key role in PCR optimisation. Low or no yields are often the result of using the wrong buffer.

However, the choice of a correct enzyme is the most important parameter for a successful PCR reaction. *Taq* polymerase is a thermostable polymerase. It possesses relatively high processivity and is now relatively cheap. *Taq* DNA polymerase generates PCR products with single deoxyadenosine overhangs on the 3′-ends. These overhangs allow easy cloning into "T"-vectors (for example, Promega's pGEM-T Easy Vector) which possess "T" overhangs complementary to those on the PCR product. Enzymes which have 3′-->5′ exonuclease ("proofreading") activity are sometimes recommended to ensure accurate amplification of the PCR product. *Pfu* DNA polymerase is often used instead of, or in conjunction with, *Taq* polymerase. This enzyme is more thermostable and proofreads DNA, resulting in a lower error rate.

Deoxynucleotides (dNTPs) are used as "building blocks" by the DNA polymerase in order to build the new strands. It is important that the four dNTPs are in equimolar concentrations or else the fidelity of PCR can be affected. Ideally, the concentration of dNTPs should be 50-100μM. If it is higher, then the fidelity of the process will be affected because *Taq* polymerase will misincorporate at a higher rate than normal, whereas if it is lower it may affect the efficiency of PCR.

Selecting the appropriate primer is one of the most important steps in designing a PCR experiment. Primers should be designed to hybridise efficiently at the target sites

in the template sequence with as little hybridization as possible to other sequences that are also present in the sample or to other sites in the template. The guidelines for efficient primer design are described in a later section (Section 4.2.10.2).

2. Efficient denaturation and annealing temperatures

It is important that the template is efficiently denatured in order to provide the single-stranded templates for PCR. This is achieved during the initial denaturation phase (usually 5 minutes) when the sample is heated to 92-96°C. This must be adapted for the thermal cycler and tubes being used. For example, longer times are required for denaturation in 500µl tubes than in 200µl tubes. In theory, if the denaturation temperature is too low, then incompletely melted DNA molecules occur, thus giving no access to the primers. A longer denaturation time or higher denaturing temperature is required for GC-rich template DNA.

For most purposes, annealing temperature is optimised empirically. The choice of the primer annealing temperature is the most critical factor in designing a high specificity PCR. If the temperature is too high, no annealing occurs, but if it is too low, non-specific annealing will increase dramatically. Primer-dimers will form more readily at low temperature and if the primers have one or more complementary bases pairing between the two primers can occur. Usually, synthesis of the new DNA strand by elongation of the primer molecules occurs at about 72°C (the temperature optimum of the thermostable DNA polymerase).

3. Cycle Number and length

The number of cycles of PCR should be kept to the minimum required to generate sufficient product for further analysis or manipulation. This reduces the likelihood of errors arising and of non-specific products accumulating. *Taq* DNA polymerase can add approximately 60 bases per second at 72°C. A 45-second extension is sufficient for fragments up to 1 kb. For extension of fragments up to 3 kb, about 45 seconds per kb is desirable. However, these times may need to be adjusted for specific templates.

4. PCR Additives

PCR additives, defined as chemical substances that when added in small amounts increase the yield, specificity and consistency of PCR reactions are also important in PCR optimisation. However, it is impossible to predict which additives would be useful in a particular context. Because of this they are empirically tested for each combination of template and primers under different conditions, such as annealing temperature. Compounds that are known to be useful in PCR reactions include: Dimethyl Sulfoxide (DMSO) up to 10% (Seto 1990), formamide at 5% (Sarkar *et al.*, 1990), BSA (bovine serum albumin) (Taylor *et al.*, 1997).

5. Template Quality

PCR may be inhibited by many compounds present in the biological specimens or reagents used to extract the DNA. For example, detergents are often used for cell lysis and protein denaturation. Extraction protocols often use proteinase K (Section 4.3.1) to digest denatured proteins. It is important to remove or inactivate this protease before PCR, as *Taq* polymerase is susceptible to digestion. Often PCR reactions are

performed on relatively crude DNA preparations that contain inhibitory substances and PCR controls are essential to eliminate the possibility of inhibition. If inhibition is occurring, it is often useful to dilute the DNA sample. This will have the effect of diluting template DNA but it will also dilute the inhibitor.

4.2.2.4 Reamplification and PCR product purification

The best way of obtaining pure PCR product is to optimise reaction conditions to yield only one product; however, there are still circumstances where the resulting product might be of low yield, it may contain a number of secondary bands, or the quality of the PCR product is not good for cloning (Section 4.2.6). One method to circumvent this problem is by reamplifying PCR products. A small aliquot of the product of the first PCR is used as a template in a second PCR experiment, using the same primers and reaction conditions, but with smaller number of cycles and increased annealing temperatures.

Reamplification of PCR products does not always ensure that a single PCR product will be produced, therefore PCR products need to be purified before any other experiments (e.g. cloning, sequencing). The advantages of purifying PCR products include removal of excess primers, nucleotides, buffer components, non-target amplification products and compounds that may inhibit ligation reactions prior to cloning. Purification may also increase the concentration of the product. However, loss of product is also possible since no purification protocol has 100% recovery rate (McPherson and Møller 2000).

4.2.3 Gel electrophoresis

Following PCR, it is usual to identify the products of the reaction by some form of detection system. The most common procedure for this is gel electrophoresis. It provides information on the size of the DNA molecule and under certain conditions can be used to discriminate different sequences of the same size. Gel electrophoresis separates molecules based on physical characteristics such as size, shape, or isoelectric point.

For DNA electrophoresis, the most common media used are polyacrylamide and agarose. These media are very flexible because varying the concentration and for polyacrylamide, the degree of crosslinking, can alter the size range which can be discriminated. Polyacrylamide gels are more useful for separating smaller fragments of DNA (under 300–500 base pairs) (Bartlett 2003) and for applications where high resolution is required (such as analysis of microsatellites) because they are capable of resolving size differences of as little as 1 bp. Polyacrylamide gels can be run faster and at higher temperature than agarose gels. However, liquid polyacrylamide is a neurotoxin and presents a safety hazard. Furthermore, using polyacrylamide gels requires, in general, the use of labelled nucleotides, although silver staining may be applied.

Agarose gels are more robust and easy to prepare. Although resolution is poorer, some modern forms of agarose have separation ability similar to that of polyacrylamide. The major strength of agarose gels is the greater range of separation. Conventional agarose electrophoresis can separate DNAs from 200 to 50,000 base-

pairs. Adaptations of agarose electrophoresis, for example, those using pulsed electric fields, can be used to separate DNA fragments of up to 10 Mbp (Bartlett 2003).

4.2.4 Staining methods

Fluorescence-based detection of nucleic acids is an important part of many molecular biology procedures. The fluorescent stain most commonly used for detecting nucleic acids in electrophoresis gels is ethidium bromide (EtBr), which has the property of fluorescing under UV light when it is intercalated with DNA. Ethidium bromide is reported to have a sensitivity limit of 1ng/band for double-stranded DNA (dsDNA) in agarose gels (Sambrook *et al.*, 1989) and is even less sensitive for detecting single-stranded DNA (ssDNA). Other fluorescent dyes are sometimes used including SYBR Gold. This is more sensitive than ethidium bromide and can be used to quantify DNA. A popular method of visualising DNA is silver staining, which was first developed for proteins (Switzer *et al.*, 1979). Silver staining has now been perfected and extended to the study of other biological molecules including DNA that have been separated by electrophoresis in a variety of supports (Merril 1990; Rabilloud 1990). The procedure is based on photochemically-derived silver stain (Goldman and Merril 1982) in which silver nitrate is the impregnating agent and formaldehyde (in an alkaline environment) is the reducer.

4.2.5 Cloning

The process of molecular cloning involves isolating a DNA sequence of interest and generating multiple copies of it in an organism, usually a bacterium, which is capable of growth over extended periods. Large quantities of the DNA molecule can be then isolated in pure form for detailed molecular analysis. The ability to generate virtually an unlimited number of copies (clones) of a particular sequence is the basis of recombinant DNA technology.

A typical cloning experiment involves the ligation of PCR products into a bacterial vector. Commonly used vector types include plasmids, Lambda phage, cosmids and the yeast and bacterial artificial chromosome (YAC and BAC respectively). Subsequently, the recombinant DNA enters into the host cell by the process of transformation and multiplies. Normal *E. coli* and other host cells find it difficult to take up plasmid DNA from the medium. For this reason, competent cells are used which are cells that have been treated to alter the cell walls so that DNA can pass through easily. Competent cells that have been transformed with plasmid are grown in the presence of X-gal which is used to indicate whether the bacterium expresses the β-galactosidase enzyme, which is encoded by the lacZ gene on the plasmid. X-gal is cleaved by β-galactosidase yielding galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter product is then oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product. Thus, if X-gal and an inducer of β-galactosidase, such as IPTG, are contained within an agar medium on a culture plate, one can easily distinguish which colonies have taken up a functional LacZ gene. Transformant colonies are blue in the case of a vector with no inserted DNA and white in the case of

a vector containing a fragment of cloned DNA which disrupts the lacZ gene and prevents its functioning. This is the basis of the detection system known as "blue-white screen selection". Selected single white colonies are introduced into liquid media containing a specific antibiotic. Following growth of the culture the recombinant plasmid molecule is extracted from the bacterial host cells and can be subject to further investigation such as sequencing of the insert.

4.2.6 Restriction digestion

Plasmids can be inspected for correct inserts by restriction digestion. Restriction enzymes are endodeoxyribonucleases that recognise short, specific sequences within DNA molecules and then catalyse double-strand cleavage of the DNA. A given restriction enzyme cuts DNA segments within a specific nucleotide sequence. These recognition sequences are typically only four to twelve nucleotides long. Once the cuts are made with the restriction enzymes, the plasmid samples are loaded into a gel for electrophoresis. A specific marker is also loaded on the gel, indicating the amount of base pairs in each segment of the plasmid.

4.2.7 Sequencing

DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. Currently, almost all DNA sequencing is performed using the chain termination method developed by Frederick Sanger and co-workers (Sanger *et al.*, 1977). This technique uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates.

In chain terminator sequencing, extension is initiated at a specific site on the template DNA by using a short oligonucleotide primer complementary to the template at that region. The oligonucleotide primer is extended using a DNA polymerase. Included with the primer and DNA polymerase are the four deoxynucleotide bases, along with a low concentration of a chain terminating nucleotide (usually a di-deoxynucleotide). Limited incorporation of the chain terminating nucleotide by the DNA polymerase results in a series of related DNA fragments that are terminated only at positions where that particular nucleotide is used. The fragments are then size-separated by electrophoresis in a polyacrylamide gel, or more usually now, in a narrow glass tube (capillary) filled with a viscous polymer.

PCR products can either be sequenced directly or the product can be cloned and sequenced within the vector using vector specific primers. The advantage of direct sequencing is that the vast majority of the amplified product will consist of the correct sequence. If cloning is used, it is possible that the actual cloned sequence may have incorporated some *Taq* errors (Kobayashi *et al.*, 1999).

Direct sequencing and cloning have relative advantages and disadvantages (Sterling 2003). Direct sequencing is a simple procedure that can be easily standardized and only a single sequence needs to be determined for each sample unlike cloning where several clones need to be checked for each sequence. However, direct sequencing is rarely successful unless several requirements are fulfilled. PCR reactions must be optimised to yield only a single product. If the same oligos are used to sequence as in a PCR amplification, they will also give sequence from any non-specific products. Primer-dimers may not interfere with the identification of a PCR product on agarose

gel electrophoresis, but they are an efficient template for sequencing, resulting in noise in sequence data close to the primer. In addition, primers may be acceptable for PCR amplification, but the same primers may fail in sequencing. This is because PCR is an exponential process and as it is usually carried well beyond completion, even rather poor primers will produce amplification in a PCR reaction. However sequencing is strictly linear and poor primers are less well tolerated.

4.2.8 RT-PCR

RT-PCR is based on the ability of the enzyme reverse transcriptase, an RNA-dependent DNA polymerase, to generate a complementary strand of DNA (first-strand cDNA) using the mRNA as a template. The reverse transcriptase reaction is performed on either total cytoplasmic RNA or on purified mRNA. It is important that no genomic DNA is present, as this will also provide a template for the PCR amplification step. An appropriate control for any contaminating DNA is a control reaction in which the reverse transcriptase step is omitted. RT-PCR can be carried out using either two-step or one-step formats. Two-step RT-PCR is useful for detecting multiple messages from a single RNA sample. One-step RT-PCR is ideal when processing large numbers of samples and when very high sensitivity is needed. cDNA synthesis is an important process as it forms the basis of many molecular biology techniques including cloning and gene expression analysis.

4.2.8.1 RNA Extraction

RNA is more susceptible to degradation than DNA, due to the ability of the 2′ hydroxyl groups adjacent to the phosphodiester linkages in RNA to act as intramolecular nucleophiles in both base- and enzyme-catalysed hydrolysis. Working with RNA requires strict precautions: Gloves must be worn at all times as skin is an abundant source of ribonucleases. Solutions must be prepared using autoclaved glassware and then autoclaved after they are prepared. When weighing out chemicals, the tip of the spatula should be flamed. Also, pre-sterilised water must be used and then after the solution is prepared it must be re-autoclaved. Most important, a separate area for RNA work should be maintained that has its own set of pipettes and solutions.

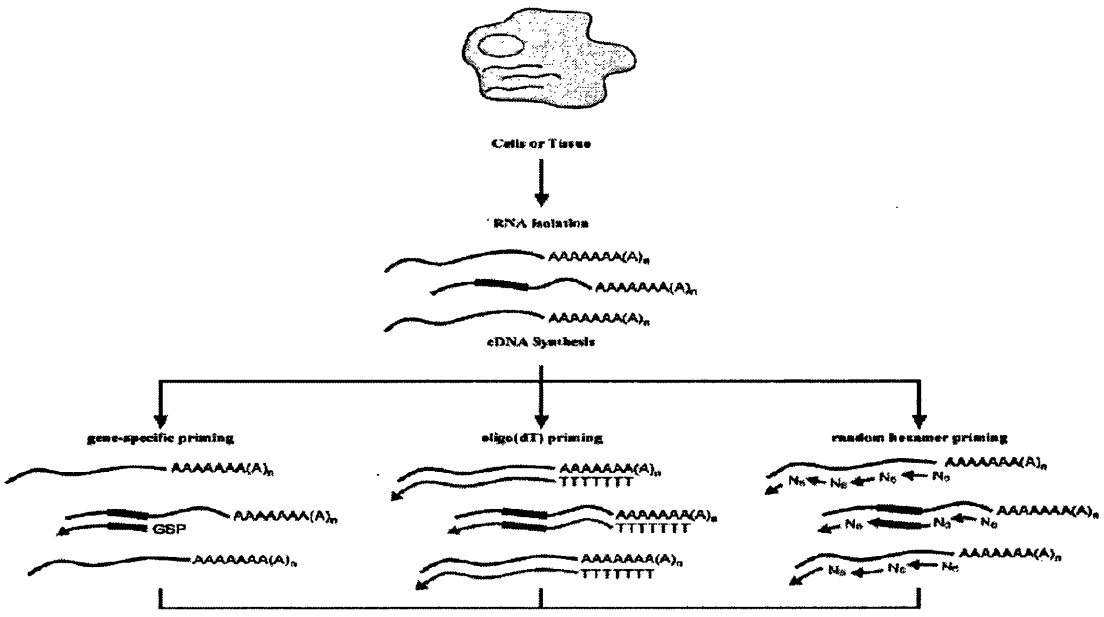
4.2.8.2 First-strand cDNA synthesis

This step involves copying the RNA to first-strand cDNA with reverse transcriptase. cDNA contains only the coding part of the sequence, which is complementary to its corresponding mRNA transcript. Reverse transcriptase is primer-dependent. This means that DNA extension is from a primer. The type of primer that is selected depends on the later application. Primer types include random primers, oligo(dT) primers, or gene-specific primers.

Random primers are most useful for generating cDNA from RNA templates with secondary structure. They are used to synthesise cDNA from an entire RNA extract and are generally a good choice for RT-PCR, but will also synthesise cDNA from

rRNA. They provide the least specificity because they anneal randomly to the RNA template and they generate shorter cDNAs (Lee *et al.*, 2003). Oligo (dT) primers are more specific than random primers. They hybridise to the poly (A) tails found at the 3′ ends of most eukaryotic mRNAs (Frohman *et al.*, 1988). Since poly (A)-RNA constitutes approximately 1–2% of the total RNA population, the amount and complexity of cDNA is considerably less than when random primers are used. Alternatively, a gene specific primer can be used. Gene-specific primers are oligonucleotides that hybridise to specific RNA target sequences. Because they are gene specific, these primers can be designed to anneal more tightly than oligo(dT) primers, thus raising the annealing temperature of the reaction. This may be particularly useful when the target mRNA is at a low copy-number. (Lee *et al.*, 2003). The mechanism of action of these three different types of primers used for cDNA synthesis are summarised in Figure 4.3.

Figure 4.3 Diagrammatic representation of three types of primers used in cDNA synthesis (adapted from Techniques for Optimizing RT–PCR Reactions. Lee *et al.*, 2003).



4.2.8.3 Amplification of cDNA

This step is to amplify the cDNA by PCR. Appropriate upstream and downstream primers are used and these are either specific to the target gene or for cDNA library construction these are non-specific. Due to the single-stranded nature of the first-strand cDNA, the early cycles of the PCR involve linear amplification as the first strand can only act as template for one of the primers. Exponential amplification from both primers occurs once sufficient copies of the second strand have been generated.

4.2.9 Single Stranded Conformational Polymorphism analysis (SSCP)

Single-stranded conformation polymorphism analysis (SSCP) is a simple method that allows one to rapidly determine whether there are sequence differences between relatively short stretches of DNA. Coupled with sequence analysis, SSCP is an extremely useful method for both identifying and characterising genetic polymorphisms and mutations. SSCP was originally described by Orita (1989). The principal advantage of the method is that it is very rapid to perform and that many individual PCR products may be screened for variation simultaneously. If properly optimised the method is very sensitive and different mutations within a fragment can often be distinguished on the same gel (Glavac and Dean 1993).

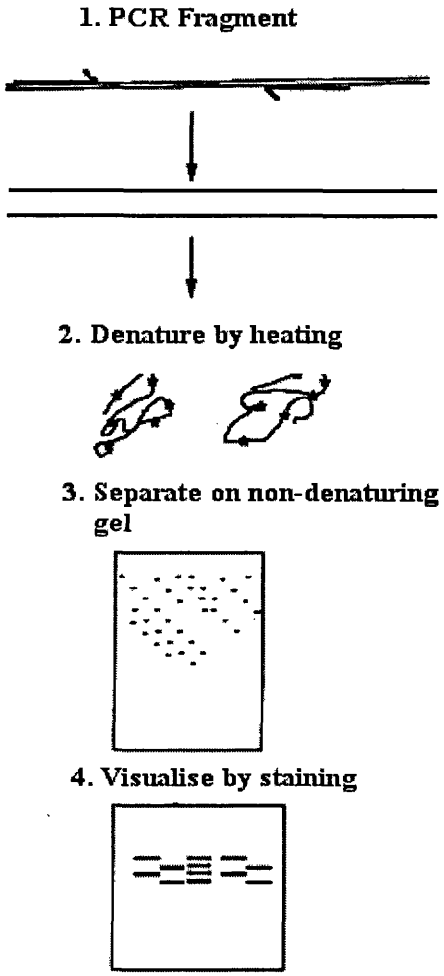
The theory of SSCP is that the sequence and length of a single stranded DNA fragment determine its conformation. Even single-base differences can cause different secondary conformations and this results in different gel migration rates of the DNA

strands. Under low temperatures and non-denaturing conditions DNA strands fold into structures that migrate according to their shape (Gasser and Chilton 2001). DNA strands of different sequence generally do not assume the same shape and therefore have distinct gel mobilities. The sensitivity of this technique is inversely proportional to the size of fragment and fragments at least as large as 775 bases long can be analysed successfully (Orti *et al.*, 1997).

Bands on SSCP gels can be visualised in several ways. These include silver-staining (Atkinson and Adams 1997; Sommer and Tichy 1999) and fluorescent dyes (Turenne *et al.*, 2000; Zumstein *et al.*, 2000). Ethidium bromide can detect SSCP variation (Sunnucks *et al.*, 2000) although it does not stain single-stranded DNA well; however, SYBR gold can be used (Small and Gosling 2000).

PCR samples to be used for SSCP are denatured (Figure 4.4) to single stranded molecules by mixing with an equal volume of loading buffer containing formamide to hinder reannealing of the DNA. Samples are then kept on ice in order to prevent single-strand molecules reannealing to double stranded DNA. Subsequently the samples are run on non-denaturing polyacrylamide gels which are then stained to reveal the DNA fragments.

Figure 4.4 Basic steps in SSCP procedure: 1. The DNA fragment of interest is amplified by PCR. 2. The PCR amplified-labelled DNA fragment is denatured by heating. 3. Fragments are separated on an acrylamide gel. 4. Patterns are revealed by staining (Adapted from Han and Robinson 2003).



4.2.10 Primer Design

4.2.10.1 Introduction

The specificity and sensitivity of PCR largely depends on the efficiency of primers (He *et al.*, 1994). The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors including: a) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures; b) duplex stability of mismatched nucleotides and their location; and c) the efficiency with which the polymerase can recognize and extend a mismatched duplex. The primers which are unique for the target sequence to be amplified should fulfil certain criteria such as primer length, GC%, annealing and melting temperature, 5' end stability, 3' end specificity (Dieffenbach *et al.*, 1995).

Many reviews of PCR optimization (Erlich *et al.*, 1991; Dieffenbach *et al.*, 1995; Roux 1995) consider different parameters of PCR but generally do not discuss basic concepts of PCR primer design, which is perhaps the most critical parameter for successful PCR. Poorly designed primers can result in a PCR reaction that will not work. The primer sequence determines several things such as the length of the product, its melting temperature and ultimately the yield. A badly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation.

4.2.10.2 Guidelines for primer design

When designing PCR primers, a number of factors are important. Since both specificity and the temperature and time of annealing are at least partly dependent on primer length, this parameter is critical for successful PCR (Wu *et al.*, 1991). It is generally accepted that the optimal length of PCR primers is 18-22 bp. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature. Primers with long runs of a single base should generally be avoided. It is especially important to avoid four or more G's or C's in a row.

Melting Temperature (T_m), the temperature at which one half of the DNA duplex will dissociate to become single stranded is an important factor as it indicates duplex stability. The optimal melting temperatures for primers is in the range 52-58°C. Primers with melting temperatures above 65°C should be avoided because of potential for secondary annealing. A good working approximation of this value (generally valid for oligos in the 18–30 base range) can be calculated using the formula of Wallace *et al.*, (1979), $T_m = 2(A+T) + 4(G+C)$ where A, T, C and G are the numbers of the nucleotides in the primer.

GC% is also an important characteristic of DNA relevant to primer design which provides information on the strength of annealing. Primers should have GC content between 45 and 60 percent (Dieffenbach *et al.*, 1995). For primers with a GC content of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 50°C. GC

content, melting temperature and annealing temperature are strictly dependent on one another (Rychlik *et al.*, 1990).

Another important factor relevant to primer design relates to the observation that the 3' terminal position in PCR primers is essential for the control of mis-priming (Kwok *et al.*, 1990). Primers should be "stickier" on their 5' ends than on their 3' ends. A "sticky" 3' end as indicated by a high GC content could potentially anneal at multiple sites on the template DNA. A "G" or "C" is however desirable at the 5' end. This 5' GC "clamp" reduces false secondary bands (Sheffield *et al.*, 1989).

The presence of secondary structures produced by intermolecular or intramolecular interactions can also lead to poor or no yield of the product. These adversely affect primer template annealing and thus the amplification. They greatly reduce the availability of primers to the reaction. Hairpins are formed by intramolecular interaction within the primer and should be avoided. If this state exists, a primer will fold back on itself and result in an unproductive priming event that decreases the overall signal obtained (Breslauer *et al.*, 1986). Hairpins that form below 50°C generally are not such a problem. Self dimers are formed by intermolecular interactions between the two same sense primers, where the primer is homologous to itself. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they reduce the product yield. Cross dimers are formed by intermolecular interaction between sense and antisense primers, where they are homologous. However, sometimes these secondary structures are harmless when the annealing temperature does not allow them to form. For example, some dimers or

hairpins form at 30°C while during the PCR cycle, the lowest temperature only drops to 60°C.

4.3 Methods

4.3.1 DNA Extraction

For members of the genus *Callopora*, the best method for DNA extraction was by extracting from larvae. The protocol used for this method is a modified version of the one published by Sutherland *et al.* (1998). Larvae were extracted from ovicells of brooding colonies under a stereomicroscope (Olympus SZ60, magnification ca. x32) using a pair of fine forceps (TAAB Type 5). Prior to lysis, larvae were washed with filtered sterilised sea water (0.2µm Acrodisc Supor membrane syringe filters) and transferred with a P10 Gilson Pipette into 0.2ml ABGene microtubes containing 15µl lysis solution. Samples were incubated for 90 minutes at 37°C followed by 10 minutes incubation at 99°C during which the proteinase K was inactivated. The lysed material was kept at –20°C and 2 µl of this solution was used directly for subsequent PCR reactions.

