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Reproductive compatibility and speciation in *Celleporella hyalina* Ssensu Lato

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**REPRODUCTIVE COMPATIBILITY AND
SPECIATION IN *CELLEPORELLA HYALINA* SENSU
LATO**

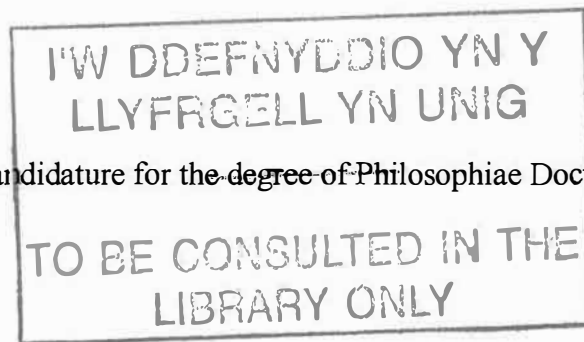
A Thesis submitted to the University of Wales, Bangor

by

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Summary.

The site of sperm storage in *C. hyalina* was investigated using thymidine-methyl-³H. Failure of sperm donor colonies to take up the radiotracer and incorporate it into spermatozoa appears to have prevented the identification of the site of storage of exogenous sperm in this species. Potential ways of overcoming this problem in future studies are discussed.

Considerable variation in the ability of colonies to self-fertilise was observed among twenty one geographically isolated populations of *C. hyalina*. Results ranged from a complete lack of self-fertilisation activity in isolated colonies from most populations to comparable levels of embryo production by isolated and grouped colonies from the Woods Hole population. This study also included colonies of two other *Celleporella* species, *C. angusta* and *C. carolinensis*. Isolated colonies of *C. carolinensis* were found to suppress the development of female zooids, and therefore to be incapable of self-fertilisation. All isolated colonies of *C. angusta*, however, were found to produce embryos and release abundant viable larvae in numbers comparable to those released by grouped colonies.

Mating trials to assess the level of reproductive compatibility between geographically isolated populations revealed the presence of at least seven good biological species, and perhaps several incipient species within *C. hyalina* sensu lato. All mating trials between populations occupying separate phylogenetic clades (based on mitochondrial DNA sequencing) were unsuccessful. Mating trials between populations occupying the same phylogenetic clade gave results ranging from complete reproductive compatibility to complete reproductive incompatibility.

Morphological analysis appeared to reveal the presence of at least four morphologically distinct populations within *C. hyalina* sensu lato, and revealed a number of potentially useful taxonomic characters. However, the presence of cryptic species within *C. hyalina* sensu lato could not be ruled out. The problems

associated with the failure to recognise cryptic species are discussed. These studies suggest that the use of a concordant suite of unrelated morphological, reproductive and molecular genetic characters may be necessary for the discrimination of species in bryozoan groups.

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Chapter 1.

General Introduction.

Bryozoans are sessile, colonial, filter-feeding, aquatic coelomates of which there are over 4,000 known extant species and as many as 4 times this number of extinct species preserved as fossils (Ryland, 1970). A few species of bryozoans inhabit freshwater lakes and rivers, though the vast majority are to be found in the marine environment where they are a common component of the faunal assemblages of most shorelines. Bryozoan colonies are frequently found colonising marine macroalgae and other substrata such as rocks and shells (Ryland, 1970; Ryland and Hayward, 1977; Hayward and Ryland, 1979; 1985; Hayward, 1985), often occurring in very high numbers (up to thirty species have been recorded encrusting a single *Pinna* shell – Ryland, 1970). Marine bryozoans are by no means limited to the shoreline, and may be found ranging from the intertidal to the abyssal zone into depths of over 8,000 m (Ryland and Hayward, 1977), although they are most abundant, and their diversity at its greatest, in the shallow seas of the continental shelf (Ryland, 1970; Ryland and Hayward, 1977; Hayward and Ryland, 1979).

The phylum Bryozoa is divided into three classes (for a full description of each of the major divisions of this phylum see Ryland, 1970). The first of these is the Phylactolaemata, which is exclusively freshwater in distribution. The second class, the Stenolaemata, is divided into four orders, the Cyclostomata, the Cystoporata, the Trepostomata, and the Cryptostomata, only one of which, the Cyclostomata, contains extant species. The third class, the Gymnolaemata, is an important invertebrate class containing over 3,000 extant species (Ryland, 1970). It is divided into two orders, the Ctenostomata, and the larger Cheilostomata. Early in the last century the Cheilostomata were further divided into two suborders, the Anasca and the Ascophora (Hayward and Ryland, 1979). Members of the suborder Anasca are characterised by having a non-calcified upper surface known as the frontal membrane, whereas members of the suborder Ascophora have an entirely calcified frontal surface. This difference in