4.3.2 PCR

A typical PCR reaction (25µl) that was used in this study consisted of the following:

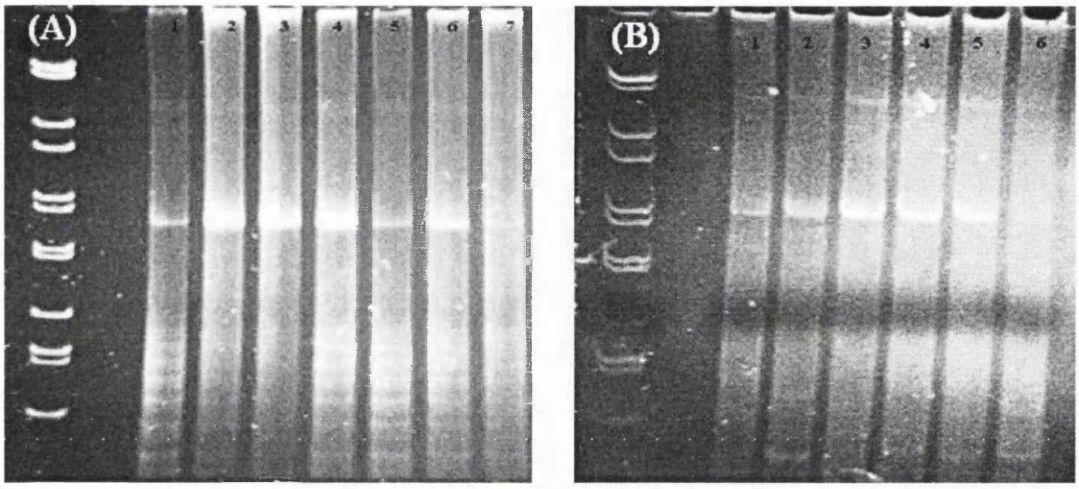
- 5 µl dNTP 0.1mM (Promega)

- 2.5 µl Buffer I (10x) (ABGene 500mM KCl, 100mM Tris-HCl pH8.3, 15mM $MgCl_2$)
- 1.5 µl $MgCl_2$ (25mM) (ABGene)
- 0.8 µl of each primer (5nmol) (Sigma-Genosys)
- 1.5 units *Taq* Polymerase (ABGene)
- 80ng template DNA or 1.5µl of either larval lysed DNA or gel purified PCR product.

For all PCR reactions the denaturing step was at 95°C for 30 seconds. The annealing temperature differed according to the primer set used as well as the template DNA. Extension was at 72°C for 1min 50s. These steps were repeated 33 times followed by a final extension step at 72°C for 10 minutes. When reamplification of PCR products took place, the annealing temperature was raised by 4 degrees compared with the original and the number of cycles was reduced to 20. An automated thermal cycler (MJ Research PTC-225 Peltier Thermal Cycler) was used for all PCR reactions.

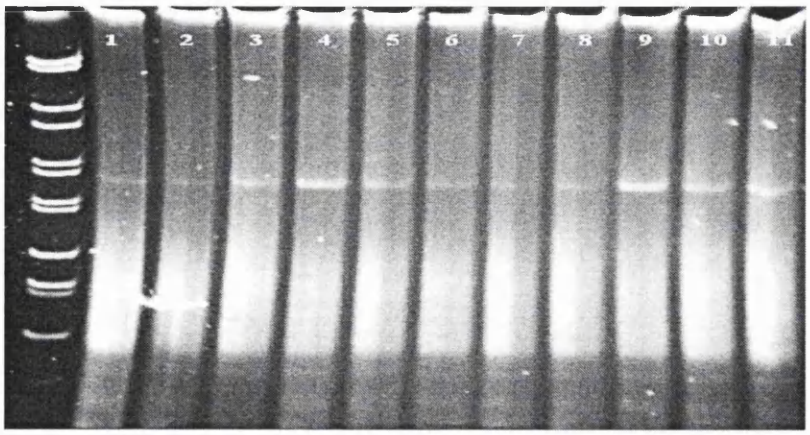
PCR optimisation was routinely performed during the course of this study. In one set of experiments, temperature gradient PCR reactions took place in order to establish the optimum temperature at which the primers would work (Figure 4.5A). A temperature gradient from 50 to 59°C was used. A second series of optimisation experiments involved a magnesium chloride gradient. As already mentioned, in the absence of adequate free magnesium, *Taq* DNA polymerase is inactive whereas excess free magnesium reduces enzyme fidelity. For these reasons, it was important to determine empirically the optimal $MgCl_2$ concentration for each reaction. To do so, a reaction series containing 0-3.0mM Mg^{2+} was prepared in 0.5mM increments (Figure 4.5B).

Figure 4.5 (A) Temperature gradient experiment (50°C lane 1 to 59°C lane 7) showing the most efficient annealing temperature (lane 3); (B) $MgCl_2$ gradient experiment (0mM lane 1 to 3.0mM lane 6) showing the most efficient $MgCl_2$ amount (lane 3). Marker VI, Boehringer Mannheim with lengths (bp) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220 and 154 is also shown.



In the present study, in order to obtain a pure PCR product, reactions were optimised experimentally. However, in some instances secondary bands were observed which changed the quality of the PCR product such that it could not be used for further experiments. In other instances the yield of the PCR product was not adequate enough (Figure 4.6).

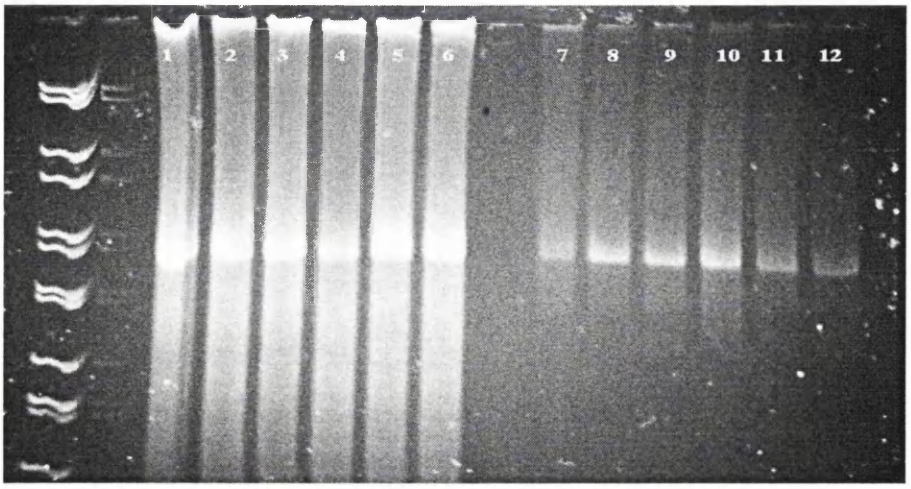
Figure 4.6 A gel showing low yield PCR reactions (lanes 1, 2, 6, 7 and 8) as well as amplification with secondary products (lanes 3, 4, 5, 9, 10 and 11). Marker VI, Boehringer Mannheim with lengths (bp) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220 and 154 is also shown.



One method to circumvent this problem was by reamplifying PCR products. The fragments of interest were dissected from the gel with sterile scalpel blades, transferred to Spin-X columns (Corning Inc) and kept at -20°C overnight. The columns were subsequently centrifuged at 10000rpm for 10minutes and 1µl of the eluted DNA solution was used as a template using the reamplification conditions described previously.

However, even after reamplifying a PCR product, there were still circumstances where artefacts were present. For this reason, PCR products had to be purified from gels. Two methods were employed in this study for this purpose. The first one utilised the Promega Wizard PCR preps DNA Purification System with which DNA was recovered from acrylamide gels and the other was the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) with which DNA was recovered from agarose gels. Examples of PCR products before and after purification are shown in Figure 4.7.

Figure 4.7 PCR product purification: Lanes 1-6 represent uncleaned PCR products showing nucleic contamination streaking throughout lane and lanes 7-12 represent same PCR products after purification. Marker VI, Boehringer Mannheim with lengths (bp) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220 and 154 is also shown.



4.3.3 Gel electrophoresis

1% agarose gels were prepared in order to separate and analyse DNA by mixing in a flask the following ingredients:

- 0.4g agarose
- 40ml TBE x1

Subsequently the contents were heated for about 1 minute in a microwave followed by a 5 minute cooling period. The gel was then poured slowly into a tray (Pharmacia Gel Electrophoresis Apparatus LKB GPS 200/400) and any bubbles that were present were pushed away to the side using a disposable tip. A comb was then inserted and the gel was left to set for at least 30 minutes. After the gel had set, 360ml of TBE x1 running buffer was poured into the gel tank to submerge the gel. It was important to use the same buffer (1x TBE) at this stage as was used to make the gel. Gels were left running at 65V for at least 60 minutes.

The marker ladder depended on the type of experiment. For example, for restriction digests (section 4.2.6) λ Hind III Fragments (500 μg/ml) (Invitrogen) were used as a marker ladder. For checking and purifying PCR products, DNA Molecular Weight Marker VI (0.25 μg/μl) (Roche) was used as a marker ladder.

Polyacrylamide gels (5-6%) were made by mixing in a flask the following ingredients:

- 4.5ml TBE X 10
- 8.0ml Acrylamide (37.5:1).
- 32ml distilled water.

Subsequently, the contents were degassed for a few minutes and the following were added to the mixture:

- 45µl Temed.
- 220µl 10% APS

The mold plates were cleaned with ethanol and the gel solidified in 20 to 30min. Each gel was then placed in a gel tank (Mini-Protean 3 cell system, BIO-RAD) and the electrodes were plugged in Power Pac 300 (Bio-Rad). Gels were run under constant voltage (5V/cm) for about 70 minutes.

4.3.4 Staining methods

When ethidium bromide was used as a stain, 35µl of a 10mg/ml solution was added to a tank containing 1 litre of distilled water and the gel was placed in the tank for 15 to 20min in complete darkness. After staining, the gel was transferred into distilled water to remove the ethidium bromide and the DNA bands were visualised using a High Performance Ultraviolet Transilluminator (Ultra Violet Products). SYBR gold stain was made by mixing 10µl SYBR gold in a tray containing 100ml TBE X1. The gel was covered and left to stain for 15 to 20 min and subsequently was viewed under ultraviolet light. Staining the Polyacrylamide gel with silver stain was performed using the following protocol:

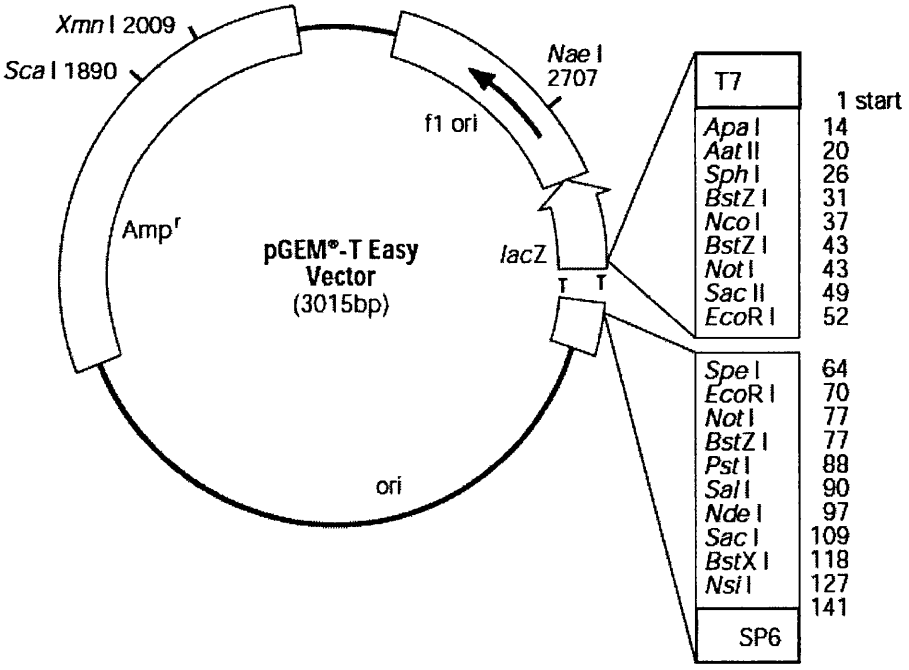
- The gel was fixed by washing it 2 times for 3 minutes in Solution A (Appendix A)
- Stained for 10 minutes in Solution B
- Washed twice in distilled water to remove excess silver nitrate and
- Developed for 10 minutes in Solution C

The tray containing the gel, while in solutions B and C, was covered to keep it as dark as possible as these steps were photosensitive. Upon completion of staining, the gel was placed back in solution A to prevent expansion.

4.3.5 Cloning

For this study, the pGEM-T Easy Vector System (Figure 4.8) was selected as it is a convenient system for the cloning of PCR products.

Figure 4.8 pGEM-T Easy Vector circle map and sequence reference points (Adapted from Promega manual pGEM-T and pGEM-T Easy Vector Systems Technical Manual No. 042).



The first step in the cloning experiment was to ligate the PCR product to the vector followed by transformation of the ligated product into the host bacteria. Promega's JM109 *E. coli* competent cells were used.

Ligation reactions were prepared according to the following protocol:

- The pGEM-T Easy Vector tube was centrifuged briefly to collect the content at the bottom of the tube.
- The 2X Rapid Ligation Buffer was vortexed vigorously before each use.

Then the following ingredients were added in this exact sequence to a 0.5ml centrifuge tube:

- 5 µl of 2X Rapid ligation buffer.
- 1 µl of pGEM-T Easy Vector (50ng).
- 1 µl of PCR product.
- 1 µl of T4 DNA Ligase (3 Weiss units/µl).
- Deionised water to a final volume of 10 µl.

All tubes and solutions were kept on ice while preparing the ligation reactions and were mixed by pippeting gently. Reactions were subsequently incubated at 4°C overnight.

Transformation reactions were subsequently prepared using the following protocol:

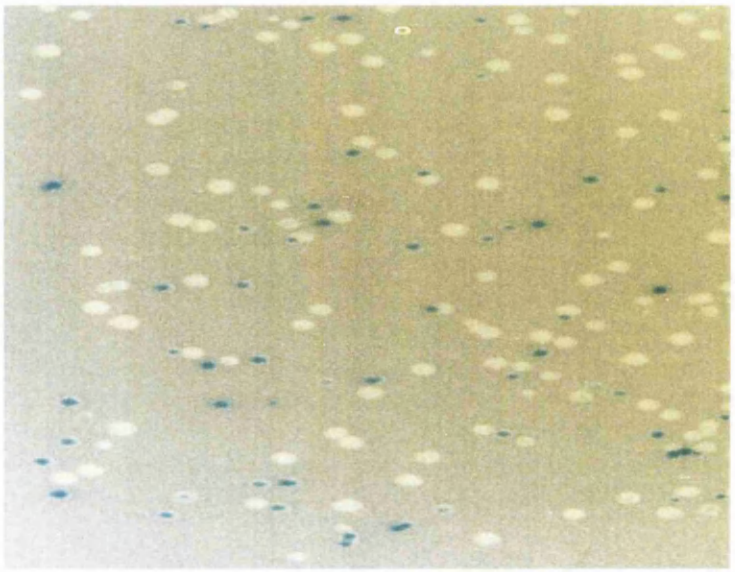
- Tubes containing the ligation reactions were centrifuged briefly to collect contents at the bottom of the tube.
- 2 µl of each ligation reaction was then added to a sterile 1.5ml centrifuge tube on ice.

- Tubes of frozen JM109 High efficiency competent cells were removed from –70°C storage and placed in an ice bath until just thawed (about 5 minutes). Cells were mixed by gently flicking the tubes.
- 50µl of cells were transferred into each tube containing 2 µl of each ligation reaction.
- Tubes were gently flipped to mix and placed on ice for 10 mins.
- Cells were heat-shocked for 45–50 seconds in a water bath at exactly 42°C. Tubes were immediately returned on ice for a further 2 mins incubation.
- 950µl of room temperature SOC medium was added to the tubes containing cells transformed with the ligation reactions. This was followed by 1 hr incubation at 37°C with shaking (~225rpm).
- 100µl of each transformation culture was then plated onto LB/ampicillin/ IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium was used for plating.

Agar plates were prepared using a commercial ready mix (S-Gal/LB Agar Blend). In addition, the antibiotic ampicillin (25µg/ml) was added to the agar to prevent the growth of any bacteria other than ones with ampicillin resistance.

Plates were subsequently placed in the incubator (37°C) for about 16 hours and checked the following day. Successful cloning of an insert in the pGEM-T Easy was verified according to the blue-white screening method. Clones that contained PCR products produced white colonies (Figure 4.9).

Figure 4.9 Blue and white colonies.



White colonies were then picked with a P10 Gilson pipette and put to grow in 25ml universal tubes containing liquid LB broth and the antibiotic ampicillin (25µg/ml) in the incubator at 37°C on a stirring plate at 260rpm overnight for 16 hours.

The final part in the cloning experiment involved plasmid DNA extraction. Plasmids were extracted using the Wizard Plus SV Minipreps DNA Purification System as it provides a simple and reliable method for rapid isolation of plasmid DNA. The entire procedure was carried out according to the following protocol.

- Tubes containing bacterial cultures were centrifuged for 10 minutes at 4000rpm in a tabletop centrifuge (IEC Centra CL3R).
- The supernatant was poured off and any excess liquid was removed with a pipette.

- 250 µl of Cell Resuspension solution was then added and the cell pellet was resuspended by gently pipetting. Resuspended cells were transferred to a sterile 1.5ml eppendorf.
- 250 µl of Cell Lysis Solution was added to each sample and mixed by inverting gently 4 times.
- 10µl Alkaline Protease Solution was added, mixed and incubated for 5min at room temperature.
- 350µl of Neutralization Solution was added, mixed and the tubes were centrifuged at 14,000 rpm for 10min.
- Plasmid DNA purification units were prepared by inserting one spin column into one 2ml Collection Tube for each sample.
- The clear lysate (approximately 850µl) was transferred to the prepared Spin Column by decanting, while making sure not to transfer any of the white precipitate with the supernatant.
- Supernatant was centrifuged through the spin column at 14,000rpm in a microcentrifuge for 1 minute at room temperature. The spin column was removed from the tube and the flowthrough was discarded from the collection tube.
- 750 µl Wash Solution (ethanol added) was added to the spin column and centrifuged at 14,000 rpm for 1min. The flowthrough was discarded and the column was reinserted into the collection tube.
- The wash procedure was repeated using 250 µl of Wash Solution and spin columns were centrifuged at 14,000rpm for 2 min. The flowthrough was discarded and the column was reinserted into the collection tube.

- Spin columns were transferred to new, sterile 1.5 ml eppendorfs and the plasmid DNA was eluted by adding 50µl of nuclease-free water to the spin column.
- A final 1 minute of centrifuging at maximum speed followed and the spin columns were discarded while the tubes with the purified plasmid DNA were stored at -20°C.

4.3.6 Restriction digestion

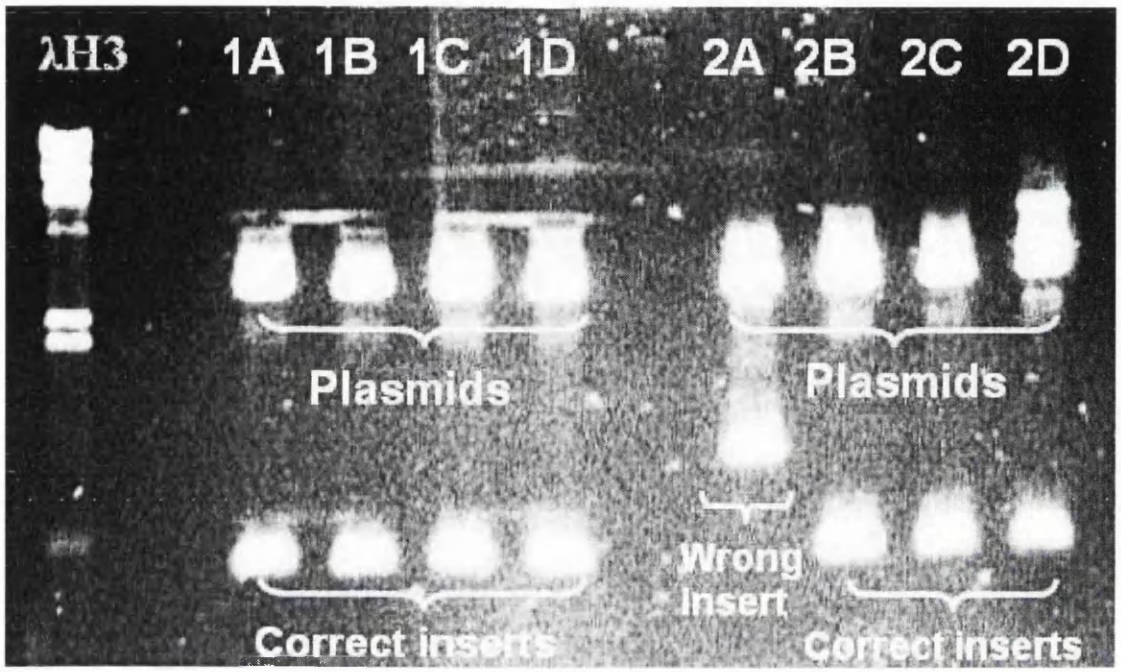
Plasmids were inspected for correct inserts by restriction digest with *EcoRI*, which has the recognition sequence 5'-GAATTC-3'. Cut sites for this enzyme flank the insert (Figure 4.8).

Restriction digestion reactions were prepared by adding in 0.5ml eppendorfs the following components:

- 6µl filter sterilised water.
- 2µl purified plasmid DNA.
- 1 µl *EcoRI* (10u μl^{-1}) (Invitrogen).
- 1 µl 10x Reaction buffer 3 (Invitrogen).

Tubes were mixed briefly in a tabletop centrifuge (Model IEC Micromax) and placed in the incubator at 37°C for 2 hrs. The digested plasmid DNA samples were run in an agarose gel (Figure 4.10) and the gel was stained with ethidium bromide followed by visualisation under ultraviolet light to check for inserts and their size. The molecular marker used was λ Hind III Fragments (500 µg/ml) (Invitrogen).

Figure 4.10 Plasmid restriction digest. Samples 1A-1D and 2B-2D represent plasmids with correct sized inserts; sample 2A represents plasmid with wrong sized insert. λH3 represent the molecular marker used (λ Hind III) with lengths (bp) 23.310, 9.415, 6.557, 4.361, 2.322, 2.027, 564 and 125.



4.3.7 Sequencing

Sequencing reactions were prepared in 0.2ml thin walled tubes by adding the following:

- 2µl purified plasmid DNA with PCR product insert (denatured at 96°C for 1 min).
- 2 µl sense or antisense primer (M13 primers, complementary to vector regions flanking the ligated PCR product).
- 8µl filter sterilised water.
- 8µl DTCS mix.

The above components were mixed briefly by centrifuging before thermal cycling. Although, mixing occurs as the temperature cycles during the thermal cycling steps, it is not effective as mixing by hand prior to cycling (CEQ 2000 Dye Terminator Cycle

Sequencing Chemistry Protocol). Sequencing reactions were kept on ice in a container in the PCR hood.

The tubes were then placed in the thermal cycler and the following conditions used:

- 96°C for 20sec (Denaturing).
- 50°C for 20sec (Annealing).
- 60°C for 4min (Extension).

Repeated 30 times (30 cycles).

- 4°C once finished.

Tubes were subsequently stored at -20°C once the program was completed. Plasmid samples with the correct insert were sent for sequencing on a Beckman Coulter CEQ 2000XL DNA analysis system according to the manufacturer's instructions.

4.3.8 RNA extraction from single larva and First Strand Synthesis

RNA extraction from single larva was performed according to the following protocol:

- 0.5ml tubes with 50 µl of lysis buffer were prepared.
- Larvae were added to the tubes (one larva per tube) and 50 µl of acid phenol (Ambion) was also added.
- Tubes were incubated at 65°C for 2 hrs and vortexed gently every 15-20mins in order to mix the phases.
- Tubes were put on ice for 10 mins, followed by the addition of 1/10 volume of 3M Sodium Acetate. Tubes were then centrifuged for 10 mins at 10,000 rpm.

- Two extractions of phenol/chloroform and one with only chloroform were then performed and the supernatant was precipitated in 2.5 volumes of cold absolute ethanol using glycogen (Ambion) as a carrier molecule.
- Tubes were subsequently spun for 20 mins at 10,000rpm at room temperature and the supernatant was poured off.

The pellets were then left to dry at room temperature and then were resuspended in 40 µl of RNAse free sterile water.

First strand synthesis was performed using ABGene's Reverse-IT kit according to the following protocol:

- 12 µl RNA template (0.1µg to 5µg) and 1µl first strand primer, which could be either a COI specific primer (final concentration 0.5-2µM) or a random decamer primer (400ng/µl) which was supplied with the kit were added to 0.5ml microcentrifuge tubes.
- Samples were heated for 5 minutes at 70°C in order to remove any secondary structures that may be present and were then placed on ice.
- 4 µl of 5X First Strand Synthesis Buffer DTT (100mM), 2 µl of dNTP and 1 µl of Reverse IT RTase Blend were added and samples were mixed well by spinning gently.
- Samples were incubated for 30 minutes at 47°C during which cDNA synthesis took place, followed by 10 minutes incubation at 70°C during which the RTase was deactivated.
- Samples were afterwards stored at -20°C.

The next step was to amplify the cDNA by PCR. A typical PCR protocol involved mixing (for 50µl reaction) the following components together in thin wall tubes:

- 5µl of 10x one-step buffer, 15mM $MgCl_2$.
- 2µl of 5mM dNTP mix.
- 2 µl of upstream primer (to final concentration of 0.2 µM).
- 2 µl of downstream primer (to final concentration of 0.2 µM).
- 0.25 µl of *Taq* DNA polymerase, 5U/ µl.
- Distilled water to 45 µl.
- 5 µl of 1st strand reaction mix.

And the recommended cycling parameters were as follows:

- Initial denaturation: 1 cycle at 94°C for 2 minutes, followed by 35 cycles of:
- Denaturation: 94°C for 30 seconds.
- Annealing: 50-65°C for 30 seconds.
- Extension: 72°C for 30 seconds.
- And a final extension of 1 cycle at 72°C for 5 minutes.

A 10µl aliquot of the RT-PCR reaction was then analysed using acrylamide gel electrophoresis.

4.3.9 Single Stranded Conformational Polymorphism (SSCP)

In a basic SSCP protocol, PCR products obtained using optimum conditions and purified with the methods described, were mixed with Stop solution containing 95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The samples were heated at 95°C to denature for 10 minutes in a thermal cycler and subsequently kept on ice for 10 minutes prior to loading onto the polyacrylamide gel.

Electrophoresis was carried out on a dual cooled gel electrophoresis unit (Hoefer SE600) with 18 x 16 cm plates and 1cm wide spacers. Buffer (TBE X 1) was prepared in advance and kept cold at 4°C before use. Refrigerated water from a thermostatically controlled circulator (Hoeffer Cooling System, Circulator Bath RCB 500) was passed through a vertical cooling chamber to maintain the upper buffer and gel at the constant temperature of 10°C throughout the run.

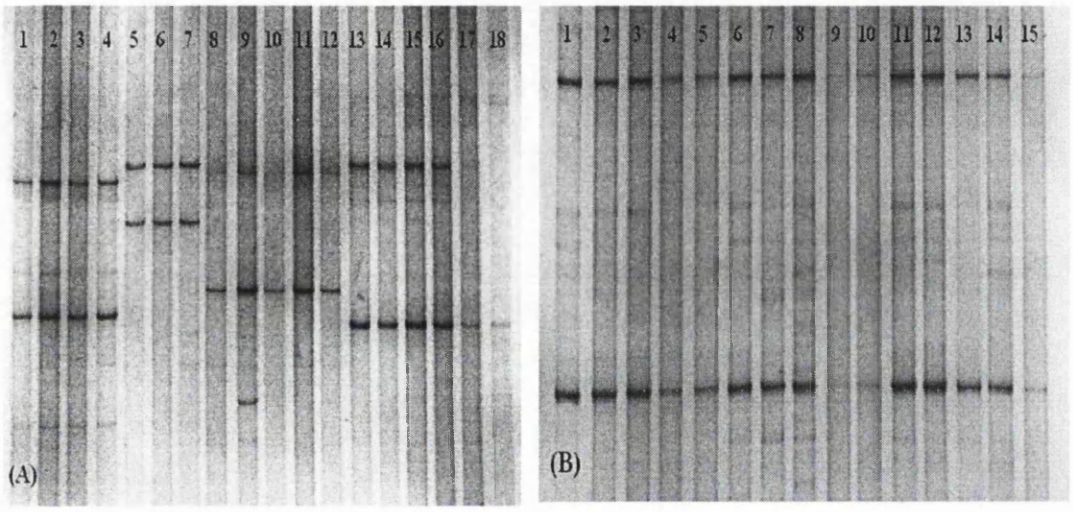
The Polyacrylamide gel (10%) used for SSCP was made according to the following protocol:

- 3.75ml TBE X 10.
- 13ml Acrylamide (37.5:1).
- Water added to 38ml.
- 33µl Temed.
- 350µl APS 10%.

Upon setting, the gel was kept cold before loading the samples. The gel was run at constant voltage of 300V for 17 hours and stained with silver stain (Section 4.2.5). A

typical SSCP gel is as shown in Figure 4.11A, B. The two main bands in each lane represent the two strands of the DNA double helix. Bands that differ between lanes indicated sequence differences.

Figure 4.11 (A) A typical SSCP gel showing band variation from different samples from *Callopora dumerilii*. Lanes 1-4 represent DNA samples from colony 1, lanes 5-7 from colony 2, lanes 8-12 from colony 3, lanes 13-16 from colony 4, lanes 17-18 from colony 5. (B) A typical SSCP gel showing no band variation from different samples from different species. Lanes 1-5 represent DNA samples from *Callopora dumerilii*, lanes 6-10 represent DNA samples from *Callopora lineata* and lanes 11-15 represent DNA samples from *Callopora rylandi*.



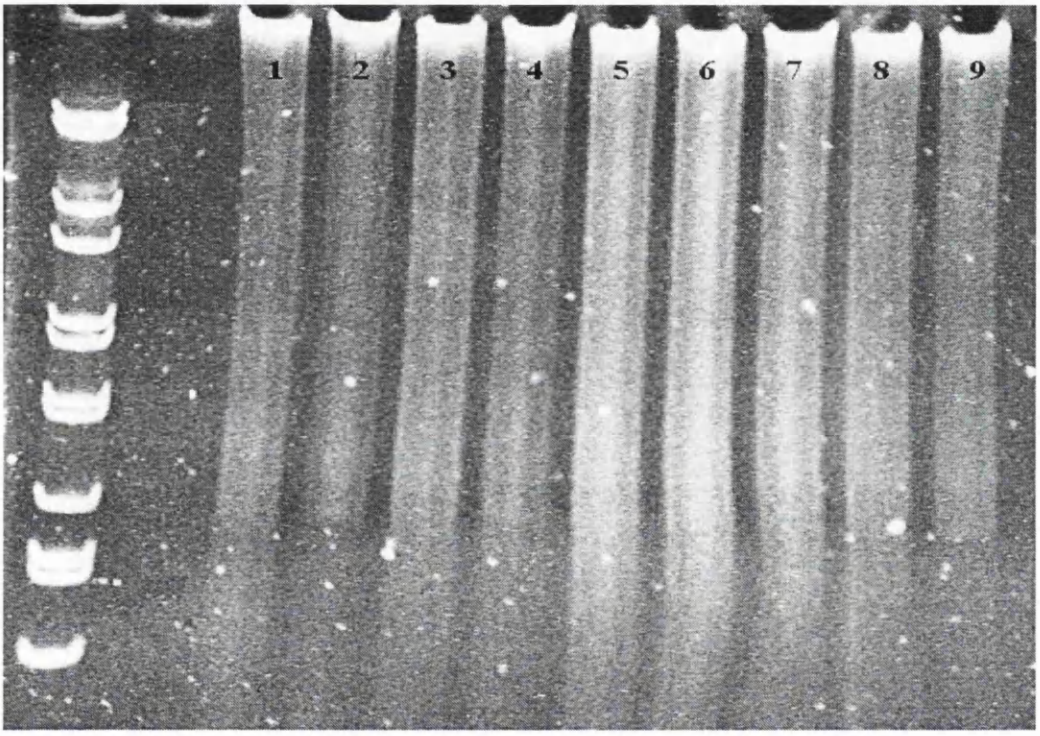
4.4 Primer Development and Design

PCR analysis of mitochondrial DNA (mtDNA) from a relatively little studied group such as bryozoa was hampered by lack of prior sequence data and by the small amount of tissue available in many species. This hindered design of species-specific PCR primers.

Primer development started in October 2003. Before that a range of cytochrome oxidase I (COI) primers designed by Dr Porter (pers. Communication) were used. The

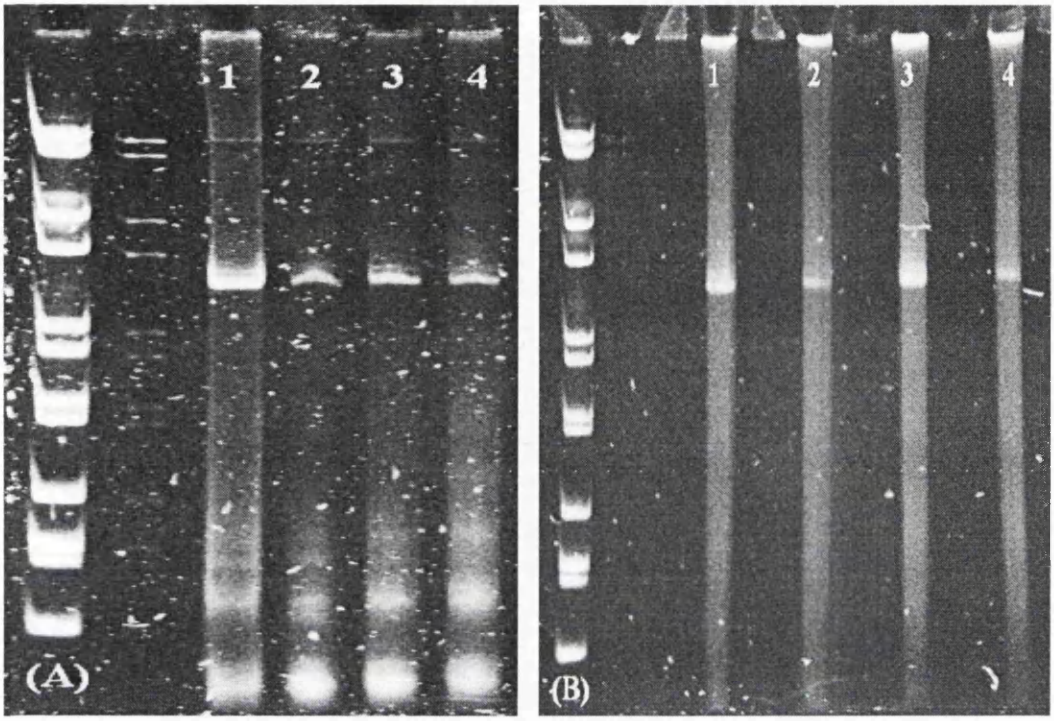
results were disappointing, with no products of the expected size being amplified (Figure 4.12) using DNA extracted from larvae of any of the three *Callopora* species.

Figure 4.12 Gel showing unsuccessful PCR amplifications using a pair of previously developed bryozoan primers. Lanes 1-3 represent DNA samples from *Callopora dumerilii*, lanes 4-6 represent DNA samples from *Callopora lineata* and lanes 7-9 represent DNA samples from *Callopora rylandi*. Marker VI, Boehringer Mannheim with lengths (bp) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220 and 154 is also shown.



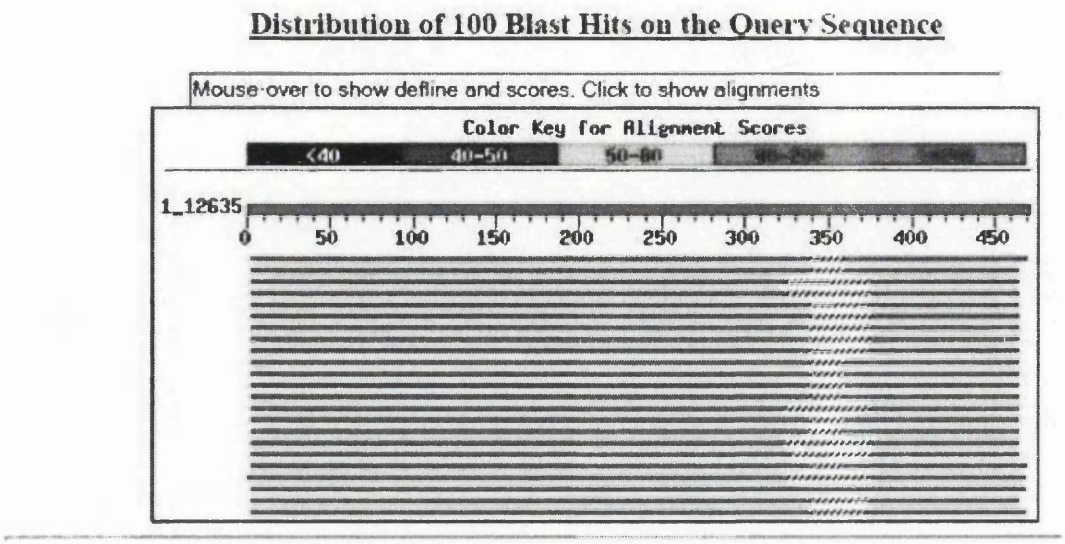
Subsequently, universal primers developed by Folmer *et al.*, (1994) were used for the COI gene. Use of this set of primers proved successful as DNA from lysed *Callopora* larvae gave very good PCR products (Figure 4.13A). The size of the PCR product at 710bp was in line with that expected. These PCR products were purified (Figure 4.13B), cloned and sequenced.

Figure 4.13 Folmer *et al.* (1994) primers amplifying larval DNA. (A) bands of the expected size (~710bp) using *Callopora lineata* larvae as template DNA prior purification (B) after purification. Marker VI, Boehringer Mannheim with lengths (bp) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220 and 154 is also shown.



Sequence identity was checked using BLAST in Genbank (Altschul *et al.*, 1997) but the BLAST results gave no bryozoan hits (Figure 4.14).

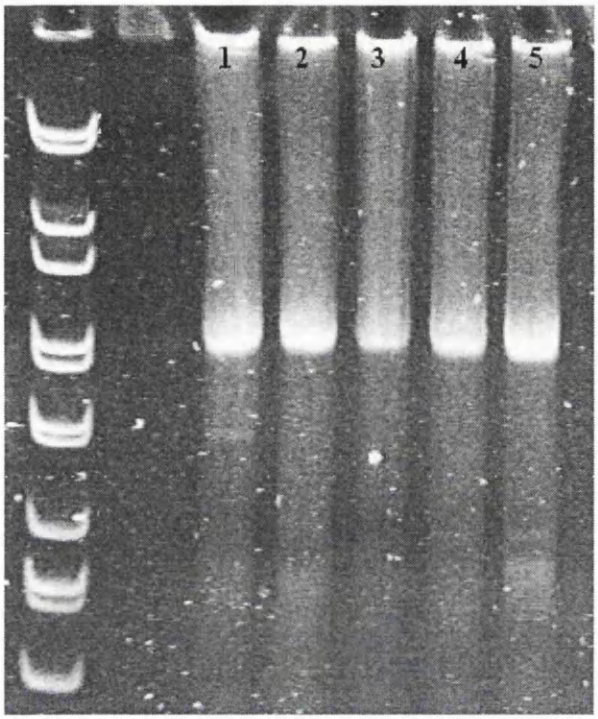
Figure 4.14 A typical output of the BLAST program showing no bryozoan hits.



Sequences producing significant alignments:	Score (bits)	E Value	N
gi|18157560|gb|AF385091.1|AF385091 Lampsilis perovalis ...	181	4e-55	3
gi|30140187|emb|AJ436905.1|MGR436905 Malacodbella gross...	180	7e-55	2
gi|5107809|gb|AF156526.1| Villosa vanuxemensis UMMZ 265...	181	7e-55	3
gi|5107808|gb|AF156525.1| Villosa vanuxemensis UMMZ 265...	181	7e-55	3
gi|18157592|gb|AF385107.1|AF385107 Lampsilis altilis UA...	181	7e-55	3
gi|18157590|gb|AF385106.1|AF385106 Lampsilis altilis UA...	181	7e-55	3
gi|18157588|gb|AF385105.1|AF385105 Lampsilis altilis UA...	181	7e-55	3
gi|18157586|gb|AF385104.1|AF385104 Lampsilis subangulat...	181	7e-55	2
gi|18157582|gb|AF385102.1|AF385102 Lampsilis subangulat...	181	7e-55	2
gi|18157576|gb|AF385099.1|AF385099 Lampsilis australis ...	181	7e-55	2

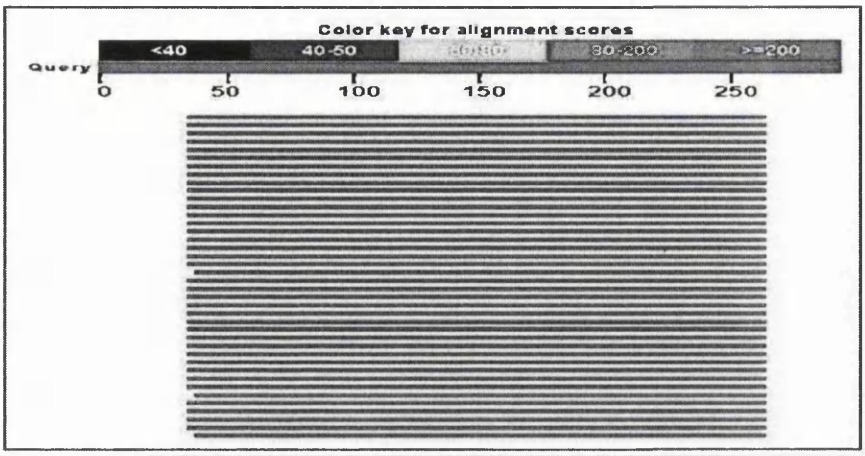
Waeschenbach (2003) designed bryozoan specific COI primers from sequences amplified with the universal primers (Folmer *et al.*, 1994). This set of primers, (referred to as COI-1) which was used to amplify DNA from *Bowerbankia imbricata* by Waeschenbach (2003) was surprisingly found to amplify DNA from *Callopora lineata* (Figure 4.15) and gave bands of the expected size.

Figure 4.15 Waeschenbach (2003) COI-1 primers amplifying larval DNA. Bands of the expected size (~517bp) using *Callopora lineata* larvae as template DNA are shown. Marker VI, Boehringer Mannheim with lengths (bp) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220 and 154 is also shown.



Sequence data obtained using this set of primers, checked using BLAST (Figure 4.16), returned bryozoan sequences deposited by other researchers. There were no *Callopora* sequences in the database and the closest match was a *Bugula dentata* sequence.

Figure 4.16 A typical output of the BLAST program showing bryozoan hits.



Sequences producing significant alignments:		Score (Bits)	E Value
gi|17025898|gb|AAL32111.1|	cytochrome c oxidase subunit I [Bugul	136	3e-31
gi|54401647|gb|AAV34593.1|	cytochrome oxidase subunit 1 [Bugula	136	3e-31
gi|54401639|gb|AAV34589.1|	cytochrome oxidase subunit 1 [Bugu...	135	5e-31
gi|17025894|gb|AAL32109.1|	cytochrome c oxidase subunit I [Bugul	135	6e-31
gi|17025888|gb|AAL32106.1|	cytochrome c oxidase subunit I [Bugul	134	2e-30
gi|17025884|gb|AAL32104.1|	cytochrome c oxidase subunit I [Bugul	134	2e-30
gi|17025886|gb|AAL32105.1|	cytochrome c oxidase subunit I [Bugul	134	2e-30
gi|17025892|gb|AAL32108.1|	cytochrome c oxidase subunit I [Bugul	133	3e-30
gi|27657940|gb|AAQ18236.1|	cytochrome oxidase subunit 1 [Bugula	133	3e-30
gi|6670919|gb|AAF23067.1|	cytochrome c oxidase subunit I [Bugula	133	3e-30

However, these results give support to the view that the primers were amplifying *Callopora* DNA rather than DNA from contaminating organisms.

Having evidence that the sequence obtained was a bryozoan one a phylogenetic tree was built (Figure 4.17) to confirm the validity of this finding. The tree was constructed from other bryozoan sequences from GenBank, as well as sequences obtained from Waeschenbach (2003). Also included in the tree were sequences from more distantly related organisms which were added for comparative purposes and to highlight any bryozoan groups that did occur.

Figure 4.17 Verification of sequence validity using phylogenetic reconstruction. Neighbour-joining tree based on COI sequences. Numbers above branches are bootstrap values (500 replicates). For GenBank accession numbers, see Table 4.1. The sequence obtained for Callopora is 13CF.

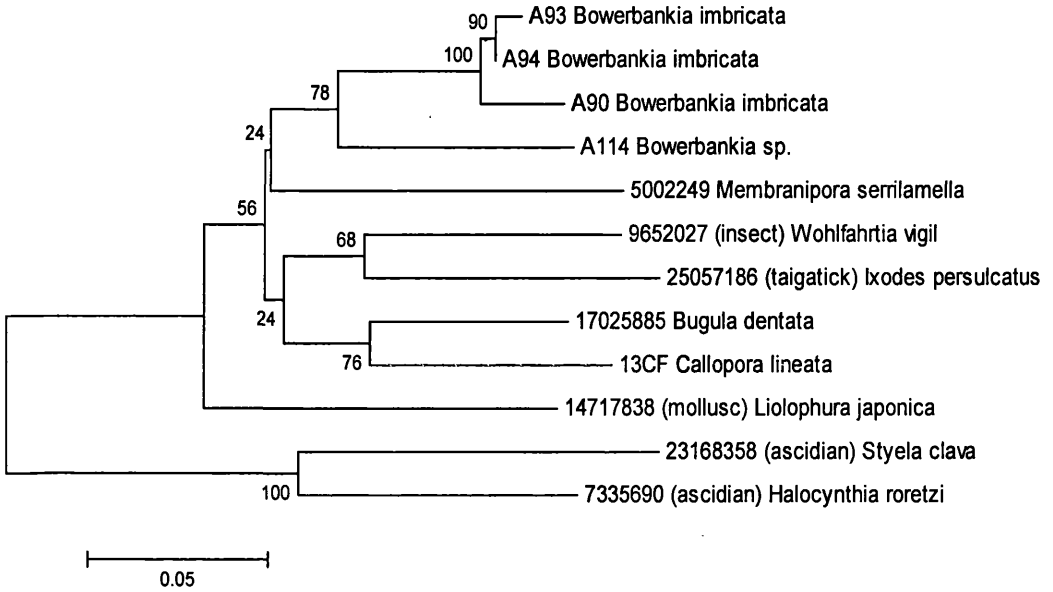


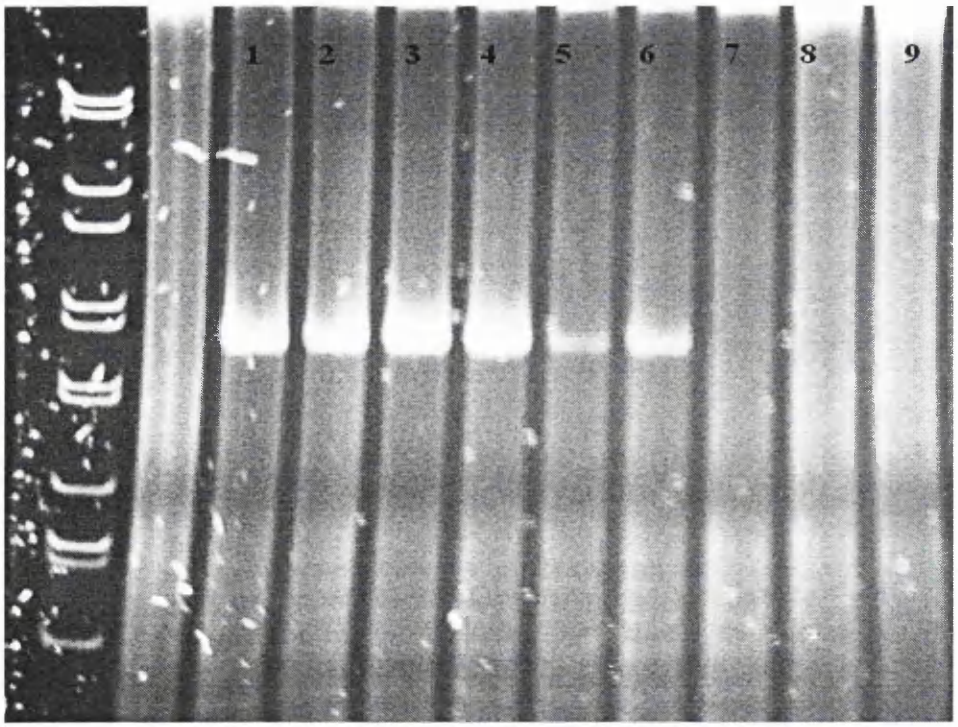
Table 4.1 Sequences used in phylogenetic trees; GenBank accession numbers where available are denoted (N/A means those sequences have not yet been submitted to GenBank). Sequences A90, A93, A94 and A114 are from Waeschenbach (2003) and sequence 13CF is from this study.

Species name	Accession number	Sequence ID
Bowerbankia imbricata	N/A	A93
Bowerbankia imbricata	N/A	A94
Bowerbankia imbricata	N/A	A90
Bowerbankia sp.	N/A	A114
Membraniporra serrilamella	5002249	*Membranipora serrilamella*
Wohlfahrtia vigil	*9652027*	(insect) *Wohlfahrtia vigil*
Ixodes persulcatus	25057186	(taigatick) *Ixodes persulcatus*
Bugula dentata	17025885	*Bugula dentata*
Callopora lineata	N/A	13CF
Liolophura japonica	14717838	(mollusc) *Liolophura japonica*
Styela clava	23168358	(ascidian) *Styela clava*
Halocynthia roretzi	7335690	(ascidian) *Halocynthia roretzi*

The *Callopora lineata* sequence (13CF) groups with a *Bugula dentata* sequence. This is a positive finding which is supported by a moderate bootstrap value. Hence it was concluded that there is no strong reason to doubt that the sequence obtained using the COI 1 primers designed by Waeschenbach (2003) was of bryozoan origin and is the first *Callopora* sequence obtained to date.