morphology between the anascans and ascophorans leads to each having a different method of everting the feeding apparatus, or lophophore (see below). In the suborder Anasca, parietal muscles, each attached to the frontal membrane, contract pulling the frontal membrane inwards and increasing the hydrostatic pressure inside the coelom. This increase in pressure, coupled with the relaxation of a pair of occlusor muscles, causes the operculum to open allowing the lophophore to protrude (Ryland, 1970). In the suborder Ascophora, the parietal muscles are attached to the underside of an underlying sac, the ascus. When contracted they cause the ascus to dilate and water from outside the zooid enters the ascus to compensate for the decreased coelomic volume. The increase in size of the ascus causes the lophophore to be protruded, with the operculum opening as before (Ryland, 1970).

Bryozoan colonies are built of modular units termed zooids. All bryozoan colonies contain modules known as autozooids that contain a polypide consisting of a U-shaped gut and a crown of ciliated tentacles termed the lophophore, used for capturing planktonic particles such as microalgae that the colony feeds upon. When everted into the feeding position lateral cilia on the tentacles beat metachronally, generating a water current that enters the top of the lophophore and passes out between the tentacles forming a central stream directed towards the mouth (Ryland, 1970). When the particles reach the mouth the pharynx acts as a suction pump drawing them in (Riisgård and Manríquez, 1997). Each zooid is able to communicate physiologically with its neighbouring zooids. In the Phylactolaemata, the main body coeloms of zooids are confluent, but, in the other two classes each zooid is clearly separated from its neighbours by bounding walls and physiological communication occurs via pores in these walls (Gordon and Hastings, 1979). In all three classes a fluid-filled mesenchymal strand, termed the funiculus, connects the stomach wall to the other parts of the zooid (Gordon *et al.*, 1987), and in the Gymnolaemata it forms a colonial network via the pores in the bounding walls (Lutaud, 1982: cited by Gordon *et al.*, 1987). The funiculus is believed to act as a pathway for transport of metabolites, allowing nutrients to pass from the feeding autozooids to non-

feeding polymorphic zooids (Best and Thorpe, 1985). All bryozoan colonies are hermaphroditic (Ryland, 1970; McKinney and Jackson, 1989). In most species all zooids will be functional hermaphrodites whereas in others separate male (androzooids), female (gynozooids), and sterile feeding zooids can be found (McKinney and Jackson, 1989).

A bryozoan colony starts life as a sexually produced larva or, in the case of the Phylactolaemata, an asexually produced dormant body termed a statoblast. Metamorphosis of the larva (or statoblast) produces a primary zooid, termed the ancestrula, which gives rise to one or more daughter zooids by the asexual process of budding. Colony growth then continues radiating from the ancestrula with the newly produced daughter zooid, or zooids, producing further buds and, therefore, further zooids. This iterative process may be prolonged, often leading to the production of many thousands of zooids in a single colony. Bryozoan colonies may be either encrusting, in which case they grow as above spreading out across the substratum, or erect. Erect colonies may either develop directly from an upright ancestrula, or in some cases an initial crust will give rise to the erect zooids (Ryland, 1970).

The chosen subject for the current study is the encrusting, ascophoran cheilostome *Celleporella hyalina* (L.), a member of the family Hippothoidae. Much work has been devoted to the problems associated with the classification of members of the Hippothoidae (Ryland and Gordon, 1977; Hastings, 1979; Morris, 1980; Moyano and Gordon, 1980). However, following the revisions of Ryland and Gordon (1977) and Hastings (1979), *Celleporella* was redefined and accepted as a valid genus within the family Hippothoidae along with the genera *Hippothoa* and *Plesiothoa* (Gordon and Hastings, 1979). *C. hyalina* appears to have a worldwide distribution amongst the polar and temperate waters of the northern and southern hemispheres (Ryland and Gordon, 1977; Hastings, 1979; Ryland, 1979; Morris, 1980; Moyano, 1986; Fernández Pulpeiro and Reverter Gil, 1992), where it is found growing predominately on macroalgae and occasionally other hard substrata (Hayward and Ryland, 1979; Morris, 1980). *C.*