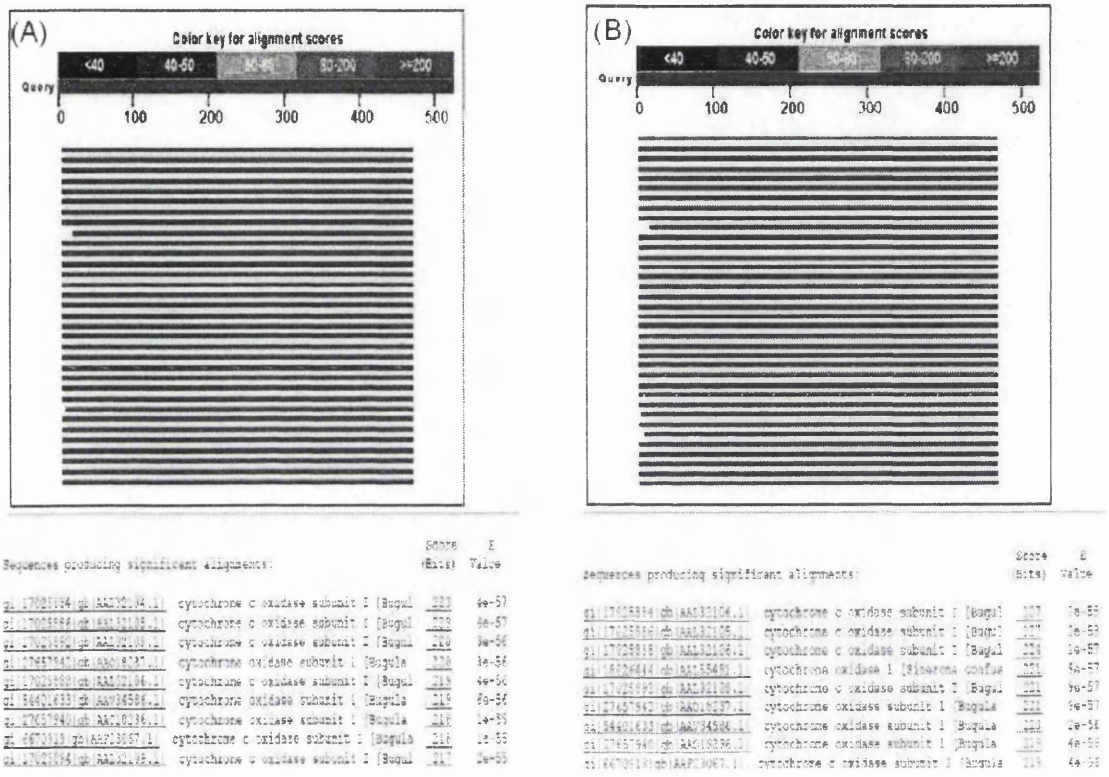
Using this specific *Callopora lineata* sequence, a *Callopora* specific set of primers was designed (called COI-2). Using this new set of primers, DNA from lysed *Callopora lineata*, *Callopora dumerilii* but not *Callopora rylandi* larvae was found to amplify successfully (Figure 4.18).

Figure 4.18 COI-2 *primers* amplifying larval DNA. Bands of the expected size (~470bp) using *Callopora lineata* larvae as template DNA (lanes 1-3), *Callopora dumerilii* (lanes 4-6) and *Callopora rylandi* (lanes 7-9). Marker VI, Boehringer Mannheim with lengths (bp) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220 and 154 is also shown.



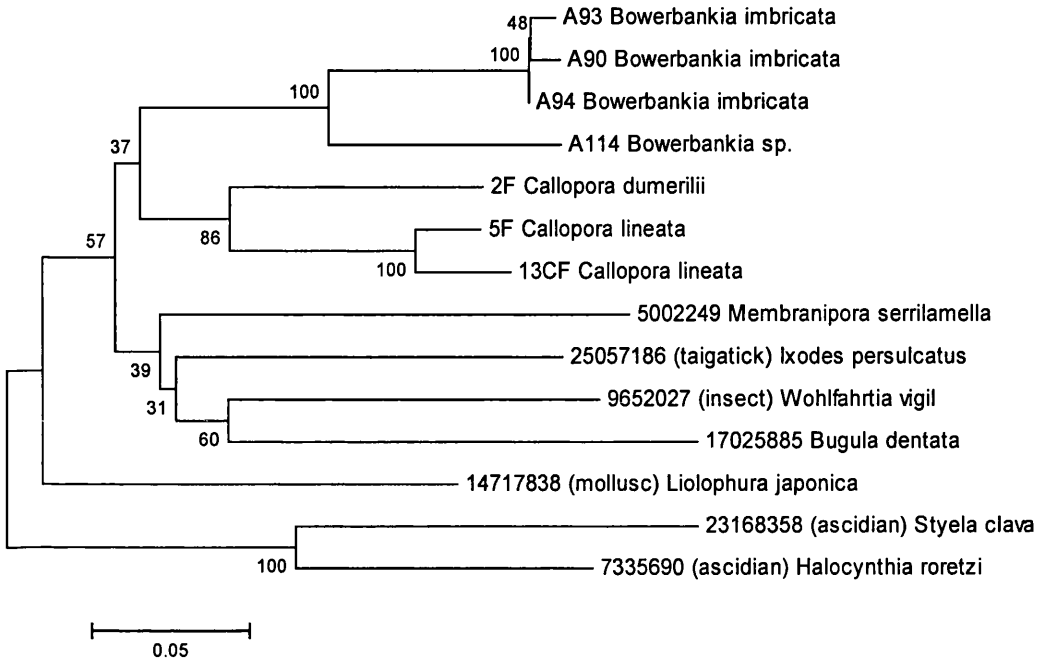
Again, the sequence data obtained using this set of primers was checked using BLAST (Figure 4.19).

Figure 4.19 Blast results for (A) *Callopora lineata* sequence and (B) *Callopora dumerilii* sequence



Both *Callopora lineata* and *Callopora dumerilii* sequences that were obtained using the COI-2 set of primers, matched with *Bugula dentata* sequences. A phylogenetic tree was again built (Figure 4.20) in order to confirm the bryozoan nature of the sequences that were obtained. The same data set (Table 4.1) was used as in the previous phylogenetic tree (Figure 4.17).

Figure 4.20 Verification of sequence validity using phylogenetic reconstruction. Neighbour-joining tree based on COI sequences. Numbers above branches are bootstrap values (500 replicates). For GenBank accession numbers, see Table 4.1. Sequences 2F and 5F are from this study.

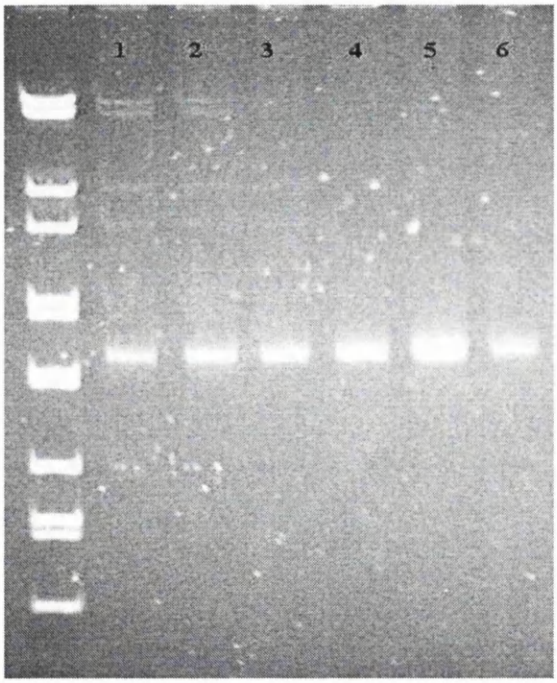


The *Callopora* sequences that were obtained by using the COI-2 set of primers formed a clade that was supported by a high bootstrap value and that clade in turn grouped with a clade that was composed with the *Bowerbankia* sequences that were obtained by Waeschenbach (2003) using COI-1 set of primer, although the support for this large clade was not strong. *Bugula dentata* does not group with bryozoan sequences, but the support in this region of the tree is not strong. These findings supported the view that the sequences obtained using the COI-2 set of primers were of bryozoan origin and consequently these primers have been routinely used to obtain *Callopora dumerilii* and *Callopora rylandi* sequences.

The partial success of the COI-2 set of primers, (*Callopora rylandi* DNA samples could not be amplified using this set) required the design of a new set of primers that would work equally well with all three *Callopora* species. Indeed, the third set of

primers that was designed (COI-3) from sequences obtained using COI-2 primers proved successful in amplifing DNA from *Callopora rylandi,* but also amplified DNA from *Callopora dumerilii* and *Callopora lineata* (Figure 4.21). A characteristic of this set of primers is the cleanliness of the products indicating the specificity of the primers for the three species.

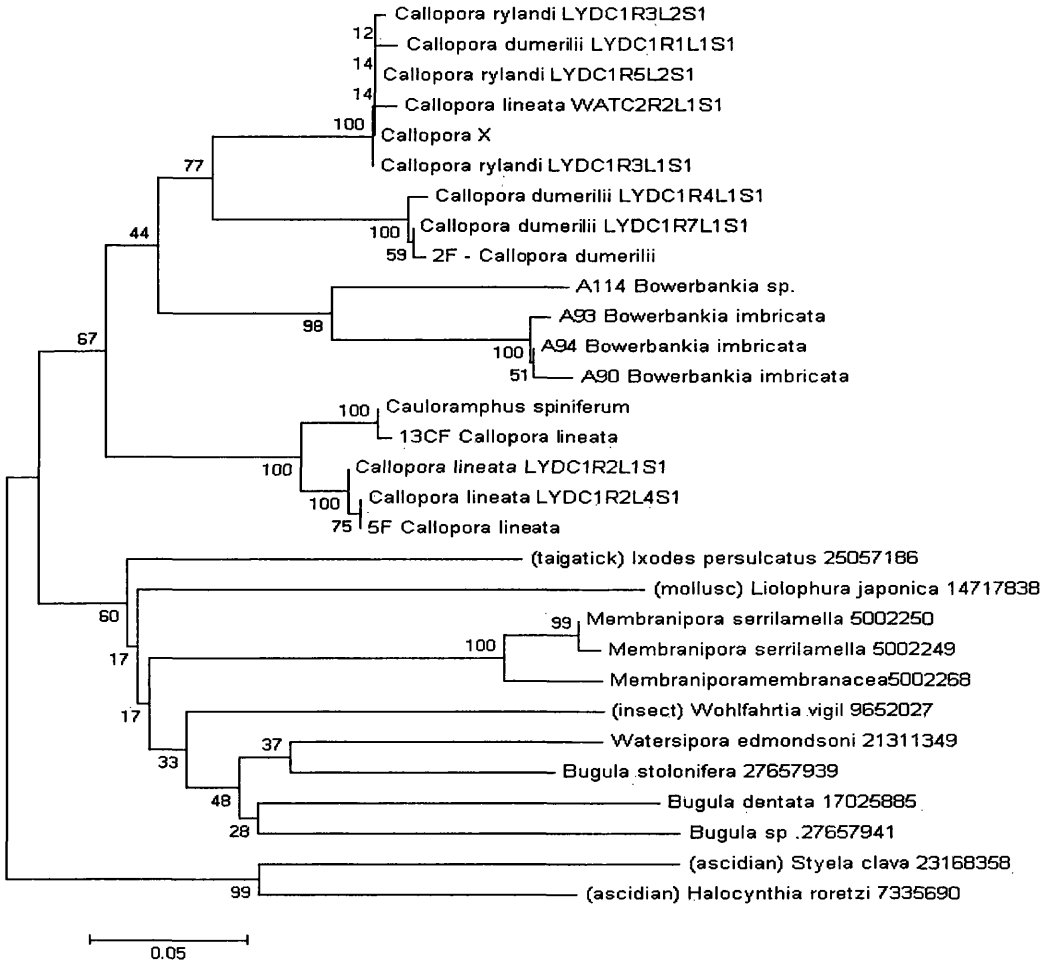
Figure 4.21 COI-3 *primers* amplifying larval DNA; Bands of the expected size (~470bp) using *Callopora lineata* larvae as template DNA (lanes 1-3), *Callopora dumerilii* (lanes 4-6) and *Callopora rylandi* (lanes 7-9). Marker VI, Boehringer Mannheim with lengths (bp) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220 and 154 is also shown.



Sequence data obtained were once again checked using BLAST with results suggesting that the sequences were of bryozoan origin. Phylogenetic reconstruction (Figure 4.22) was also used again. The sequences of Table 4.1 were used together with an additional sequence for *Cauloramphus spiniferum* (family Calloporidae, genus *Cauloramphus*) that was obtained with the COI-3 primers. An additional

Callopora sequence (marked *Callopora X*) from zooids with unusual morphology (see Chapter 2, section 2.6), which might represent a previously unknown species, which was obtained with COI-3 primers too, was also included.

Figure 4.22 Verification of sequence validity using phylogenetic reconstruction. Neighbour-joining tree based on COI sequences. Numbers above branches are bootstrap values (500 replicates). For GenBank accession numbers, see Table 4.1. Sequences A90, A93, A94 and A114 are from Waeschenbach (2003), sequences *Callopora rylandi* (LYDC1R3L2S1, LYDC1R3L1S1, LYDC1R5L2S1), *Callopora lineata* (WATC2R2L1S1, 5F, 13CF, LYDC1R2L4S1, LYDC1R2L1S1), *Callopora dumerilii* (2F, LYDC1R1L1S1, LYDC1R7L1S1, LYDC1R4L1S1), *Cauloramphus spiniferum* and *Callopora X* are from this study and sequences *Membranipora serillamella* (5002249, 5002250), *Membranipora membranacea* (5002268) and *Watersipora edmondsoni* (21311349) are from GenBank.



The *Callopora* sequences that were obtained using the COI-3 set of primers formed clades with *Callopora* sequences obtained using the previous primers. The grouping together of all the *Callopora* sequences in the tree provide good support for the view that these are genuine bryozoan sequences rather than from a contaminating organism that happens to be shared by larvae from different *Callopora* samples and species. The grouping apart of *Callopora* from the most distantly related organisms in the tree demonstrate at least that a widely distributed contaminant is not being amplified. It should be noted that the deep branches in all the three trees (Figures 4.16, 4.19 and 4.21) are not very well supported. This is to be expected given that trees are constructed from relatively short sequences. This feature is also shown by trees constructed from COI sequences in the Barcoding of Life database (http://www.barcodinglife.com) (Hebert *et al.*, 2003; Ward *et al.*, 2005). Short sequences are however valuable for species identification or providing support near the terminal branches of trees.

CHAPTER 5

POPULATION GENETIC STRUCTURE AND PHYLOGENETIC RELATIONSHIPS IN *CALLOPORA*

5.0 POPULATION GENETIC STRUCTURE AND PHYLOGENETIC RELATIONSHIPS IN *CALLOPORA*

5.1 Population genetics and population genetic structure

Population genetics is the discipline that is concerned with the study of the allele frequency distribution and change under the influence of four evolutionary forces: natural selection, genetic drift, mutation and migration. Mayr (1963) recognised that the mechanisms of evolution, which are important for influencing diversity in different populations, are fundamental in the formation of new phylogenetic lineages and ultimately new species. The genetic constitution of a population is referred to as the gene pool, which is all the alleles carried by the members of a population. As the frequencies of alleles within the gene pool change, the population evolves. If the allele frequencies remain constant, then the population does not evolve; it is said to be in a state of genetic equilibrium. Genetic equilibrium, however, can only be a hypothetical state because various evolutionary forces are always present which lead to change.

In the absence of selection, population genetic structure is determined by the balance of forces that tend to produce local genetic differentiation and forces that tend to produce genetic homogeneity (Slatkin 1987). Mutation and genetic drift due to finite population size, as well as natural selection favouring adaptations to local environmental conditions tend to differentiate local populations. On the other hand, gene flow, mediated by migration or dispersal of gametes, may either constrain evolution by preventing adaptation to local conditions or promote evolution by spreading new genes and combinations of genes throughout a species' range.

5.2 Models of dispersal and population genetic structure

Dispersal and migration used by species can affect the spatial scale and distribution of genetic structure in different ways (Slatkin 1989). The island model (Wright 1931) assumes that all populations are equal in size, with N individuals and make equal contributions to the migrant pool; all populations are equally close to all other populations (i.e. no isolation by distance); there is equilibrium and there is no selection or mutation. On the other hand, the stepping-stone model (Kimura and Weiss 1964), which is an adaptation of Wright's isolation by distance species concept (Wright 1943), assumes that migration occurs at a constant rate but only between adjacent subpopulations in either one or two dimensional arrays. At equilibrium, dispersal is more likely to occur among adjacent than distant populations. As a result, levels of genetic structure among populations may be positively correlated with geographic distance between populations (Slatkin 1993). In addition to these two models, a third was proposed by Levins (1970), which is called the metapopulation model. Levins used this term to describe a model of population dynamics of insect pests in agricultural fields, but the idea has been also applied to species in naturally or artificially fragmented habitats. This model assumes a set of subpopulations, (a 'population of populations' as was originally described), all of which exist in a balance between extinction and recolonisation and are linked to one another by dispersal and gene flow.

Dispersal refers to those processes by which a species maintains or expands the geographic distribution of a population. It has a key role in the establishment and

maintenance of biological diversity and has a significant contribution at ecological, biogeographic, genetic and evolutionary levels. Dispersal implies movement; either away from an existing population (population expansion), or away from the parent organisms. Dispersal is a one-way process and in aquatic organisms often is controlled by water currents. Water has the ability to be a supportive medium and to circulate rapidly over great distances. This allows many newly hatched young to be dispersed over large distances, often as much as hundreds of kilometres. The distance dispersed is partially correlated with time spent in the plankton (Crisp 1976). This has significant consequences for the geographical range and genetic structure of populations (Crisp 1978; Jackson 1986; Scheltema 1989). Dispersal potential can affect biological interactions such as competition or reduction of predation and may allow a species to persist in heterogeneous environments by exploring a variety of potential environments (Palmer and Strathman 1981; Strathman 1974). Dispersal may also increase the geographic range of species by exploitation of new habitats, reduction of inbreeding and maintenance of genetic continuity between metapopulations (Wilson and Hessler 1987).

Marine species are often characterised by large population sizes, high dispersal potential and in many cases wide geographic distributions. These factors combined with the continuity of the marine habitat tend to oppose isolation and divergence of populations (Palumbi 1996b). Species that differ in their life-histories and in their reproductive strategies have also differences in their dispersal capabilities. Differences in dispersal may have an impact on genetic exchange among populations within a species, which would therefore influence the way in which gene flow, genetic drift and natural selection interact in order to produce geographic variation in

gene frequencies. Broad dispersal homogenises populations genetically, as immigrants originate in different selective environments, whereas limited dispersal resulting from geographic restricted gene flow results in increased differentiation among populations as the small number of immigrants arriving from adjacent populations tend to resemble resident individuals genetically (Wright 1943; Kimura and Weiss 1964). Levels of genetic subdivision are higher for those species with limited dispersal than for similar species with broad dispersal. The reason for this is that fewer migrants move among populations and that the homogenising effects of gene flow reduce as the separation of populations increases (Slatkin 1981).

In many benthic marine organisms interspecific differences in dispersal are determined by the ontogeny of larvae. Many species have adult stages that are sessile or sedentary and larvae with different ontogenies spend time developing and dispersing in the plankton. The larvae of some species spend many weeks in the plankton (Strathman 1987) and can be found far away offshore (Scheltema 1971). However, planktonic dispersal does not guarantee the ability to survive a very long trip across the ocean or to settle in new habitats successfully. The majority of planktonic larvae die in the water column; others arrive in inappropriate habitats; others starve or are eaten by predators. The larvae of other species settle very quiclkly. For example, the mean larval dispersal of the solitary scleractinian coral *Balanophyllia elegans*, which has non pelagic planulae, based on laboratory observations and field experiments by Gerrodette (1981) is less than 0.5 metres from the parent. In addition to this study, a study by Jackson (1986) showed that larvae tend to settle within the parental microhabitat and thus raising the possibility that patches of substratum are colonised by family groups. This is particularly true for

some marine bryozoans which have been shown to be strongly philopatric, i.e. settling very close to the maternal colony and failing to colonise or having low rates of colonisation of suitable substrate of less than 1m away (Keough and Chernoff 1987). In particular, larvae of *Bugula neritina* are able to recognise kin and preferentially settle among them (Keough 1984).

Most bryozoans brood short-lived larvae which settle within seconds to hours of release by their parents. In particular, most cyclostomes and cheilostomes release short-lived lecithotrophic larvae with limited dispersal capabilities (Jackson 1985, 1986; Temkin and Zimmer 2002). Thus it is extremely unlikely that brooded larvae could be transported more than a very short distance. Laboratory experiments showed that coronate larvae of many colonial bryozoans settle within a few hours of release and settlers rapidly colonise available substrates (Jackson 1985; Keough 1989). For example, *Celleporella hyalina* broods lecithotrophic larvae that, once released from the maternal colony, swim for up to about 4 hours but usually settle within 1 hour in the presence of suitable substrata (Orellana *et al.*, 1996). Larvae of *Crisia eburnea* settle within 15 minutes of release in the laboratory (Nielsen 1970). On the other hand, *Electra pilosa* releases planktotrophic larvae that swim for several weeks before the larvae are ready to settle (Schopf 1977). The *Callopora* species studied in this thesis shed lecithotrophic larve with a presumed short swimming period, probably settling very soon after release.

In general larval settlement behaviour affects in different ways larval dispersal. For instance, larvae that settle randomly are expected to settle immediately after they acquire the capability to do so, whereas larvae that utilise settlement signals are

expected to spend more time in the plankton before they come across the suitable signal (Toonen and Pawlik 1994).

5.3 The use of molecular markers in population genetics

5.3.1 Molecular marker characteristics

Population genetics studies require large number of polymorphisms to be identified as markers. Before the advent of DNA techniques, only a small number of markers were available. Analysis was based on the study of gene products, the majority of which were protein polymorphisms and few of these were available for most organisms. However, the DNA revolution in population genetics has caused a dramatic change in the way analyses of polymorphisms are performed. The advantages of using DNA markers are many. For example, there is more potential information on polymorphisms in DNA sequences than in proteins, techniques such as PCR or sequencing are in principle the same for any DNA segment and different types of DNA polymorphism are available each of which has its advantages.

Depending on the type of analysis, molecular markers should meet some criteria that fit them for particular types of analysis. Ideally, the molecular marker should be present as a single copy in the haploid genome rather than be a sequence that is repeated within individuals. Thus, single-copy genes can be used. This is critical because if the molecular marker is present in more than one repeated copy within

individuals, such as nuclear ribosomal genes, then the sequences obtained from different individuals might be from different copies of the gene.

When DNA sequences are used in population genetics, the ease of alignment is an important consideration. It is generally accepted that deletions, duplications or both can occur which results in alteration of the sequence length. Therefore, sequences need to be aligned and gaps removed before analysis. For protein coding genes this is straightforward since gaps always occur in groups of three corresponding to codons, otherwise there would be a frameshift mutation in the alignment. In ribosomal genes the above does not apply and alignments could prove much more difficult. Either regions with poor alignment are removed (Gatesy *et al.*, 1993) or secondary structure information is used to aid alignment (Kjer 1995).

DNA sequences should also have a mutation or substitution rate that is high enough to provide a sufficient number of variable sites. However, substitution rate should be low enough to avoid large numbers of multiple substitutions. If the rate is too high, then there will be sites that have two or more substitutions and further substitutions will mask previous ones. This results in what is known as saturation which could cause problems when constructing phylogenetic trees.

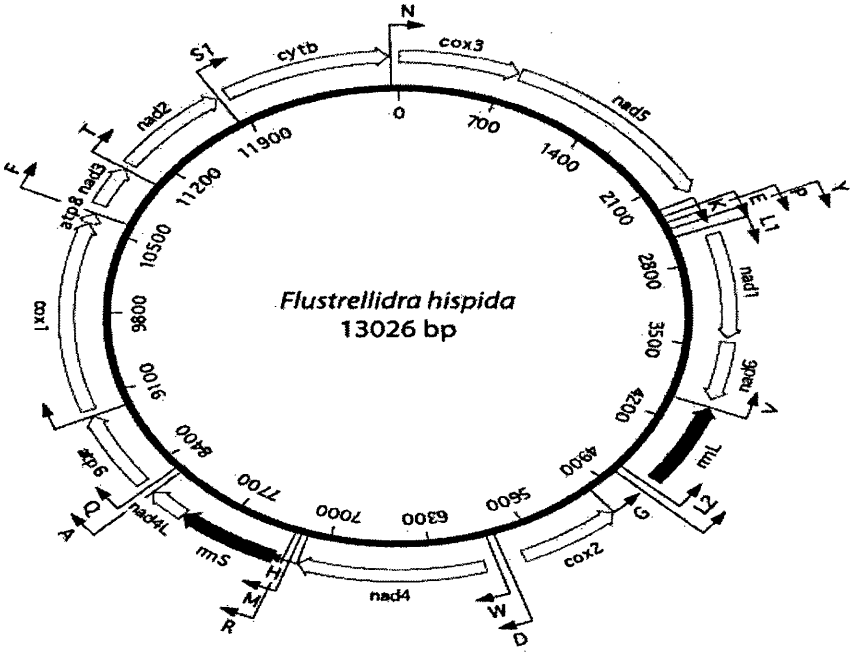
In this study, the mitochondrial DNA has been selected as the marker of interest since the mitochondrial genome of animals represents a better target for analysis than the nuclear genome because of its lack of introns, its limited recombination and its haploid mode of inheritance. In addition, there are good PCR primers that enable the

amplification of specific segments of the mitochondrial genome from a wide range of animals (e.g. Folmer *et al.*, 1994).

5.3.2 Mitochondrial DNA (mtDNA)

Animal mitochondrial DNA is a small, duplex, covalently closed circular molecule the size of which varies in different species (16-20kb) averaging approximately about 16kb. There are some exceptions, for example the mitochondrial DNA of the deep-sea scallop *Placopecten magellanicus*, which is approximately 34kb in length (Snyder *et al.*, 1987). The mitochondrial DNA genome of some bryozoans has been estimated to be approximately 16-20kb (Porter *et al.*, 2001), however, Waeschenbach *et al.* (2006) sequenced the complete mitochondrial genome of *Flustrellidra hispida* concluding that the genome is relatively small at 13026bp long (Figure 5.1).

Figure 5.1 Mitochondrial genome map of *Flustrellidra hispida*. Protein-coding genes are shown as open arrows, ribosomal RNA genes as closed arrows and tRNAs as thin arrowed lines. Transcription direction is indicated by arrow direction. (Adapted from Waeschenbach *et al.*, 2006).



In many animals mitochondrial DNA has 37 genes coding for 22 transfer RNAs, 2 ribosomal RNAs (12S and 16S) and 13 proteins that are involved in the electron transport and oxidative phosphorylation of the mitochondria. In addition to these genes, a region known as the "D-Loop" (in vertebrates and echinoderms) or "A+T-rich region" (in Drosophila) about 0.8kb long appears to exercise control over mitochondrial DNA replication and RNA transcription (Avise *et al.*, 1987). A characteristic of the mitochondrial genome is its efficient arrangement with little noncoding DNA. Gene arrangement is stable although differences in gene order appear in some organisms. For example, the mitochondrial DNA of two nematodes *Caenorhabditis elegans* and *Ascaris suum* (Okimoto *et al.*, 1992) contains the genes for 2 ribosomal RNAs, 22 transfer RNAs and 12 protein genes which are the same as the 12 of the 13 protein genes found in other metazoan mitochondrial DNAs. A gene for ATPase8 that precedes the ATPase6 gene in other metazoan mtDNAs has not been located in either of the two nematode mtDNAs. In *A. suum* and *C. elegans* mtDNAs, all genes are transcribed in the same direction. This again contrasts to the situations found in other totally sequenced metazoan mtDNAs where different proportions of protein and tRNA genes are transcribed in opposite directions. In addition, unusual features regarding nematode mitochondrial tRNA genes and mitochondrial protein gene initiation codons have been found. In the *C. elegans* and *A. suum* mitochondrial-genetic codes, AGA and AGG specify serine, TGA specifies tryptophan and ATA specifies methionine, which is different from other organisms. Similar differences in gene arrangements have been found between sea urchin and human mitochondrial DNA (Roberts *et al.*, 1983). Restriction fragment cross-hybridization analysis showed that the 16S rRNA and cytochrome oxidase subunit 1 genes are adjacent in sea urchin mtDNA. These two genes are separated in human and

other mammalian mtDNAs by the region containing unidentified reading frames 1 and 2.

In *Flustrellidra hispida* (Figure 5.1) all but one of the 36 genes typically found in metazoan genomes were identified. The gene order is unique amongst metazoan mitochondrial genomes so far characterised. There are 17 regions of non-coding nucleotides, ranging in size from 1 to 195nt, totalling 506 nucleotides. Two of these offer possible origins of replication. The small proportion of non-coding regions and numerous regions of overlap between genes result in a highly compact genome. Initiation codons are ATA, ATG, TTG and ATT. Initiation codons of the invertebrate mitochondrial code not used are ATC and GTG. Termination codons are TAA, TAG and the abbreviated stop codon T. A total of 21 of the typical complement of 22 tRNA genes were identified.

As a molecular marker mitochondrial DNA has many advantages that make it a sensitive marker for detecting population subdivision in marine invertebrate species. For example Avise *et al.* (1992) used mtDNA to characterise genetic differentiation between populations within and outside the Gulf of Mexico in a range of marine species. Sharp discontinuities were observed in terms of haplotype frequencies and deep splits were observed on evolutionary trees constructed from the haplotypes. A similar situation was found in the study of Quesada *et al.* (1995). It was shown that a major genetic break in mtDNA haplotypes between Atlantic and Mediterranean populations of the mussel *Mytilus galloprovincialis* was detected in the east side of the Alborán Sea (Almeria-Orán oceanographic front). This study clearly fits with the

distinction between Mediterranean and Atlantic populations and demonstrates the passive dispersal of pelagic larvae of marine species through the Straits of Gibraltar.

There have also been studies comparing different types of markers. Hurst and Skibinski (1995) used allozyme and restriction enzyme analysis of mtDNA to study variation in samples from British populations of the marine limpet *Patella vulgata* in two regions. Allozyme analysis revealed significant differences in allele frequencies among samples. Population differentiation values were found higher within regions rather than between them, indicating that genetic heterogeneity was localised and not related to geographic separation.

A very interesting feature of mitochondrial DNA is that it has been reported to evolve faster than nuclear DNA (Brown 1985). In fact, mitochondrial DNA appears to evolve at 5 -10 times the rate observed for single-copy nuclear DNA (Wilson *et al.*, 1985). The higher rate of mitochondrial DNA evolution is mainly attributed to transitions (replacement of a pyrimidine base by the other or a purine base by the other), which often outnumber transversions (replacement of a pyrimidine by a purine or vice versa) by 10 to 1 in intra-specific comparisons (Meyer 1993). The predominance of transitions is greatest in comparisons of closely related sequences and it decreases as sequences diverge, until no bias is detectable. However, there is evidence for major variability in relative rates among taxa. For example, the mitochondrial DNA and single-copy nuclear DNA in sea urchins and flies evolve at similar rates (Vawter and Brown 1986; Martin and Palumbi 1993). Another interesting feature of mitochondrial DNA for population genetic studies is the fact that in most organisms it does not appear to undergo recombination. Even though it contains 37 genes, it can be

regarded as a single genetic marker with multiple alleles; therefore genotypes for mitochondrial DNA are referred to as haplotypes that correspond to non-recombining characters.

However, recent studies challenge the universal view that mitochondrial DNA does not recombine. In 1999 two population studies have raised the possibility of recombination in primate mitochondrial DNA. One of them was based on a high frequency of homoplasies (Eyre-Walker *et al.*, 1999) and the other was based on a negative correlation between linkage disequilibrium and physical distance along the molecule of mitochondrial DNA variants (Awadalla *et al.*, 1999). However, these two studies have received much criticism about the validity of the assumption that mitochondrial DNA recombines (Arctander 1999; Merriweather and Kaestle 1999; and Kivisild *et al.*, 2000). In addition, Ladoukakis and Zouros (2001) presented evidence for mitochondrial DNA recombination in mussels from direct recovery of recombinant molecules in the gonads of several males of the mussel *Mytilus galloprovincialis*. In this species two lineages of mitochondrial genomes exist, each with a different mode of transmission, a phenomenon known as doubly uniparental inheritance of mitochondrial DNA, therefore the possibility existed that mitochondrial DNA recombination was a special property of doubly uniparental inheritance. In addition to this study, Burzynski *et al.* (2003) presented evidence for recombination of mitochondrial DNA in the marine mussel *Mytilus trossulus*, by demonstrating the existence of two recombinant mitochondrial DNA variants from within the non-coding region. Apart from these studies, direct evidence for recombination events exists in three more animal species. These are the nematode *Meloidogyne javanica* (Lunt and Hyman 1997), the flatfish *Platichthys flesus* (Hoarau *et al.*, 2002) and most

recently, humans (Kraytsberg *et al.*, 2004). The latter is a unique case of biparental inheritance of skeletal mitochondrial DNA and has been reported in a man suffering from a form of mitochondrial myopathy. Although several mechanisms could be involved in mtDNA recombination such as intramolecular and intermolecular crossing-over and nonhomologous and homologous recombination, it is homologous recombination that has strong implications for the evolution of mitochondrial genomes.

To a population geneticist the most interesting feature of mtDNA is its uniparental mode of inheritance through the egg cytoplasm (maternal inheritance). Thus, unlike the situation for nuclear DNA, the mitochondrial DNA mutations arising in different individuals are not recombined during sexual reproduction. Exceptions to this maternal mode of inheritance appear to be the biparental inheritance in mussels (Zouros *et al.*, 1992; Fisher and Skibinski 1990) as well as the paternal inheritance in mice (Gyllesten *et al.*, 1991). In marine Bryozoa there is currently no evidence to suggest biparental inheritance; however this does not mean it does not occur.

Recombination and biparental inheritance of mtDNA are factors that would affect the interpretation of many studies using this molecule. Another limitation to the use of mitochondrial DNA is the condition called heteroplasmy, which is defined as the presence of more than one mitochondrial DNA type within an individual. Evidence for heteroplasmy has appeared over the years in literature for organisms like *Drosophila* (Hale and Singh 1986), fish (Bentzen *et al.*, 1988) and others. There are two kinds of heteroplasmy. The first one is called length heteroplasmy, where two mitochondrial DNA molecules of different length exist in the same individual and is

caused by insertions, deletions or both in the D-Loop (Moritz and Brown 1986). The second type of heteroplasmy, which is much rarer than length heteroplasmy is called restriction heteroplasmy and is a state where mitochondrial DNA molecules with different restriction sites occur in the same individual. Evidence for restriction heteroplasmy have been reported in many organisms, for example *Drosophila* (Solignac *et al.*, 1984); mussels (Fisher and Skibinski 1990) and in anchovy (Magoulas and Zouros 1993).

An ideal molecule for phylogenetic use would be free from reversals as well as parallel or convergent evolutionary changes. Mitochondrial DNA, however, is characterised by a remarkably high level of within-species homoplasy. Homoplasy can occur by convergence or by parallelism. Convergence describes similarities between two species that evolved independently from different features in their common ancestor, whereas parallelism occurs when two groups independently develop similarities from the same structures. In DNA, the phenomenon of homoplasy is mainly attributed to recurrent transitional base substitutions at some nucleotide sites (Aquadro and Greenberg 1983). Homoplasy may cause problems in population genetic analysis as it can affect measures of genetic diversity, gene flow, genetic distances (both between individuals and populations), assignment methods and phylogenetic analysis (Estoup *et al.*, 2002).

A potential problem with mitochondrial genes is that genes can be transferred from the mitochondrion to the nucleus. These genes, called numts (nuclear copies of mitochondrial genes) or mitochondrial pseudogenes, can be amplified and sequenced by mistake and this can lead to erroneous analyses (Bensasson *et al.*, 2001).

Currently, reports of numts have been observed in several vertebrates (Lopez *et al.*, 1994; Arctander 1995) and invertebrates (Zhang and Hewitt 1996; Sunnucks and Hales 1996; Gellissen *et al.*, 1983). The characteristic features of this phenomenon can be summarised as follows (Zhang and Hewitt 1996). Many mitochondrial regions have been found to be integrated into the nuclear genome, including protein-coding, rRNA-coding and non-coding regions, the control regions, rRNA genes, cytochrome *b* and genes for the subunits I and II of the COI gene, with the latter being the most commonly described. The nuclear insertion can be a large mitochondrial fragment and may have a high copy number (Lopez *et al.*, 1994). Nuclear insertions show various degrees of homology with their mitochondrial counterparts, depending on the region and the taxa involved. Numts can be easily identified as PCR ghost bands, extra bands in restriction profiles, sequence ambiguities, frameshift mutations, stop codons and unpredicted positions on phylogenetic trees. The first step to deal with numts is by utilising techniques such as single stranded conformation polymorphism (SSCP) analysis, constant denaturant capillary electrophoresis (CDCE), cloning and sequencing of PCR products in order to establish whether or not more than one mtDNA sequence has been amplified. Numts can be avoided by increasing the amount of mtDNA amplified. To do that purifying mitochondria prior to DNA extraction by long PCR amplification or by using tissue that is rich in mtDNA relative to nuclear DNA is essential. A more efficient way to deal with numts is by using RT-PCR. Using this technique cDNA is produced from mRNA, which is devoid of introns and other regulatory sequences thus making the amplification much simpler.

In this study the cytochrome oxidase I (COI) has been selected as the target gene of interest. COI has been widely employed in addressing questions of animal genetic

diversity, population genetic structure and dynamics, phylogeography and population evolution. One of the advantages of using COI is that the universal primers for this gene are very robust, enabling recovery of its 5' end from the representatives of most, if not all, animal phyla. COI has been extensively used in the DNA Barcoding project as the core of a universal bioidentification system for animals. It was found that COI profiles usually assigned newly analysed taxa succesfully to the appropriate phylum or order and that species-level identifications could be obtained using large datasets of COI profiles. It was shown that a model COI profile, based upon the analysis of a single individual from each of 200 closely allied species of lepidopterans, was 100% successful in correctly identifying subsequent specimens (Hebert *et al.*, 2003). Application of COI Barcoding was successfully used to identify fish species (Ward *et al.*, 2005).

5.4 Previous population genetics studies in Bryozoa

Studies similar to this one which are concerned with aspects of population genetics of a particular marine bryozoan species using mitochondrial DNA, are relatively scarce. One of them is the mitochondrial DNA study of Schwaninger (Schwaninger 1999) who examined genetic relationships among allopatric populations and sympatric morphs of the cheilostome algal epiphyte *Membranipora membranacea*, which has long-lived planktotrophic larvae. Allozymes were used to clarify the relationships among four widely separated populations, two in the North Pacific and two in the North Atlantic Ocean. Allozymes and mitochondrial DNA sequencing were then used to shed light on the genetic relationships among three sympatric morphs that might

correspond to the species *Membranipora vilosa* and *Membranipora membranacea* in the North-Eastern Pacific. Results from this study suggested that high levels of gene flow occur between populations on the west coast of North America.

In another study, Hoare *et al.* (1999) used microsatellite loci to analyse British populations (Menai Strait and Plymouth) of *Celleporella hyalina*, a cheilostome algal epiphyte with lecithotrophic larvae. Their results showed that there were no significant differences between populations up to 8 km apart, whereas there was a considerable genetic subdivision between populations from the Menai Strait and Plymouth (500 km apart) indicating possible isolation by distance. It was suggested that dispersal which was likely to explain such genetically homogeneous populations on a scale of up to 8 km, might be due to rafting of the algal substratum.

Goldson *et al.* (2001) used the RAPD technique to evaluate the interaction between larval dispersal and hydrography in the population genetic structure of the two marine bryozoans *Celleporella hyalina* and *Electra pilosa.* Their analysis showed an unexpectedly high degree of constraint exerted by hydrographic factors on the dispersal of pelagic larvae. Samples of *Celleporella hyalina* showed genetic differentiation over distances as small as 10m, which agrees with their limited mode of dispersal, whereas *Electra pilosa* showed higher levels of genetic heterogeneity over larger distances as expected from their mode of dispersal, which is much higher than *Celleporella hyalina* due to the long-lived planktotrophic larvae. In addition, they also suggested that the population differentiation observed between samples of *Electra pilosa*, collected from sites 70 km apart, is consistent with coastal water currents and frontal systems that restrict the exchange of water masses between the

two sites. In other words, gene flow is constrained more when larvae have a relatively short pelagic phase, but the effect is still significant even when the pelagic phase is much longer. Hydrography and heterogeneous distribution of suitable substrata therefore are likely to create many opportunities for local genetic differentiation.

A similar study, where the genetic structuring in two marine bryozoans with different modes of reproduction and dispersal of sexually derived larvae was examined is the one by Porter *et al.*, (2002). In this study, the RAPD technique was used to investigate the relationship between reproductive mode and genetic population structure in two species of *Alcyonidium* (*Alcyonidium gelatinosum* and *Alcyonidium mytili*) with contrasting larval modes and adult habitats and to compare patterns of genetic diversity and structure between the two marine species and a freshwater bryozoan (*Cristatella mucedo*). The results from this study suggested high levels of population genetic substructure in the populations of *Alcyonidium gelatinosum*, which is due to the short-lived lecithotrophic larvae with limited dispersal ability. A highly significant level of population substructure was also observed in *Alcionidium mytili*, which is perhaps due to the low levels of water exchange in rias and estuaries. In addition, this study showed that bryozoans with different reproductive strategies show distinguishing levels of population genetic structure. In other words, the marine bryozoan species showed greater clonal diversity compared to the freshwater one and that a species with pelagic larvae demonstrated lower levels of intrapopulation similarities compared to the one with lecithotrophic larvae. This could have been attributed to lower levels of dispersal than predicted by the planktotrophic species or higher levels of dispersal due to rafting of the lecithotrophic species.

All the above studies mentioned suffered the limitation that replicate developmental types from similar environments were not considered. However, a very recent study by Watts and Thorpe (2006) made replicate comparisons of the population-genetic structure of species of bryozoa that have contrasting larval developmental modes. Their study characterised the pattern of variation at allozyme loci among British populations of four species of bryozoa; two species with cyphonate larvae (*Electra pilosa* and *Membranipora membranacea*) and two species with coronate larvae (*Schizoporella unicornis* and *Cryptosula pallasiana*). The results of their study showed variation in the estimates of genetic differentiation among similarly separated populations that may be a consequence of non-equilibrium genetic conditions which result in from infrequent migration, perhaps through dispersal by rafting on macroalgae. However, similar to the previous studies, the level of genetic differentiation between populations as well as the pattern of migrant exchange is linked with the larval developmental mode. In other words, species with coronate larvae show higher levels of genetic heterogeneity between populations and significant isolation by distance genetic structure whereas distance has little or no effect upon the amount of genetic differentiation among bryozoan populations with cyphonautes larvae. In conclusion, it appeared that for some cheilostome bryozoans at least genetic differentiation between populations was closely associated with the type of larval development.

5.5 Aims

The main objective of this chapter is to examine the genetic structure in populations of *Callopora dumerilii*, *Callopora rylandi* and *Callopora lineata* using the mtDNA COI gene to test hypotheses concerning levels of population differentiation and intrapopulation variation. The relationships of mtDNA lineages within and between species will also be examined to clarify the phylogenetic relationships of the three species and to search for possible phylogenetic subdivisions within species.

5.6 Materials and methods

5.6.1 The methods

Obtaining DNA sequence data was an essential part in this study. The theory and the protocols of the molecular methods that have been used were described in detail in Chapter 4. Here only the basic outline that led to the acquisition of sequence data will be presented.

Initially, DNA extraction was performed using the larval lysis method (Section 4.3.1). In cases where sequence data were suspected to be "contaminated" with numts, cDNA synthesis was performed (Section 4.3.8). PCR amplification using specific primers (Section 4.4) then followed to amplify the gene of interest, followed by product purification (Section 4.2.2.4). SSCP analysis was then routinely used to screen PCR products for variation (Section 4.3.9). Once SSCP haplotypes on gels were identified, PCR products corresponding to those haplotypes were cloned (Section 4.3.5) and subsequently sequenced (Section 4.3.7). This procedure allowed assigning sequences to SSCP samples without the necessity to sequence every sample.

5.7 Data analysis

5.7.1 BLAST

Prior to any analysis, sequences were loaded into the NCBI website and the BLAST tool (Altschul *et al.*, 1990) was employed (Section 4.4). BLAST (Basic Local

Alignment Similarity Search Tool) is a set of programs designed to perform similarity searches on all available sequence data. BLAST uses an algorithm that seeks out local alignment, in other words the alignment of some portion of two sequences, in contrast to global alignment i.e. the alignment of two sequences over their entire length. By searching for local alignments, BLAST is able to identify regions of similarity within two sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. Each hit is assessed by a score value, which is known as the E-value and which is a statistical calculation based on the score that gives the number of hits of this score that this search would return by chance using a database of this size.

The following are some of the programs that are included in BLAST and were used in this study:

1. **Protein-protein BLAST (blastp)**: this program, given a protein query, returns the most similar protein sequences from the protein database.
2. **Nucleotide 6-frame translation-protein (blastx)**: this program uses the BLAST algorithm to compare the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.
3. **Nucleotide 6-frame translation-nucleotide 6-frame translation (tblastx)**: this program compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database. This program is the slowest of all the blast programs and is used is to find very distant relationships between nucleotide sequences.

4. **Protein-nucleotide 6-frame translation (tblastn)**: This program compares a protein query against the six-frame translations of a nucleotide sequence database.

The program Blastx was found to be more efficient at returning bryozoan sequences rather than the other methods such as the Blastp.

5.7.2 Translation and sequence alignment

Having ensured sequence validity, sequences were subsequently translated to amino acid sequences by using the online tool Emboss-Transeq (Rice *et al.*, 2000). This tool translates nucleic acid sequences to the corresponding protein sequence. It can translate in any of the three forward or three reverse sense frames or in all three forward or reverse frames or in all six frames. Nucleotide sequences were translated to proteins using the invertebrate mitochondrial DNA code and then reloaded into the NCBI website but this time in order to determine the reading frame.

Each forward and reverse complemented sequence were aligned using Clustal X, version 1.83, (Thompson *et al.*, 1997) and a consensus sequence was derived. This was done for all sequences for all three species from all populations. The sequences from the three populations were then separated into haplotypes. The consensus sequences from each population separately were loaded in MEGA, version 3.1, (Kumar *et al.*, 2004) and trimmed to equal lengths for further analysis. When trimmed to equal length the consensus length was 333bp.

5.7.3 Arlequin analysis

Data were analyzed using the Arlequin software package (Version 3.1) developed by Excoffier *et al.*, (2005). Molecular diversity was estimated as both haplotype and nucleotide diversity based on the complete gene sequences and default settings. Genetic differentiation between populations was estimated using the analysis of molecular variance (AMOVA), in addition to pairwise population comparisons (F_{ST}). AMOVA is a method of estimating population differentiation directly from molecular data and testing hypotheses about such differentiation. The AMOVA takes account of both haplotype frequencies and the DNA sequence divergence of the haplotypes. In this study, AMOVA analyses were performed using both haplotype frequencies (conventional F-statistics) as well as taking account of sequence divergences.

5.7.4 Sample diversity measures

5.7.4.1 Nei's haplotype diversity (*h*)

Mitochondrial DNA diversity was estimated in the form of haplotype diversity (*h*) for each location. This was calculated within populations for each species and overall for each species. Haplotype diversity gives the probability that two haplotypes chosen at random are different in a population. Haplotype diversity was estimated according to the method of Nei (Nei 1987) with the formula:

$$h = N\,(1 - \Sigma x_i^2)/\,(N-1)$$

where x_i is the frequency of each haplotype and N is the sample size.

This is analogous to heterozygosity or gene diversity that is used in the study of polymorphisms of diploids.

5.7.4.2 Nucleotide diversity (*Π*)

Nucleotide diversity (*Π*) was calculated as the number of nucleotide differences between a pair of sequences, averaged across all possible pairs of sequences in the sample. Nucleotide diversity (*Π*) was first introduced by Nei and Li (1979) and is calculated according to the formula: $\Pi = \sum_{ij} x_i x_j \pi_{ij}$ where π_{ij} is the proportion of different nucleotides between the *ith* and *jth* types of DNA sequences and x_i and x_j are the respective frequencies of these sequences.

5.7.5 Tests of neutrality

According to the neutral mutation hypothesis, variation within populations as well as between populations is due to neutral or nearly neutral mutations (Ohta *et al.*, 1992). This hypothesis can be tested by analysing DNA sequence variation within and between populations. Many such tests have been developed. In this study the following have been used:

1. Tajima's D test statistic (Tajima 1989)
2. Fu's F_s test statistic (Fu 1997)
3. Comparing rates of non-synonymous and synonymous substitutions
4. The McDonald-Kreitman test (McDonald and Kreitman 1991)

5.7.5.1 Tajima's (D) test statistic

Tajima's test statistic compares the number of segregating sites per sequence with the nucleotide diversity. Under neutral theory, the expected amount of genetic variation per nucleotide is equal to $\theta = 4N_e\mu$ (where N_e is the effective population size and μ is the neutral mutation rate). θ can be estimated using either the number of segregating sites (S) (i.e. the number of variable nucleotide sites in a number of sequences) or the average number of pairwise differences between sequences in a sample (i.e. nucleotide diversity). Useful information on evolutionary forces can be obtained by comparing the results of different estimators of θ. This is the basis of the Tajima test. In neutral theory, $\theta = \frac{S}{a}$, where S is the number of segregating sites and (a) is a parameter that is related to the sample size. The Tajima test statistic (D) has the quantity $(\Pi - \frac{S}{a})$ as numerator. In neutral theory, it is expected that D equals zero. Purifying selection refers to selection against disadvantageous mutations and will cause many rare alleles. Thus S will be higher than Π and D will be negative. On the other hand balancing selection, which refers to selection that works to maintain polymorphism in a population, results in elevation in the value of Π, but a decrease in the number of segregating sites, thus giving D a positive value.

5.7.5.2 Fu's test statistic (F_s)

Analogous to Tajima's D test statistic is the test developed by Fu based on what is called Fu's test statistic (F_s). This test has greater sensitivity compared to Tajima's

test for detecting excesses of rare mutations and thus, neutrality can be more easily rejected when there is an excessive number of rare mutations. Fu's (F_s) test as well as the Tajima's D test were applied to test the neutral mutation hypothesis and to test for a population expansion which can affect the values of these statistics. In both cases, significance was assessed by 1000 simulated samples under the hypothesis of selective neutrality and population equilibrium as implemented in Arlequin.

5.7.5.3 Comparing rates of nonsynonymous and synonymous substitutions

Another group of neutrality tests that can detect selection but are not sensitive to demographic changes such as population expansion include the d_N/d_S ratio test and the McDonald-Kreitman test (Section 5.7.5.4). Examining rates of nonsynonymous (d_N) and synonymous (d_S) substitutions when two sequences are aligned and compared can lead to valuable evolutionary information. If the sequences have diverged entirely because of genetic drift, then the ratio d_N/d_S equal one. This statement, however, assumes that the neutral mutation rates are the same for both nonsynonymous and synonymous sites. If purifying selection has acted on the sequences, then d_N will be reduced as a result of deleterious nonsynonymous mutations increasing in frequency less readily within populations and are thus less likely to become fixed between lineages. There will be less or no effect on d_S. Therefore, with purifying selection the ratio d_N/d_S will be less than one. On the other hand, if positive selection has acted to increase the frequency of some beneficial new amino acid changing, non synonymous, mutations within populations, then d_N but not d_S will be elevated, therefore, the ratio d_N/d_S will be higher than one. For example,

many of the genes where $d_N/d_S > 1$ code for the antigenic surface proteins of parasites and viruses (Endo *et al.*, 1996), presumably reflecting the race to escape the immune system of their hosts. Endo *et al.* (1996) examined 3595 groups of homologous sequences from the DNA databases and found only 17 (0.5%) candidate loci for positive selection which had $d_N/d_S > 1$ over the entire gene sequence.

5.7.5.4 McDonald-Kreitman test

The McDonald-Kreitman test (McDonald-Kreitman 1991) considers the relationship between polymorphism and divergence by examining whether the ratio of nonsynonymous to synonymous substitutions differs within and between species. The McDonald-Kreitman method uses a 2x2 contingency table to test the independence of one classification (polymorphic differences versus fixed) from the other (synonymous changes versus non-synonymous). A nucleotide site in the sequences is said to be polymorphic if it exhibits any variation in one or both species. Fixed refers to the condition where there is no intraspecific variation within either species but there are differences between the species. All other sites are monomorphic and are not included in the analysis.

The test is based on the following three assumptions: Only non-synonymous mutations may be adaptive, synonymous mutations are always neutral and a selectively advantageous mutation will be fixed in the population faster than a neutral mutation and therefore is less likely to be found in a polymorphic state.

Under the neutral mutation hypothesis, it is expected that the ratio of fixed non-synonymous to fixed synonymous differences will be the same as the ratio of non-synonymous polymorphisms to synonymous polymorphisms. Significant differences between the two ratios can therefore be used to reject the neutral mutation hypothesis. An excess of nonsynonymous polymorphisms would indicate purifying selection. An excess of nonsynonymous fixed differences between species would indicate positive directional selection. For example, Rand and Kann (1998) applied the McDonald–Kreitman test to mtDNA using ten species pairs from diverse animal groups. All tests showed an excess of nonsynonymous polymorphism and in five tests this was significant. These results provide general support for the nearly neutral theory and for the widespread occurrence of slightly deleterious mutations in mtDNA.

5.7.6 Phylogenetic analysis

There are numerous methods for constructing phylogenetic trees from molecular data (Nei and Kumar 2000). The methods used in this study are neighbour-joining (Saitou and Nei 1987), minimum evolution and maximum parsimony. The neighbour-joining method is related to traditional cluster analysis, but removes the assumption that the data are ultrametric. The neighbour-joining method starts with a star-tree topology and sequentially chooses the pair of OTUs (DNA sequences in this case) which when linked together minimise the total length of the tree. This process is continued until the tree is completely resolved. The main advantage of the neighbour-joining method is its efficiency. It can be used on very large data sets for which other means of phylogenetic analysis (e.g. minimum evolution, maximum parsimony and maximum

likelihood) are computationally prohibitive. In the minimum evolution method, the basic procedure is first to obtain a neighbor-joining (NJ) tree and then to search for a tree with the minimum value of the sum (S) of branch lengths by examining all trees that are closely related to the NJ tree. Once the ME tree is identified, a statistical test is conducted for the difference in S between this tree and other closely related trees. The construction of a minimum evolution tree is time-consuming because, in principle, the S values for all topologies must be evaluated. The number of possible topologies (unrooted trees) rapidly increases with the number of taxa so it becomes very difficult to examine all topologies. Maximum parsimony methods originally were developed for morphological characters. Maximum parsimony is a technique used to infer a phylogenetic tree on the basis of observed data on the similarities and differences among DNA sequences. The input data used in a maximum parsimony analysis is in the form of "characters" (nucleotides in this case) for a range of taxa. The trees used in maximum parsimony analysis can be unrooted trees (there is no indication of time in the tree, only the relations between taxa) or rooted trees where an outgroup and ancestor are specified. Maximum parsimony trees are those that allow the explanation of the observed distribution of character states across taxa with the fewest number of changes between character states on the trees.

The resulting phylogenetic trees had the reliability of their tree nodes assessed by making use of the most commonly used statistical test in phylogenies that of bootstrap analysis (Felsenstein 1985). The bootstrap technique uses resampling with replacement. In other words, the total number of sites along the sequence alignment is drawn with replacement and all sites have the same probability of being sampled. Since it is a replacement technique, some sites will be sampled more than once,

whereas other will not. For each bootstrap replicate a tree is built. After all replications are made the trees are compared and the proportion of bootstrap trees that produced a particular grouping is assigned to that branch of the tree. The logic behind the bootstrapping technique is that if the data would be resampled, leaving out a part of them and the result is a different topology, then the data should not be trusted. Bootstrap provides a measure of the internal consistency of the data set. In other words, it does not yield the probability that a certain group is present on the true tree (accuracy), but rather it gives the probability that the same group will be present if a completely different set of data will be used (repeatability).

5.8 Results

5.8.1 Sequence characteristics

A 432-bp portion of COI was sequenced from 38 *Callopora dumerilii* and 34 *Callopora lineata* colonies respectively and a 470-bp of sequence was obtained from 27 colonies of *Callopora rylandi* (Table 5.1). For *Callopora dumerilii*, 81 nucleotide sites were polymorphic, 47 of which were phylogenetically informative. A total of 12 unique haplotypes were identified in *Callopora dumerilii*. For *Callopora lineata*, 91 nucleotide sites were polymorphic, 77 of which were phylogenetically informative. A total of 15 unique haplotypes were identified in *Callopora lineata*. Finally, for *Callopora rylandi*, 54 nucleotide sites were polymorphic of which 4 were phylogenetically informative. A total of 13 unique haplotypes were identified in *Callopora rylandi*.

Table 5.1 Number of colonies collected for each species from each site.

	Callopora rylandi	*Callopora lineata*	*Callopora dumerilii*
Lydstep	10	21	16
Watwick Bay	17	13	4
Pembroke Ferry	-	-	18

5.8.2 Molecular diversity

For each of the *Callopora* species, the following measures of genetic diversity were calculated: within-population diversity was estimated by haplotype diversity *h* and nucleotide diversity by Π (Table 5.2).

Table 5.2 Molecular diversity indices for three species of *Callopora* and three sites.

Species name		Nucleotide Diversity Π	Haplotype Diversity *h*
Callopora dumerilii	Lydstep	0.082	0.952
	Watwick Bay	0.159	1.000
	Pembroke Ferry	0.067	0.889
	Overall	0.083	0.930
Callopora lineata	Lydstep	0.106	0.928
	Watwick Bay	0.071	0.600
	Pembroke Ferry	-	-
	Overall	0.103	0.935
Callopora rylandi	Lydstep	0.004	0.773
	Watwick Bay	0.035	0.806
	Pembroke Ferry	-	-
	Overall	0.018	0.838

Nucleotide sequence diversity for *Callopora dumerilii* was similar for Pembroke Ferry and Lydstep except for Watwick Bay which had the higher diversity observed. For *Callopora lineata*, nucleotide sequence diversity was slightly higher for Lydstep compared to Watwick Bay. On the other hand, for *Callopora rylandi* nucleotide diversity was much lower for Lydstep compared to Watwick Bay. Overall nucleotide diversity was similar for *Callopora dumerilii* and *Callopora lineata* compared to *Callopora rylandi* which was much lower. Haplotype diversity for *Callopora dumerilii* was similar for all three samples. For *Callopora lineata* haplotype diversity was slightly higher for Lydstep compared to Watwick Bay samples, whereas for

Callopora rylandi the haplotype diversity was very similar in both populations. Overall haplotype diversity was similar in all three species.

5.8.3 Tajima's D and Fu's F_s test statistics

Tajima's D and Fu's F_s-test statistic (Table 5.3) were applied to assess if there was evidence of population growth and purifying selection which could both result in negative values of the statistic.

Table 5.3 Tajima's D and Fu's Fs test statistics.

Species name		Tajima's D	Fu's F_s
Callopora dumerilii	Lydstep	1.908 (p= 0.991)	1.855(p= 0.723)
	Watwick Bay	-	3.970 (p= 0.661)
	Pembroke Ferry	1.245 (p= 0.930)	2.879 (p= 0.890)
	Overall	0.812 (p= 0.828)	3.176 (p=0.903)
Callopora lineata	Lydstep	1.772 (p=0.986)	1.290 (p= 0.720)
	Watwick Bay	-1.032 (p=0.188)	7.594 (p=1.000)
	Pembroke Ferry	-	-
	Overall	1.638 (p=0.975)	3.529 (p=0.026)
Callopora rylandi	Lydstep	-1.713 (p= 0.031)	-4.027 (p=0.002)
	Watwick Bay	-1.769 (p=0.015)	3.477 (p=0.931)
	Pembroke Ferry	-	-
	Overall	-2.435 (p=0.000)	-0.939 (p=0.361)

Table 5.3 shows significant negative values for Tajima's D test for *Callopora rylandi* overall as well as for each population separately, which is an indication of purifying selection. No significant values were obtained for the other species. Table 5.3 also shows a significant positive value overall for *Callopora lineata* for Fu's F_s. This is in agreement with the positive value for Tajima's D. This would be consistent with balancing selection. For *Callopora rylandi* there is one significant negative value for

F_S which is consistent with the negative value for this species for Tajima's D. It appears that for both statistics the values might thus be more negative in *Callopora rylandi* than the other species perhaps suggesting greater purifying selection or a recent population expansion in this species.

5.8.4 Population Genetic Structure analysis

An analysis of population genetic structure of the three *Callopora* species was conducted on both haplotype frequencies using conventional F-statistics using analysis of molecular variance or AMOVA which uses information for both haplotype frequencies and the sequence divergence of the actual haplotypes (Table 5.4).

Table 5.4 Analysis of Molecular Variance (AMOVA) assessing genetic differentiation between populations using (I) haplotype frequencies and (II) sequence divergences.

	Source of variation	df	SS	Variance components	% of total	P.Value
(I) Haplotype frequencies						
C.dumerilii	Among	2	1.280	0.032 Va	6.380	0.044
C.lineata	populations	1	1.361	0.104 Va	19.57	0.000
C.rylandi		1	1.480	0.106 Va	21.18	0.000
C.dumerilii	Within	16	7.457	0.466 Vb	93.620	
C.lineata	populations	22	11.000	0.437 Vb	80.430	
C.rylandi		19	7.472	0.393 Vb	78.820	
C.dumerilii	Total	18	8.737	0.498		
C.lineata		23	10.958	0.538		
C.rylandi		20	8.952	0.498		
Fixation index	FST					
C.dumerilii	0.064					
C.lineata	0.196					
C.rylandi	0.212					
(II) Sequence divergences						
C.dumerilii	Among	2	41.850	1.451 Va	10.05	0.200
C.lineata	populations	1	38.375	2.457 Va	13.12	0.096
C.rylandi		1	5.341	0.247 Vb	8.09	0.000
C.dumerilii	Within	16	207.729	12.983 Vb	89.95	
C.lineata	populations	22	357.833	16.265 Vb	86.88	
C.rylandi		19	53.278	2.804	91.91	
C.dumerilii	Total	18	249.579	14.434		
C.lineata		23	396.208	18.722		
C.rylandi		20	58.619	3.051		
Fixation index	FST					
C.dumerilii	0.101					
C.lineata	0.131					
C.rylandi	0.081					

The AMOVA results are shown in Table 5.4. AMOVA based on haplotype frequencies alone demonstrated a large percentage of genetic variation in *Callopora dumerilii*, *Callopora lineata* and *Callopora rylandi* which occurs within populations, whereas a much smaller percentage occurs among populations. However, it is important to note that for haplotype frequencies the among populations P values are significant for all species whereas when sequence divergence is taken into account only the P value for *Callopora rylandi* is significant. The percentage of the variation explained by the haplotype frequency is also greater. A significant result for among populations for haplotypes but not when sequence information is also considered could occur if populations are differentiated in haplotype frequency for haplotypes which are very similar in sequence but have similar frequencies for haplotypes which are quite different in sequence. The biological significance of this difference is difficult to interpret.

Population pairwise F_{ST} values for the three *Callopora* species were also calculated as shown in Tables 5.5, 5.6 and 5.7.

Table 5.5 Population pairwise F_{ST} values for *Callopora dumerilii*. In bold are the values from haplotype frequency analysis and in italics the respective values using sequence divergences.

	Lydstep	Pembroke Ferry	Watwick Bay
Lydstep			
Pembroke	**0.069 *(p=0.018)***		
ferry	*0.021 (p= 0.234)*		
Watwick	**0.034 (p=0.649)**	**0.065 (p=0.432)**	
Bay	*0.071 (p=0.447)*	*0.313 (p=0.05)*	

Estimates of F_{ST} for *Callopora dumerilii* (the classic measure of population subdivision) obtained when conducted using haplotype frequencies alone showed

significant subdivision between Pembroke Ferry and Lydstep populations whereas when carried out using sequence divergence information and haplotype divergence showed no significant population subdivision between the populations.

Table 5.6 Population pairwise F_{ST} values for *Callopora lineata*. In bold are the values from haplotype frequency analysis and in italics the respective values using sequence divergences.

	Lydstep	Watwick Bay
Lydstep		
Watwick Bay	**0.196 (p=0.000)** *0.131 (p=0.045)*	

Table 5.7 Population pairwise F_{ST} values for *Callopora rylandi*. In bold are the values from haplotype frequencies analysis and in italics the respective values using sequence divergences.

	Lydstep	Watwick Bay
Lydstep		
Watwick Bay	**0.212 (p=0.000)** *0.081(p=0.018)*	

Estimates of F_{ST} for both *Callopora lineata* and *Callopora rylandi* showed significant population subdivision when conducted using both haplotype frequencies and sequences divergences between Lydstep and Watwick Bay populations. The results of the AMOVA are in line with those of the pairwise F_{ST} values in showing significant genetic differentiation of populations. The pairwise F_{ST} values show however that for *Callopora dumerilii* only Pembroke Ferry and Lydstep are significantly differentiated and for haplotype frequencies only.

5.8.5 Tests based on the d_N/d_S ratio

The most direct method for showing the presence of positive or purifying selection is to show that the number of nonsynonymous substitutions per nonsynonymous site (d_N) is significantly larger or smaller than the number of synonymous substitutions per synonymous sites (d_S). Table 5.8 shows the values within populations for the synonymous and nonsynonymous distances and the d_N/d_S ratio and Table 5.9 shows between species distances. The Nei-Gojobori method (Jukes-Cantor) method of correcting for multiple substitutions was used for this purpose. This method computes the numbers of synonymous and nonsynonymous substitutions and the numbers of potentially synonymous and potentially nonsynonymous sites (Nei and Gojobori 1986).

Table 5.8 Within population d_N/d_S values calculated according to Nei and Gojobori (Jukes Cantor model).

	d_N	d_S	d_N/d_S	
Callopora dumerilii	0.029	0.357	0.081	Lydstep
	0.030	0.846	0.035	Watwick bay
	0.022	0.299	0.073	Pembroke ferry
	0.014	0.478	0.029	Overall
Callopora lineata	0.028	0.505	0.055	Lydstep
	0.015	0.354	0.042	Watwick bay
	-	-	-	Pembroke ferry
	0.015	0.652	0.023	Overall
Callopora rylandi	0.004	0.004	1.000	Lydstep
	0.012	0.151	0.079	Watwick bay
	-	-	-	Pembroke ferry
	0.006	0.082	0.073	Overall

Values of d_N for *Callopora dumerilii* and *Callopora lineata* were similar and higher compared to *Callopora rylandi*. For *Callopora dumerilii*, d_N/d_S appeared similar for

Lydstep and Pembroke Ferry and higher compared to Watwick Bay. Similarly, the two *Callopora lineata* populations showed similar d_N/d_S values, whereas in *Callopora rylandi*, the Lydstep population appeared to be close to neutrality ($d_N/d_S=1$), whereas the Watwick Bay population was under purifying selection ($d_N/d_S<1$).

Table 5.9 Between species d_N/d_S values calculated according to Nei and Gojobori (Jukes Cantor model).

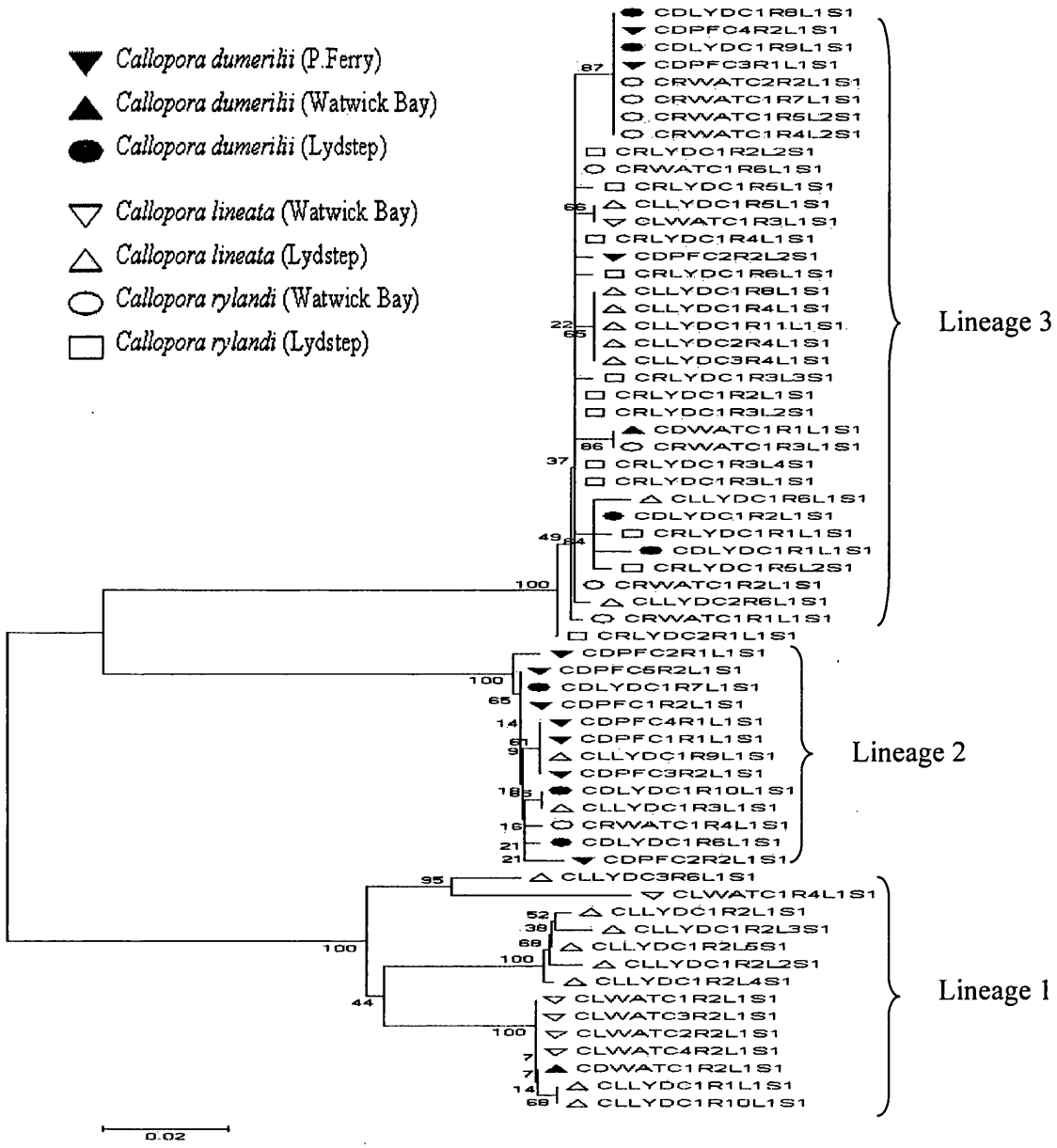
		Callopora dumerilii	*Callopora rylandi*	*Callopora lineata*
	Callopora dumerilii			
d_N	*Callopora rylandi*	0.012		
d_S		0.459		
d_N/d_S		0.026		
d_N	*Callopora lineata*	0.017	0.014	
d_S		0.761	0.668	
d_N/d_S		0.022	0.021	

The between species d_N/d_S values for the three species appeared similar for the three species. The values are low and less than one indicating purifying selection. In general, in this study d_N/d_S values are less than one suggesting purifying selection.

5.8.6 Phylogenetic relationships among species of *Callopora*

Reconstruction of phylogenetic relationships among morphologically defined species of *Callopora* was performed by using the neighbour-joining tree under the Kimura 2-parameter model (Figure 5.2). Kimura's two parameter model takes into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites.

Figure 5.2 Phylogenetic reconstruction among species of *Callopora*. Neighbour-Joining tree based on COI sequences using Kimura 2-parameter model. Bootstrap values were generated from 1000 replicates and are shown near the branches.



It can be seen in the tree that the three species of *Callopora* are arranged in three major lineages that are very well supported by the bootstrapping values. Lineage 1 contains sequences from *Callopora lineata* but there is also one sequence of

Callopora dumerilii. Lineage 2 contains primarily sequences of *Callopora dumerilii*, but there are also sequences of *Callopora lineata* as well as *Callopora rylandi*. Lineage 3 contains sequences from all three species in different proportions. These lineages do not correspond to species and may provide evidence for interbreeding between these three *Callopora* populations. Interbreeding could be caused since sperm is dispersed over only short distances, which results in self-fertilisation or cross fertilisation between closely related lineages. (Freeland *et al.*, 2000).

To test the tree structure other methods were used. Initially, a phylogenetic tree based on the Minimum Evolution tree was constructed, followed by a Maximum Parsimony tree (Figures 5.3 and 5.4 respectively).

Figure 5.3 Phylogenetic reconstruction among species of *Callopora*. Minimum-evolution tree based on COI sequences using Kimura 2-parameter model. Bootstrap values were generated from 1000 replicates and are shown near the branches.

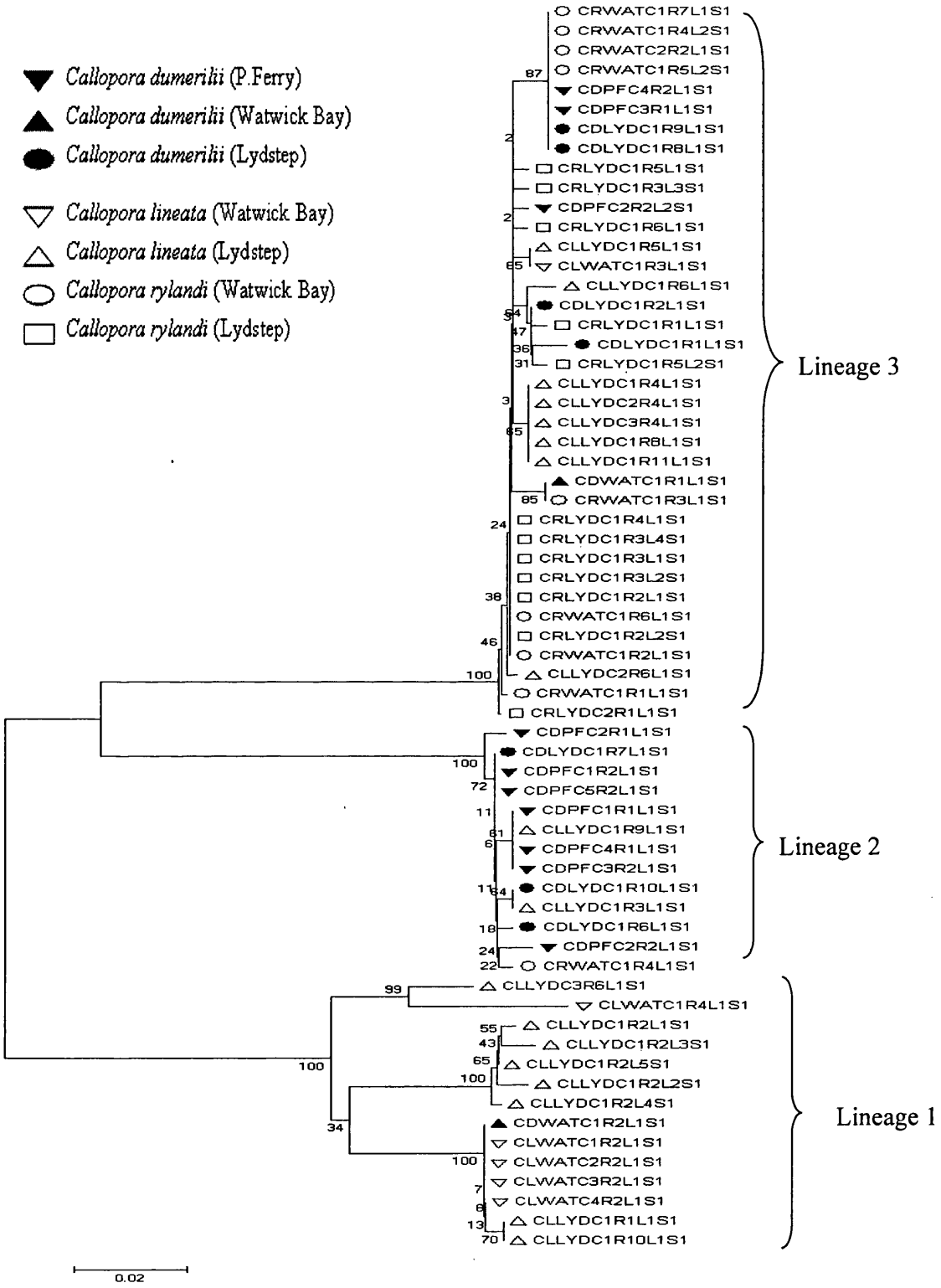
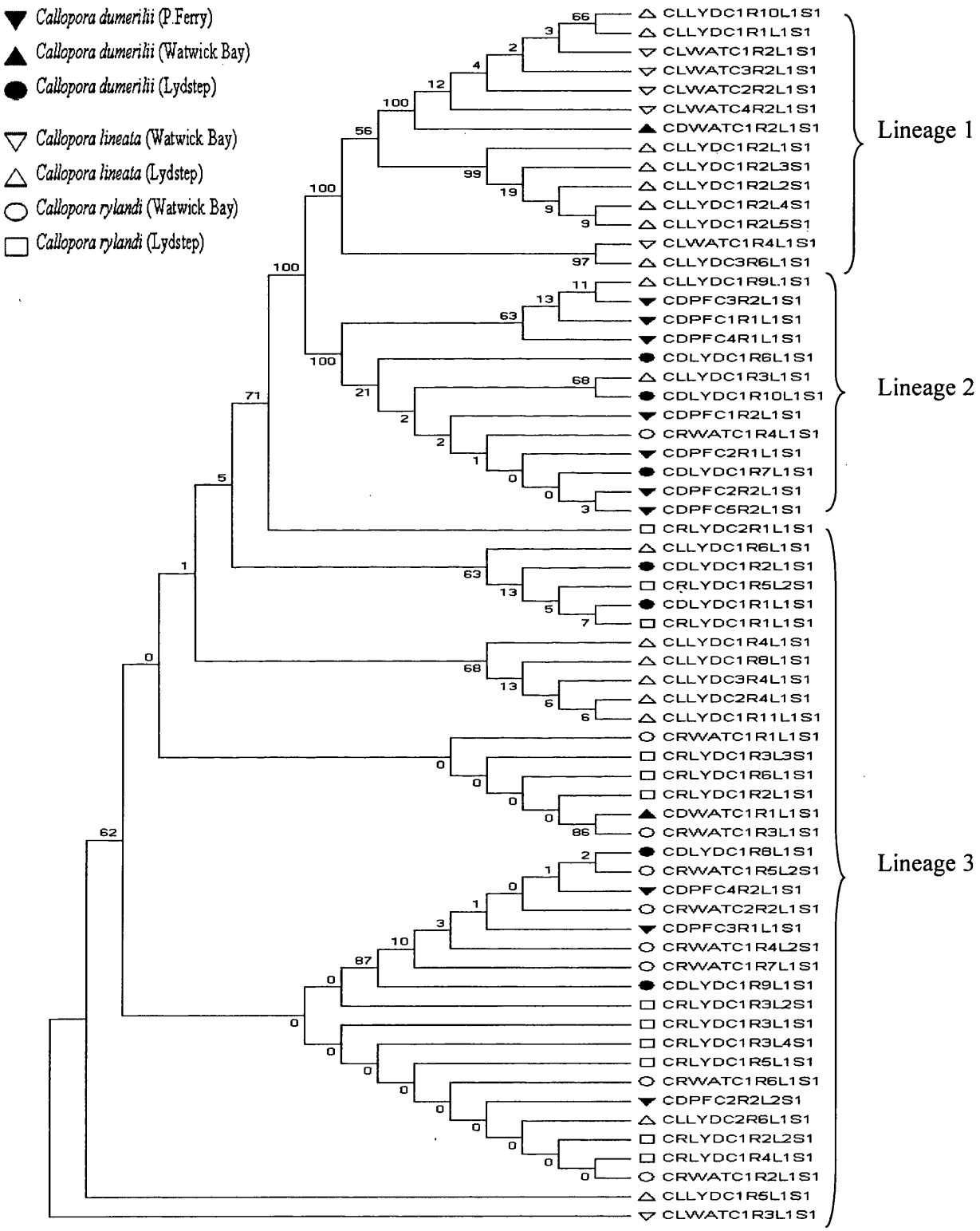


Figure 5.4 Phylogenetic reconstruction among species of *Callopora*. Maximum-parsimony tree based on COI sequences using Kimura 2-parameter model. Bootstrap values were generated from 1000 replicates and are shown near the branches.



It appears that all three methods of tree reconstruction result in the same conclusion. That is, the three species of *Callopora* are arranged in three major lineages that are very well supported by the bootstrapping values. The three lineages suggest evidence for interbreeding between the three *Callopora* species, therefore a further investigation of the three lineages separately is essential.

5.8.7 Tests of neutrality for the three lineages

Because the phylogenetic analysis resulted in a tree with three main branches, it was decided to carry out neutrality tests on these branches. As in preceding sections, Tajima's D test and Fu's F_s (Table 5.10) test statistic were used followed by tests based on the d_N/d_S ratio (Tables 5.11, 5.12) and the McDonald-Kreitman test (Table 5.13). The latter should be carried out on separate lineages. It would not have been appropriate to carry out the test on the three species as they are a mixture of distinct lineages.

Table 5.10 Tajima's D and Fu's Fs test statistics for the three lineages.

	Tajima's D	Fu's F_s
Lineage 1	0.128 (p=0.591)	-5.486 (p=0.010)
Lineage 2	-1.369 (p=0.088)	-17.765 (p=0.000)
Lineage 3	-1.816 (p=0.009)	-26.864 (p=0.000)

Table 5.10 shows significant negative values for Fu's F_s test for all three lineages which is an indication of purifying selection and a significant negative value for Tajima's D test for lineage 3, which again is consistent with purifying selection. Although such results could also be consistent with a population expansion, it is more

usual to involve purifying selection unless there is clear evidence that a population expansion might have actually occurred.

Table 5.11 Within lineages d_N/d_S values calculated according to Nei and Gojobori (Jukes Cantor model).

	d_N	d_S	d_N/d_S
Lineage 1	0.011	0.135	0.081
Lineage 2	0.005	0.006	0.833
Lineage 3	0.006	0.009	0.667

Table 5.11 shows the average number of synonymous d_S and non synonymous d_N substitutions as well as the within d_N/d_S ratio for each of the three lineages. Nonysynonymous to synonymous means appeared similar for lineages 2 and 3 which were significantly larger compared to lineage 1. However, the values for all three lineages were less than 1, an indication of purifying selection. It should be noted that purifying selection for lineage 1 might be stronger since the ratio d_N/d_S is lower. This could reflect a higher level of purifying selection in the past if individuals in this lineage experienced a harsher environment where purifying selection against deleterious mutations was particularly strong.

As with the within d_N/d_S comparisons for the lineages, Table 5.12 shows the between lineages d_N/d_S means ratio for the three lineages.

Table 5.12 Between lineages d_N/d_S values calculated according to Nei and Gojobori (Jukes Cantor model).

		Lineage 1	Lineage 2	Lineage 3
	Lineage 1			
d_N	Lineage 2	0.018		
d_S		1.063		
d_N/d_S		0.017		
d_N	Lineage 3	0.019	0.018	
d_S		1.094	0.764	
d_N/d_S		0.017	0.024	

It appeared that all three lineage comparisons have very low similar d_N/d_S ratios indicating strong purifying selection between lineages. The generally lower values of d_N/d_S in Table 5.12 compared with Table 5.11 might suggest strong purifying selection in the past when the lineages diverged than in more recent times.

Table 5.13 gives the results of the McDonald-Kreitman test for the possible comparisons between the three lineages with P values for contingency tests of the 2x2 tables.

Table 5.13 McDonald-Kreitman test for the three lineages.

		Fixed	Poly	P
Lineage 1 – Lineage 2	Nonsynonymous	2	16	0.002
	Synonymous	37	33	
Lineage 1 – Lineage 3	Nonsynonymous	0	21	0.000
	Synonymous	40	38	
Lineage 2 – Lineage 3	Nonsynonymous	3	15	0.0004
	Synonymous	37	12	

A highly significant deviation from neutrality is observed in all tests. A deficit of nonsynonymous fixed differences is clear with many fewer fixed nonsynonymous substitutions between lineages than expected. That is nonsynonymous mutations within lineages were selected against and had little possibility of being fixed between lineages. This result is again consistent with purifying selection acting against nonsynonymous mutations within the lineages.

5.9 Discussion

The aim of this chapter is to discuss the genetic structure for three *Callopora* species based on the mtDNA COI gene and to test hypotheses concerning levels of population differentiation and intrapopulation variation. In addition the relationships of mtDNA lineages within and between species are examined to clarify the phylogenetic relationships of the three species and to search for possible phylogenetic subdivisions within species.

Overall nucleotide diversity was similar for *Callopora dumerilii* and *Callopora lineata* compared to *Callopora rylandi* where it was much lower. For *Callopora dumerilii* nucleotide diversity was twofold higher in Watwick Bay populations compared to Pembroke Ferry and Lydstep which in turn were similar to each other. For *Callopora lineata* nucleotide diversity was similar for both Lydstep and Watwick Bay populations, whereas for *Callopora rylandi* Watwick Bay showed a ninefold higher nucleotide diversity compared to Lydstep Beach. The very low overall nucleotide diversity for *Callopora rylandi* could be attributed probably to a relatively small long-term effective population size rather than any severe bottleneck during evolution. On the other hand, overall haplotype diversity estimates were similar between the three species as well as for populations of each species separately.

An important aspect of this chapter was to test the neutral theory of molecular evolution in the three *Callopora* populations. For this purpose the Tajima's D test and the more sensitive Fu's F_s test was performed to examine whether samples from

different localities are at neutral equilibrium with respect to mitochondrial DNA variation. Significant deviations from neutrality could be attributed to recent population expansions or bottleneck in situations where no selective forces acting on existing haplotypes may exist (Rand 1996). Tajima's D test statistic showed significant negative values for *Callopora rylandi* overall as well as for each population separately. This could indicate recent population expansion but also might indicate strong purifying selection. However, no significant values were obtained for the other two species. Analogous to the Tajima's D test, for Fu's F_s a significant positive value for *Callopora lineata* was observed, which is in agreement with the positive value for Tajima's test for this species. This is consistent with the theory of balancing selection, in which selection actively maintains high diversity within species. For *Callopora rylandi* there was one significant negative value for F_s which was consistent with the negative value for this species for Tajima's D. Overall, for both statistics the values might thus be more negative in *Callopora rylandi* than the other species. The significant negative D values for the individual populations of *Callopora rylandi* as well as the total population might be the result of purifying selection or a population bottleneck. Although purifying selection might generate a negative D value, it would be expected to be observed more significantly in all the populations analysed and this was not the case in this study. Therefore, the explanation based on a population bottleneck might be more acceptable. The bottleneck explanation could be better supported if there were independent evidence to suggest that the population size of *Callopora rylandi* had expanded in the fairly recent past following bottleneck.

When AMOVA was conducted using sequence divergences, results showed a large percentage of genetic variation within populations and a much smaller percentage of genetic variation among populations. These values were similar between the three *Callopora* species as one might predict from their same model of dispersal and are in agreement with the numerous studies of population subdivision in marine invertebrates which have established that species with non-pelagic larvae are characterised by high levels of population substructure. The relatively high genetic subdivision between populations of *Callopora dumerilii*, *Callopora lineata* and *Callopora rylandi* is consistent with the genetic differentiation observed for other bryozoans with coronate larvae from the western Atlantic (Schopf 1973, 1977) and the Irish Sea (Goldson *et al.*, 2001) as well as that of *Cryptosula pallasiana* and *Schizoporella unicornis* from intertidal regions on the coasts of Great Britain and the Isle of Man (Watts and Thorpe 2006). High levels of population genetic substructure have also been revealed in populations of *Alcyonidium gelatinosum* (Porter *et al.*, 2002).

However, the extent to which larval mode dictates population genetic structure is not fully understood. Perhaps some patterns of genetic subdivision in marine species do not meet expectations based on their apparent larval potential (Burton 1983; Palumbi 1994). This was shown when AMOVA was conducted based on haplotype frequencies alone. Results showed that for *Callopora dumerilii* the percentage of genetic variation between populations was threefold less compared to that for *Callopora lineata* and *Callopora rylandi* which had similar percentage of genetic variation between populations. This result is rather peculiar since the three species have the same mode of larval dispersal. One possible explanation, however, could be

that *Callopora dumerilii* colonies, which are found on kelp holdfasts, have the potential for rafting (Jackson 1986) and therefore *Callopora dumerilii* could be achieving a larger level of gene flow between populations.

The above findings were also shown from the fixation indexes (F_{ST}) of the three *Callopora* species. The fixation index, which is the most inclusive measure of population substructure, was used for examining overall genetic divergence among subpopulations and to show levels of migration. A high fixation index signifies a low amount of migration. Thus, when haplotype frequencies were used, it was shown that *Callopora dumerilii* demonstrated much higher migration compared to *Callopora lineata* and *Callopora rylandi*, whereas when sequence divergences were used, fixation indexes of the three *Callopora* species were similar. Thus the different measures of population differentiation might not show consistent results.

Some non-bryozoan studies have shown that some species, which possess larvae with high dispersive potential, show lower than expected amount of dispersal. For example, Todd *et al.* (1998) showed that the nudibranch *Adalaria proxima*, which has pelagic larva, demonstrated minimal levels of larval migration and local populations were demographically closed on very small scales. It has also been shown that closely related species with identical larval dispersal potential can exhibit fundamentally different patterns of population structure across the same geographical region. The study of Marko (2004) provides good evidence for this. He gathered mitochondrial DNA sequences and characterised the population genetic structures of two congeneric intertidal gastropods, *Nucella lamellosa* and *Nucella ostrina* which have northern range end points that are greater than 1000km and like all members of the genus lack

planktonic larvae. Results from this study showed that *Nucella ostrina* demonstrated a pattern of limited population substructuring in the northern half of its range and no relationship between geographical distance and genetic differentiation among sites across the species' entire geographical range. On the other hand, *Nucella lamellosa* showed significant relationship between genetic differentiation and genetic distance among sites and revealed significant subdivision between populations.

Under neutral evolution, the number of nonsynonymous substitutions per nonsynonymous site (d_N) and the number of synonymous substitutions per synonymous site (d_S) are expected to be equal. Since nonsynonymous substitutions change the primary amino acid structure and therefore might alter protein function, high d_N/d_S ratios greater than one can be interpreted as indicative of positive selection. If the ratios are high but less than one this could also indicate relaxed selective constraint. The overall estimated d_N/d_S for the *Callopora rylandi* population was higher than that seen for the *Callopora dumerilii* and *Callopora lineata* populations. Such a difference might be taken to suggest either increased adaptive evolution in *Callopora rylandi* relative to that in *Callopora dumerilii* and *Callopora lineata* or relaxed constraints. Relaxation of evolutionary constraints in *Callopora rylandi* would be compatible with the prediction from population genetic theory that selection against deleterious mutations is more effective in larger populations where genetic drift is weak. Between population comparisons suggest that populations are under purifying selection as well. The amount of nonsynonymous substitutions appeared similar between the three *Callopora* species. Similar studies of protein coding genes in mitochondrial DNA have found elevated levels of within-species nonsynonymous polymorphisms compared with between species nonsynonymous replacements (Rand

2001). This result contrasts with a purely neutral model of sequence evolution, but is compatible with a nearly neutral model in which most amino acid substitutions are slightly deleterious. Similarly, a study by Foltz (2003) showed that ratios of nonsynonymous to synonymous substitutions were significantly higher between species with nonpelagic larvae than with pelagic ones. His analysis revealed that the COI sequences for the four species of the genus *Patiriella* (sea star) with non pelagic larvae had a d_N/d_S ratio (0.0247), which was similar to the ratio in the *Callopora* populations in this study and which is 3.5 times higher than that for the eight species with pelagic larvae (0.0071). The increase in the rate of nonsynonymous substitutions observed in lineages with nonpelagic larvae is most likely due to reduced effective population size, which increases the amount of genetic drift and results in nonsynonymous mutations becoming more nearly neutral, a result consistent with the studies of Funk *et al.* (2001).

Mitochondrial DNA analyses of sampled *Callopora* populations from the three designated areas across West Wales indicated several distinct patterns of genetic divergence within the species distribution. Perhaps the most intriguing result to arise within this study was the presence of three mitochondrial DNA lineages within these species. These are not separate lineages from each of the three species. Instead all the species are represented in each of the three observed lineages. There are different possible explanations for this structure. First it is possible that lineages existed in the ancestor of the three species and the lineages were distributed to all the separate species following the divergence of the species. Another possibility is that initially three separate lineages evolved separately in the three species. This might have happened in allopatry. After this the species might have established contact and

interbreeding occurred between the species. The three lineages would have been retained given the mode of inheritance of mtDNA but would have been distributed among the species. It is interesting to note that in lineage 1 there are three sub-lineages with high bootstrap support. These lineages might have evolved in an ancestral population. There are however more well supported lineages than there are species. This could indicate ancient barriers to gene flow or additional species that are not recognised today.

As mitochondria are not expected to be transferred by sperm, extensive pattern of backcrossing and introgression would be needed in order to establish the lineages within populations whose individuals had been taxonomically classified as good members of the respective species.

Patterns of nucleotide substitution among the lineages can be tested for concordance with that expected for a sample at mutation-drift equilibrium. Tests of neutrality and particularly Fu's F_s test statistic indicated an excess of low frequency mutations among lineages (highly significant negative values of F_s). A strongly negative value could signify sudden population growth, which in turn could be the result of a selective sweep (i.e. the process in which a strongly beneficial mutation becomes rapidly fixed in a population) for closely related haplotypes containing advantageous mutations (Maruyama and Birky 1991). In addition, demographic expansion due to a founder or bottleneck incident could also result in the accumulation of derived haplotypes of low frequency and so resemble the consequences due to a selective sweep.

Estimates for the number of synonymous (d_S) and nonsynonymous (d_N) substitutions observed in the three lineages were calculated both within and between them. When comparisons within lineages were performed, the d_N/d_S ratio was unusually high for lineages 2 and 3, compared to lineage 1. This observation could imply two things. Lineage 2 and 3 might be under similar selection forces. Another possibility, which could be more of a practical nature, is that the data set of sequences analysed is quite small. When comparisons were performed for the d_N/d_S ratio between lineages, the ratios were very similar and less than one, suggesting intense purifying selection acting on these three lineages.

When comparing the lineage 1 with the lineage 2, nonsynonymous to synonymous ratios of 2 to 37 for fixations between lineages and 16 to 33 for polymorphisms within lineages was observed, giving a significant excess of nonsynonymous polymorphisms in the McDonald-Kreitman test. Similarly, when lineage 2 and lineage 3 where compared, nonysynonymous to synonymous ratios of 3 to 37 for fixations and 15 to 12 for polymorphisms were estimated, indicating a highly significant excess of polymorphisms as well. In addition, when lineage 1 was compared with lineage 3, the nonsynonymous to synonymous ratios are 0 to 40 for fixations and 21 to 38 for polymorphisms, a result which again indicates a highly significant excess of nonsynonymous polymorphisms as with the previous comparisons. This pattern is consistent with the values observed for the d_S/d_N ratio, as well as with the nearly neutral model of molecular evolution (Ohta 1992), suggesting that a very intense purifying selection is acting on the three lineages.

Similar findings have been observed in mtDNA lineages in other species, for example in three species in the mussel genus *Mytilus* (*Mytilus edulis*, *Mytilus trossulus* and *Mytilus galloprovinciallis*) which are widely distributed in cold and temperate regions of Europe and America. *Mytilus* is unusual in having two distinct mtDNA genomes. The F genome is inherited maternally and the M genome is inherited paternally. The three species share haplotypes, although American *Mytilus trossulus* has distinct lineages. In European *Mytilus trossulus*, the M genome is replaced by an F genome variant. *Mytilus* provides a good model for applying tests of neutrality because for many tests one mtDNA genome can be used as a control against the other. Stewart *et al.* (1996) studied 813 bp of the COIII gene in two American *M. trossulus* and two American *M. edulis* individuals.

When comparing F and M lineages for American taxa, Stewart *et al.* (1996) observed significant excess of nonsynonymous substitutions in the McDonald-Kreitman test. However, in European taxa, excesses of nonsynonymous polymorphisms occur when comparing F and M lineages, although the excess is small and non-significant in *Mytilus edulis*. Excesses of nonsynonymous polymorphisms also occur in inter-continental within-genome tests. For example, for the F genome a highly significant excess of nonsynonymous polymorphisms was observed. Moreover, all the nonsynonymous F genome polymorphisms occur in the European populations. An excess of nonsynonymous polymorphism also occurs for the M genome, but is non-significant. This complex pattern is consistent with the nearly neutral model of molecular evolution (Ohta *et al.*, 1992). This would involve somewhat more intense purifying selection on the F than the M genome, but less intense purifying selection in European taxa particularly *Mytilus edulis* compared with American taxa. These

findings are consistent with the values observed for the d_S/d_N ratios. Recent relaxation in selective constraint and changes in effective population size might have played a role in causing the geographic and species-specific differences.

Similarly, Quesada *et al.* (1998) demonstrated that these patterns occur in European populations with the exception that a significant excess of replacement polymorphism within mtDNA lineages is observed in European populations of *M. galloprovinciallis*. Moreover, European populations showed an excess of replacement polymorphism within the F but not within the M genome with respect to American *M. trossulus* as well as a consistent pattern of excess of rare variants in both genomes.

The general conclusion from the *Mytilus* work is that all the different tests, such as those carried out in this thesis suggest purifying selection but that the strength of purifying selection varies between species and different populations. This is the pattern observed in the *Callopora* species. Stronger purifying selection might be expected in harsh environment or with larger population sizes, whereas weaker selection with less harsh environment or small population sizes where the variation is more nearly neutral and more affected by drift rather than selection.

CHAPTER 6

GENERAL DISCUSSION

6.0 General discussion and future work

The goal of population genetics is to describe and quantify genetic variation in populations and to use this variation to make inferences about evolutionary processes affecting populations (Hartl and Clark 1997, Hedrick 1985). Evolutionary forces such as mutation, migration, genetic drift, selection and recombination change gene frequencies in populations and shape their genetic structure.

One of the main objectives in this study was to investigate morphological variation in *Callopora* populations and to compare variation within and between colonies and populations. Anatomical characters have traditionally been used in bryozoan studies in order to describe taxonomic units both at the inter- and intraspecific level. However, their use in population structure studies was made difficult by the fact that phenotypic variation in these characters was not only directly controlled by genetic factors, but was also dependent on environmental modifications. Documentation of phenotypic variation is essential for any group of organisms in order to develop sound species concept and to allow meaningful ecological and evolutionary inferences.

The methodological approach used consisted of taking morphological measurements that were reliably measurable at the available resolution. These were zooid length (ZL), zooid width (ZW), ovicell length (OL) and ovicell width (OW). A small number of simple characters were deliberately selected with the assumption that any significant patterns observed would be analytically and interpretatively robust and have the potential to be more pronounced for other more complex systems. The morphological measurements were analysed between sites and colonies using a

Nested ANOVA approach and a principal component analysis. Nested ANOVA results showed that for between colonies within sites comparisons there were significant differences for all morphological measurements in all three species, wheareas when comparing individual sites, it was shown that there were significant differences depending on the species on the particular site. Principal component analysis similarly showed significant differences for between colonies within site comparisions for the two factors representing size and shape, whereas between sites comparisons showed differences in the two factors depending on the species.

An important source of potential variation that has not been considered in this study is that changes with time in the environmental factors such as temperature, salinity as well as diet and oxygen concentration have not been considered. Instead colonies were collected from the designated sampling sites during the summer months as it was known that these species reach the peak of their reproduction during these months (Hayward and Ryland 1998). Several studies (Hunter and Hughes 1994; Menon 1972; O'Dea and Okamura 1999; Lombardi *et al.*, 2006) have investigated the aforementioned factors in great detail and their effects have been discussed in previous sections. It was assumed that the collected *Callopora* colonies would comply with the so called "temperature-size rule" which is a common pattern of phenotypic plasticity in which higher temperature during development results in a smaller adult body size and vice versa. Therefore, this part of the thesis would be greatly enhanced by performing experiments where temperature, oxygen concentration and diet are all monitored in controlled experiments in the laboratory, in order to have conclusive evidence as to how these factors affect the morphology of the *Callopora* species. The work of Atkinson *et al.* (2006) is a very good example. In their experiments they

examined two clones of the colonial bryozoan *Celleporella hyalina* under orthogonal combinations of two temperatures and two oxygen concentrations during ontogeny, observing effects on sizes of colonies and larvae and sizes and numbers of cells, tentacles and modules (autozooids). Their results showed that the size over number responses varied among cell types and among structures at different levels of organization, with the inverse temperature-size relationship applying only to larval parenchymal cells and colony modules.

Interestingly, it has also been found that zooids of *Cribrilina annulata* were different for colonies living on different substrates. These differences (biotopical) were stronger than geographical differences (E Yagunova pers. communication). Therefore the effect of the substratum on which the colonies grow is another parameter to consider as it influences not only the general morphology of the organism, but also the geographic range of these species as will be explained later.

Once the role of these factors in the *Callopora* species has been established, morphometric studies of *Callopora* colonies from different localities grown as replicates in controlled laboratory experiments could be performed in order to study phenotypic variation (hard part morphology) and partition this variation into its genotypic and environmental components in the same manner as the experiments of Hageman (1999). This kind of work will provide insights for different kind of studies such as systematic or ecological investigations.

Further work is also needed in the area dealing with the ovicell structure. Although anatomical and SEM-studies of the ovicells in *Callopora dumerilii a*nd *Callopora*

were undertaken to resolve a long-term controversy existing in the literature about the origin of the ovicells (Ostrovsky and Schäfer 2003), nothing is known about the factors that affect the ovicell size or shape. The effect of substratum type, the physical disturbances, the amount of food and the effect of predators needs to be investigated to test the relationship of those factors with the ovicell structure.

An important thing to note is that it is quite difficult to speculate whether the factors that cause the morphological variation observed in this study are environmental or genetic. However, Hageman *et al.* (1999) proposed possible systematic sources of within colony variation. Temporal microenvironmental differences (i.e. budding of zooids in different parts of the colony at different times under different ambient conditions), spatial microenvironmental differences (i.e. growth of different parts of the colony affected by substratum or other heterogeneities) and packing constraints based on budding interactions can all contribute to variation. The genetic factors, however, should not be omitted from these assumptions, or the interaction of the environmental and the genetic factors. Ultimately, nothing can be deduced with certainty, thus the best strategy to explore these factors and their influence on variation would be to pursue controlled laboratory experiments.

The second objective in this thesis was to investigate levels of genetic variation within and among populations and to clarify phylogenetic relationships between these *Callopora* species. The methodological approach consisted of acquiring mtDNA COI sequences. The use of this method was justified by a number of reasons. For example, due to its haploid nature and mode of inheritance, the effective population size estimated from mtDNA is smaller than that of nuclear genes, unless there is extensive

heteroplasmy or the sex ratio is heavily biased in favour of females. As a result the rate of approach to genetic equilibrium occurs rapidly and amounts of among population divergence due solely to genetic drift can be as much as four-fold greater for mtDNA than for nuclear loci (Birky *et al.* 1989). This feature of mtDNA makes it a particularly sensitive single locus for detecting genetic structure among populations.

Analysis included using the analysis of molecular variance (AMOVA) using both haplotype frequencies (conventional F-statistics) and incorporating sequence divergence. Either way, it was shown that most of variability was found within populations rather than among populations for all of the three *Callopora* species. Interestingly, for haplotype frequencies the among populations P values were significant for all species whereas when sequence divergence was taken into account only the P value for *Callopora rylandi* was significant. Population pairwise comparisons provided evidence for significant subdivision for certain localities depending on the species.

Tests of neutrality carried out for the three species showed that Tajima's D and Fu's F_s test statistic appeared more negative in *Callopora rylandi* than the other species. This demonstrates a significant excess of low frequency derived mutations among haplotypes in this species. This could be attributed to a recent demographic expansion as a result of a founder or bottleneck event (Excoffier and Schneider 1999). In addition, the possibility that this excess of low frequency mutations could be result of a selective sweep for closely related haplotypes should also be considered. Perhaps the most likely explanation is the existence of purifying selection. On the other hand, strong positive values for these tests, as in *Callopora lineata* for example, could be

due to reduction of low frequency mutations. This could be caused by balancing selection for older haplotypes or by a population admixture of formerly separate populations which results in several haplotypes being at high intermediate frequencies.

These results, although significant, were limited in some instances by the small sample size of the collected colonies. For instance, only 4 colonies of *Callopora dumerilii* were collected from Watwick Bay, compared to the 16 colonies from Lydstep Beach and 18 colonies from Pembroke Ferry. Therefore, in order to have convincing evidence for population genetic structuring a larger number of samples is essential which could be done by the acquisition of more sequences. An important parameter that should also be taken into consideration is that more localities would need to be surveyed. The three localities that were selected for the needs of this thesis are quite near to each other thus it is necessary to carry out extensive sampling and analyse from localities further away from Wales. In this way, conclusive evidence in terms of population genetic subdivision, gene flow and population structure for these three species will be gathered.

An interesting point that emerged while sampling in these localities is that some species appeared in certain localities at some time during the year and either they reappeared next reproductive period in scarce amounts or not at all. A typical example was *Callopora dumerilii*. In one of the early visits to Pembroke Ferry, 15 colonies were collected. In later visits, within a period of 2 years, only 3 more colonies were collected. The absence of the other two species in Pembroke Ferry may suggest that this particular site is unsuitable for *Callopora* species. As mentioned in a preceding

section (Section 2.7.2) Pembroke Ferry is an estuary, compared to the other 2 sites, which may suggest that the actual physical environment of this site is clearly unsuitable for these species. However, the presence of even a small number colonies of *Callopora dumerilii* in one reproductive period could be attributed to the ability to raft or foul. Watts *et al.* (1998) showed that the ability to raft and foul has a significant effect on the size of geographic range of a species. The *Callopora* species studied in this thesis are also known to be epiphytic on the holdfasts of *Laminaria* species (*Callopora dumerilii*) and in kelp holdfasts (*Callopora lineata* and *Callopora rylandi*) and may be dispersed by rafting. In addition, transport by ballast water may also be possible. Information on this mechanism is very limited although Carlton and Geller (1993) found larvae of the anascan *Membranipora membranacea* in ballast water.

A remarkable finding in this thesis was the presence of three mtDNA lineages within these species. The mixing of the three *Callopora* species, especially in lineage 3, is indicative of interbreeding. Sceptics might put forward the possibility of contamination or mix of larvae when extracting DNA from these species, especially since these species occur side by side in many instances in rocks in the marine environment. However, this possibility can be ruled out since these three *Callopora* species are reasonably distinct from each other and the possibility of misidentification or human error during extraction of larvae is to a great extent minimal.

Tests of neutrality, particularly the Fu's F_s test, generally indicated that the three lineages had an excess of low frequency derived mutations among haplotypes (negative values F_s). In agreement with this tests were the d_N/d_S ratio and the

McDonald-Kreitman tests, which provided evidence that purifying selection is acting on the three lineages.

In conclusion it can be said that patterns of genetic variation among populations and species may be used to infer recruitment and evolutionary history of marine species (Burton, 1996; Parker *et al.*, 1998). Genetic studies across widely distributed populations of *Callopora* could be useful to perhaps evidence of any possible genetic and morphological differentiation. A molecular genetic approach could exclude or consider the presence of cryptic species over a nested series of geographical scales.

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APPENDIX A

This appendix presents the lists of the major chemicals and solutions that have been used throughout the course of this thesis.

Lysis solution for DNA extraction

- 7.5mM Tris-HCl pH 8.3,
- 3.75mM NH_4Cl,
- 3.75mM KCl, 1.5mM $MgCl_2$ and
- 2μg/ml Proteinase K.

Lysis buffer for RNA extraction

- 10mM Tris pH 7.5
- 10mM EDTA
- 0.5% SDS

SOC medium (typical for 100ml)

- 2.0g Bacto-tryptone
- 0.5g Bacto-yeast extract
- 1ml 1M NaCl
- 0.25ml 1M KCl
- 1ml 2M Mg^{2+} stock, filter sterilised
- 1ml 2M glucose, filtersterilised

pH should be 7.0

LB medium (Per liter)

- 10g Bacto-tryptone
- 5g Bacto-yeast extract
- 5g NaCl

Adjust pH to 7.0 with NaOH.

(S-GAL/LB AGAR BLEND) (Per 500ml)

- 5g Tryptone
- 2.5g Yeast extract
- 5g NaCl
- 6g Agar
- 0.15g S-Gal/LB Agar Blend
- 0.25g Ferric Ammonium Citrate
- 0.015g IPTG

Autoclave and after it cools off add 1ml of ampicillin (25mg/ml)

10% APS (Ammonium Persulfate) (typical for 100ml)

- 10g $(NH4)_2S_2O_8$

Solutions for silver staining.

Solution A

- 360 ml distilled H_2O,
- 40 ml 100% ethanol,
- 2 ml glacial acetic acid

Solution B

- 200 ml distilled H_2O,
- 0.2 g $AgNO_3$

Solution C

- 300 ml distilled H_2O,
- 4.5 g NaOH,
- 0.03 g $NaBH_4$,
- 1.2 ml 37 % solution formaldehyde.

APPENDIX B

This appendix contains the sequence data that were obtained in this thesis. The consensus sequence has been included. The abbreviations used are explained as follows:

For example, for CRLYDC1R1L1S1, CR stands for *Callopora rylandi*, LYD stands for Lydstep (the sampling locality), C1R1 is a code that was used in order to identify samples and to organise the data, thus C1R1 stands for Colony 1 Rock 1, and L1S1 is again a code that is used to identify the sequence that was obtained from a particular larvae. Therefore, L1S1 stands for Larva 1 Sequence 1. Similarly, the codes CD, CL stand for *Callopora dumerilii* and *Callopora lineta*, WAT stands for Watwick Bay, PF stands for Pembroke Ferry. Caulspinif stands for *Cauloramphus spiniferum*, and CalXXX stands for the new perhaps species of *Callopora* that was found in Lydstep (Section 2.6).

All sequences are shown in the 5' → 3' direction.

CRLYDC1R1L1S1

TCAGCCCATACCAATAAAGGAGTTCGTATTAAAGTTATTAGACTACTACG
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CACCCGCCAAATGCAAAGAAAAAATAGCTAAGACAACTGAAGGACCCCT
ATGTGCCAAATTTGAAGACAAAGGAGGATAAACAGTTCACCCCGTCCCAG
CCCCTCTCTCAACCAAAGAAGATAGTAACAAAAGGGACAAAGCCGGAGG
CAATAATCAAAAACTTATATTATTTAACCGAGGAAAAGCTATATCAGGTG
CCCCCAACATTAAAGGTACCAACCAATTACCAAATCCACCAATTATAACT
GGCATAACCATAAAAAAAAATTATTATAAATGCATGGGCAGTAACAATCAC
ATTATATAATTGATCATTACCCATCAAACCCCCA

CRLYDC2R1L1S1

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GCCCCCAACATTAAAGGTACCAACCAATTACCAAATCCACCAATTATAAC
TGGCATAACCATAAAAAAAAATTATTATAAATGCATGGGCAGTAACAATCA
CATTATATAATTGATCATTACCCATCAAACCCCCA

CRLYDC1R2L1S1

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ACGCTATTTCCTCGGTTAAATAATATAAGTTGATGATTATTGCCTCCGGCT
CTCTGTCCCTTTTGTTACTATCTACTATGGTTGCAGAGAGGGGCTGGGACG
GGGTGAACTGTATTATCCTCCTATTGTCTTCAACATATTGAGCACACTATG
GGGTCCTTCCAGCCTGACATTAGACTATTACCTTCTTTGCATCTGGCGGGC
TGCATTCATACAATTTTGGGGGCCATTAATTTTATAACTACTACTATAAAT
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AGCTCGCATACGA

CRLYDC1R2L2S1

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CGAAGAGACTCCCTATGTGCCAAGATTTGAAGACATAAGGAGGATAAAC
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AAAAGCTATATCAGGTGCCCCCAACATTAAAGGTACCAACCAATTACCAA
ATCCACCAATTATAGCTGGCATAAGCCATAAAAAAAATATATAATAAATG
CATGGGCAGTAACAATCACATTATATAATTGATCATTACCCATCAAACCC

CCAATCACTAATGAATTCGCTGCCGCCTGCAGGTCGACCATATGGGAGAG
CTCCCAACACGTTGGATGCATAGCTAGACTATTCTA

CRLYDC1R3L1S1

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CRLYDC1R3L2S1

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CRLYDC1R3L3S1

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CRLYDC1R3L4S1

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CRLYDC1R6L1S1

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CRWATC2R2L1S1

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CRWATC1R3L1S1

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GCAATAATCAAAAACTTATATTATTTAACCGAGGAAAAGCTATATCAGGT
GCCCCCAACATTAAAGGTACCAACCAATTACCAAATCCACCAATTATAAC
TGGCATAACCATAAAAAAAAATTATTATAAATGCATGGGCAGTAACAATCA
CATTATATAATTGATCATTACCCATCAAACCCCCA

CLLINC1R8L1S1

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ATAGTGTCAATGTCACTGAGGTGACCGAGACAACCAGGAGGGCGGAATC
CATAACACACCCCCCTCCATTAGTTGCCCCGGGCCACTAATTCAAATGGC
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CCTGTAGCAAATCGTAGTTGTGGGAGGTTCGACGGTGACGGTCCCTCTTG
GTTACGAGACGGGCTAACTCCTGCTCCAGTCGCTGGACTGTGGGTGTCAG
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CLLINC1R5L2S1

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TCCCCGGGTCCTCTCTAGGTCACACCCTTCATTCNTTCTATAGCNCTAANT
TCGTAAAACGTTAATTAGGGGTTATAAGCAAGAAAAGAAGGGGGGGAAG
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CLLINC1R7L1S1

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CLLINC1R3L1S1

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GTGATAATTGGGGGATTTGGGAATTGACTAGTACCCTTAATATTAGGGGC
ACCGGATATAGCTTTTCCTCGGTTAAATAATATGAGGTTTTGGTTATTACC
GCCTGCGTTGTCTCTTTTATTACTATCTTCGTTGGTTGAAGGGGGAGTAGG
AACGGGGTGGACAGTTTATCCTCCTCTTACTTCAAGTTTAGCTTACAGAGG
TCCTTCAGTANATTTAGCAATTTTTTCCTCTTCATTTAGCTGGGGCATCTAC
TATTCTTATNGGGCT

CLLINC1R9L1S1

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GTGATAATTGGGGGATTTGGGAATTGACTAGTACCCTTAATATTAGGGGC
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GCCTGCGTTGTCTCTTTTATTACTATCTTCGTTGGTTGAAGGGGGAGTAGG
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TCCTTCAGTANATTTAGCAATTTTTTCCTCTTCATTTAGCTGGGGCATCTAC
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CLLINC1R10L1S1

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ATCTAGGAAGGTTCATGGAACAGCTAGCGGAAAAAACAGAACCATTGAG
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CLLINC1R11L1S1

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GGATAAACAGTTCACCCCGTCCCAGCCCCTCTCTCAACCAAAGAAGATAG
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ACCGAGGAAAAGCTATATCAGGTGCCCCCAACATTAAAGGTACCAACCAA
TTACCAAATCCACCAATTATAACTGGCATAACCATAAAAAAAATTATTAT
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AACCCCCA

CLLINC2R4L1S1

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TAGCTAAGTCAACTGAAGGACCCCTATGTGCCAAATTTGAAGACAAAGGA
GGATAAACAGTTCACCCCGTCCCAGCCCCTCTCTCAACCAAAGAAGATAG
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ACCGAGGAAAAGCTATATCAGGTGCCCCCAACATTAAAGGTACCAACCAA
TTACCAAATCCACCAATTATAACTGGCATAACCATAAAAAAAATTATTAT
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AACCCCCA

CLLINC3R4L1S1

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GGATAAACAGTTCACCCCGTCCCAGCCCCTCTCTCAACCAAAGAAGATAG
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ACCGAGGAAAAGCTATATCAGGTGCCCCCAACATTAAAGGTACCAACCAA
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AACCCCCA

CLWATC1R1L1S1

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ACCGGATATAGCTTTTCCTCGGTTAAATAATATGAGGTTTTGGTTATTACC
GCCTGCGTTGTCTCTTTTATTACTATCTTCGTTGGTTGAAGGGGGAGTAGG
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TCCTTCAGTANATTTAGCAATTTTTTCCTCTTCATTTAGCTGGGGCATCTAC
TATTCTTATNGGGCT

CLWATC1R2L1S1

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ACCTGATATAGCTTTTCCTCGATTAAATAATATGAGGTTTTGGTTATTACC
ACCGGCGTTGTCTCTTTTATTATTATCTTCGTTGGTTGAAAGGGGGGCAGG
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GTCCTTCAGTAGATTTAGCCATTTTTTCTCTTCATTTAGCCGGGGCATCTTC
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CLWATC2R2L1S1

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GTCCTTCAGTAGATTTAGCCATTTTTTCTCTTCATTTAGCCGGGGCATCTTC
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CLWATC3R2L1S1

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ACCGGCGTTGTCTCTTTTATTATTATCTTCGTTGGTTGAAAGGGGGGCAGG
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GTCCTTCAGTAGATTTAGCCATTTTTTCTCTTCATTTAGCCGGGGCATCTTC
TATTTTAGGGGCTATTAATTTTATAACCACAACTTTAAATATACGGAGTAG
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AGCAGTTTTGTTACTTCTTTCTTTGCCT

CLWATC4R2L1S1

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GTAATAATTGGGGGATTTGGGAATTGATTAGTACCTTTAATATTAGGGGC
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GTCCTTCAGTAGATTTAGCCATTTTTTCTCTTCATTTAGCCGGGGCATCTTC
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CLWATC1R3L1S1

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ATGTGCCAAATTTGAAGACAAAGGAGGATAAACGGTTCACCCCGTCCCAG
CCCCTCTCTCAACCAAAGAAGATAGTAACAAAAGGGACAAAGCCGGAGG
CAATAATCAAAAACTTATATTATTTAACCGAGGAAAAGCTATATCAGGTG
CCCCCAACATTAAAGGTACCAACCAATTACCAAATCCACCAATTATAACT
GGCATAACCATGAAAAAAAATTATTATAAATGCATGGGCAGTAACAATCA
CATTATATAATTGATCATTACCCATCAAACCCCCA

CLWATC1R4L1S1

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CCGGCTAAATGAAGAGAAAAAATTGCTAAATCTACTGAAGGACCTCTGTG
CGCTAAATTTGACGAAAGAGGAGGATAAACTGTCCACCCTGTTCCTGCTC
CCCTTTCAACCAACGAAGATAATAATAAAAAAGACAACGCAGGTGGTAA
CAACCAAAAACTCATATAATTTAATCGAGGAAAAGCTATATCCGGTGCCC
CCAATATTAAAGGCACTAATCAATTACCAAATCCCCCGATTATTACCGGT
ATAACCATAAAAAAAAATCATTATAAATGCATGAGCAGTAACAATTACATT
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CAULSPINIF

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ATATTTAAAGTTGTGGTTATAAAATTAATAGCCCCTAAAATAGAAGATGC
CCCGGCTAAATGAAGAGAAAAAATGGCTAAATCTACTGAAGGACCTCTGT
GAGCTAAGTTTGACGAAAGGGGAGGATAAACTGTCCATCCTGTTCCTGCC
CCCCTTTCAACCAACGAAGATAATAATAAAAGAGACAACGCCGGTGGTAA
TAACCAAAACCTCATATTATTTAATCGAGGAAAAGCTATATCAGGTGCCC
CTAATATTAAAGGTACTAATCAATTCCCAAATCCCCCAATTATTACCGGTA
TAACCATAAAAAAAAATCATTATAAATGCATGAGCAGTAACAATTACATTA
TAAAGTTGATCATTCCCTAATAACCCCCCGGGTTGCCTTAATTCAACCCGA
ATTAAAGCCCTAAGACCCACCCGAATTAAAGCCCTAAGACCCA

CALXXX

TCAGCCCATACCAATAAAGGAGTTCGTATTAAAGTTATTAGACTACTACG
TATATTTAAAGTAGTAGTTATAAAATTAATGGCCCCCAAAATTGAAGAAG
CACCCGCCAAATGCAAAGAAAAAATAGCTAAGTCAACTGAAGGACCCCT
ATGTGCCAAATTTGAAGACAAAGGAGGATAAACAGTTCACCCCGTCCCAG
CCCCTCTCTCAACCAAAGAAGATAGTAACAAAAGGGACAAAGCCGGAGG
CAATAATCAAAAACTTATATTATTTAACCGAGGAAAAGCTATATCAGGTG
CCCCCAACATTAAAGGTACCAACCAATTACCAAATCCACCAATTATAACT
GGCATAACCATAAAAAAAATTATTATAAATGCATGGGCAGTAACAATCAC
ATTATATAATTGATCATTACCCATCAAACCCCCAGGTTGACTCAACTCTAC
CCGAATTAAAGCCCTAAGACCCA

APPENDIX C

This appendix shows the number of sequences that were obtained for the three *Callopora* species for the three sites based on the SSCP haplotypes.

Table C-1: Number of *Callopora lineata* sequences from Lydstep

	Sequence 1	Sequence 2	Sequence 3	Sequence 4	Sequence 5	Sequence 6	Sequence 7	Sequence 8	Sequence 9	Sequence 10	Sequence 11	Sequence 12	Sequen 13
Colony 1	16	-	-	-	-	-	-	-	-	-	-	-	-
Colony 2	-	1	1	1	1	1	-	-	-	-	-	-	-
Colony 3	-	-	-	-	-	-	2	-	-	-	-	-	-
Colony 4	-	-	-	-	-	-	-	9	-	-	-	-	-
Colony 5	-	-	-	-	-	-	-	3	-	-	-	-	-
Colony 6	-	-	-	-	-	-	-	3	-	-	-	-	-
Colony 7	-	-	-	-	-	-	-	2	4	-	-	-	-
Colony 8	-	-	-	-	-	-	-	-	-	3	-	-	-
Colony 9	-	-	-	-	-	-	-	-	-	-	4	-	-
Colony 10	-	-	-	-	-	-	-	-	-	-	-	7	-
Colony 11	1	-	-	-	-	-	-	-	-	-	-	-	4
Colony 12	1	-	-	-	-	-	-	4	-	-	-	-	-
Colony 13	-	-	-	-	-	-	-	-	-	-	-	-	1
Colony 14	-	-	-	-	-	-	-	3	-	-	-	-	-

Table C-2: Number of *Callopora lineata* sequences from Watwick Bay

	Sequence 1	Sequence 2	Sequence 3	Sequence 4
Colony 1	3	-	-	-
Colony 2	-	2	-	-
Colony 3	-	2	-	-
Colony 4	-	1	-	-
Colony 5	-	1	-	-
Colony 6	-	-	5	-
Colony 7	-	-	-	1

Table C-3: Number of *Callopora rylandi* sequences from Lydstep

	Sequence 1	Sequence 2	Sequence 3	Sequence 4	Sequence 5	Sequence 6	Sequence 7	Sequence 8	Sequence 9
Colony 1	2	-	-	-	-	-	-	-	-
Colony 2	-	3	-	-	-	-	-	-	
Colony 3	-	-	1	1	-	-	-	-	-
Colony 4	2	-	-		1	1		-	-
Colony 5	-	-	-	-	-	-	1	-	-
Colony 6	2	-	-	-	-	-	-	1	-
Colony 7	-	-	-	-	-	-	-	-	1

Table C-4: Number of *Callopora rylandi* sequences from Watwick Bay.

	Sequence 1	Sequence 2	Sequence 3	Sequence 4	Sequence 5	Sequence 6	Sequence 7	Sequence 8	Sequence 9
Colony 1	3	-	-	-	-	-	-	-	-
Colony 2	-	3	-	-	-	-	-	-	-
Colony 3	-	5	-	-	-	-	-	-	-
Colony 4	-	-	3	-	-	-	-	-	-
Colony 5	-	-	-	1	1	-	-	-	-
Colony 6	-	4	-	-	-	-	-	-	-
Colony 7	-	-	-	-	-	4	-	-	-
Colony 8	-	5	-	-	-	-	-	-	-

Table C-5: Number of *Callopora dumerilii* sequences from Lydstep

	Sequence 1	Sequence 2	Sequence 3	Sequence 4	Sequence 5	Sequence 6	Sequence 7	Sequence 8	Sequence 9
Colony 1	4	-	-	-	-	-	-	-	-
Colony 2	-	5	-	-	-	-	-	-	-
Colony 3	-	-	4		-	-	-	-	-
Colony 4	-	-	-	5	-	-	-	-	-
Colony 5	-	-	-	-	4	-	-	-	-
Colony 6	-	-	-	-	-	4	-	-	-
Colony 7	-	-	-	-	-	-	3	-	-
Colony 8	-	-	-	-	-	-	-	1	-

Table C-6: Number of *Callopora dumerilii* sequences from Pembroke Ferry.

	Sequence 1	Sequence 2	Sequence 3	Sequence 4	Sequence 5	Sequence 6
Colony 1	2	3	-	-	-	-
Colony 2	-	3	-	-	-	-
Colony 3	-	-	3	-	-	-
Colony 4	-	-	-	3	1	-
Colony 5	2	-	-	-	-	-
Colony 6	-	-	3	-	-	-
Colony 7	-	-	1	-	-	-
Colony 8	2	-	-	-	-	-
Colony 9	-	-	-	-	-	1

Table C-7: Number of *Callopora dumerilii* sequences from Watwick Bay.

	Sequence 1	Sequence 2
Colony 1	3	-
Colony 2	-	13

APPENDIX D

This appendix presents the primer sequences that have been used throughout the course of this thesis. It starts with the non-specific bryozoan primers that were initially used and finishes with the highly specific Callopora primers that were designed for the purposes of this thesis. The primer sequences that have been used for the sequencing reactions are also included.

Non-specific bryozoan primers (Porter pers. com.)

1st pair

- ANA-F: YGGRATARTYGGRAGAGG (where Y=C+T and R= A+G)
- ANA-R: TGAKGTRTTSASATTWCG (where K= G+T, R=A+G, S= G+C and W= A+T)

Universal COI primers (Folmer *et al.*, 1994)

- HCO2198: TAAACTTCAGGGTGACCAAAAAATCA
- LCO1490: GGTCAACAAATCATAAAGATATTGG

COI-1 (Waeschenbach 2003)

- CO1-1F: TTGGGTTGTGGTCTGGAATG
- COI-1R: CTGCTAACACAGGCAAAGAAAG

COI-2 (This study)

- COI1-2F: GGGTCTTAGGGCTTTAATTCGGGT
- COI1-2R: CAGCCCATACCAATAAAGGAGTTCG

COI-3 (This study)

- COI-3F: GGGGGTTTGATGGGTAATG
- COI-3R: TCAGCCCATACCAATAAAGGA

M13 -primers used for sequencing reactions (New England Biolabs)

- M13-F: CGCCAGGGTTTTCCCAGTCACGA
- M13-R: AGCGGATAACAATTTCACACAGGA