hyalina forms small circular colonies, hyaline and unilaminar at first, later becoming opaque and bilaminar with frontally budded zooids originating near the colony centre (Ryland and Gordon, 1977; Hayward and Ryland, 1979). The ancestrula is schizoporelloid (lacking spines and having a sinus in the proximal margin of the orifice – Ryland and Gordon, 1977), with characteristic unilateral budding (Ryland and Gordon, 1977; Hayward and Ryland, 1979), leading to a spiral early astogeny (Hayward and Ryland, 1979). Colonies consist of three zooid types: autozooids, female zooids and male zooids (Ryland and Gordon, 1977; Hayward and Ryland, 1979). The basal layer of the colony consists mainly of sterile feeding autozooids, the sexual zooids (or gonozooids) usually being most numerous in the frontally budded layer (Ryland and Gordon, 1977; Hayward and Ryland, 1979). Male zooids are similar in appearance to the autozooids (Hayward and Ryland, 1979) but are usually smaller (Ryland and Gordon, 1977; Hayward and Ryland, 1979), occasionally being found in the basal layer of colonies as well as in the frontally budded layer (Ryland and Gordon, 1977; Cancino, 1983). Female zooids are very short and triangular in shape bearing a prominent, globular ovicell (Ryland and Gordon, 1977; Hayward and Ryland, 1979) in which brooding of the embryos occurs (see below). Female zooids are usually reported as only occurring in the frontally budded layer of colonies (Pinter, 1973; Ryland and Gordon, 1977; Hayward and Ryland, 1979; Cancino, 1983), an earlier observation of females occurring in the basal layer of *C. hyalina* (Marcus, 1938) has since been questioned, with Ryland and Gordon (1977) suggesting that Marcus “was misled by having two very similar species in his collection”. This suggestion was later proved to be correct by Ryland (1979), who identified the specimen referred to by Ryland and Gordon (1977) as being *Celleporella carolinensis*.

C. hyalina is believed to be routinely outcrossing in nature (Hunter and Hughes, 1993a; Hoare *et al.*, 1999; Manríquez, 1999 – but see Chapter 3). Male zooids consist mainly of spermatogenic tissue, and although there is no digestive tract present, the small polypide does retain a functional lophophore and well developed system for its eversion (Hughes, 1987). Recent work has shown that

sperm release in this species occurs through the protruded tentacles of the male lophophore (Hoare *et al.*, 1999; Manríquez, 1999), an observation in agreement with previous studies on sperm release in other gymnolaemate bryozoans (Silén, 1966; 1972; Bullivant, 1967; Temkin, 1994). The method of entry of sperm into the colony is unknown, however it has been suggested that sperm could be entrained by the feeding currents of extended lophophores of the autozooids (as seen in other species of bryozoan – Silén, 1966; Temkin, 1994), and access the female zooids by intra-colonial sperm migration along the funicular system (Hughes, 1987). This theory is supported by the observations of Marcus (1938), who found spermatozoa in all three zooid types, and Manríquez (1999), who demonstrated that intra-colonial sperm migration must occur in *C. hyalina*. Fertilisation is internal, occurring in the ovary, probably during vitellogenesis, or maybe even previtellogenesis (Hughes, 1987; Ostrovsky, 1998). This agrees with the observations of Temkin (1996) who studied nine other gymnolaemate bryozoans and reported that in each species sperm fuse with eggs in or near the ovaries. The resulting embryo passes from the maternal coelom into the brood chamber (ovicell) via the coelomopore (Hughes, 1987), where it receives an additional input of nutrients via a placental system (Hughes, 1987). Brooding of the embryo takes 3-4 weeks (Hughes, 1987; Cancino and Hughes, 1988). During the brooding period the embryo undergoes a 13-15 fold increase in volume (Hughes, 1987; Ostrovsky, 1998). In addition to the ovary and placental tissue contained in the female zooid, there remains a small polypide rudiment (Hughes, 1987), including a pair of short parietal muscles thought to function during larval release (Ostrovsky, 1998). The lecithotrophic larva released from the ovicell at the end of the brooding period settles on a suitable substrate within 1.5-4 hours after release, metamorphosing into the schizoporelloid ancestrula within a further two days at 15°C (Cancino and Hughes, 1988). Colony growth continues as outlined above.

The ease with which *C. hyalina* can be cultured, and propagated clonally, in the laboratory (Hunter and Hughes, 1991; Manríquez, 1999) make this species an ideal experimental organism, allowing many aspects of its reproductive biology

and morphology to be studied under controlled conditions (e.g. Hughes and Hughes, 1986; Hunter and Hughes, 1993a; 1993b; 1995; Yund and McCartney, 1994; Hunter *et al.*, 1996; McCartney, 1997; Goldson, 1998; Manríquez, 1999).

Chapter 2.

Exogenous sperm storage in *Celleporella hyalina*.

2.1. Introduction.

The storage of viable sperm by both males and females, in specialised parts of the reproductive tract, forms an integral part of the reproductive strategy employed by many animal species. Within a species the process of sperm storage may be undertaken by either the male sex, the female sex, or frequently both sexes. Good examples of this can be found in bats. Bernard (1985) reported that in the Cape horseshoe bat (*Rhinolophus capensis*) sperm storage is undertaken by the male, and this may also be the case in the Geoffroy's horseshoe bat (*Rhinolophus clivosus*) (Wessels and van der Merwe, 1997). However, Racey (1979) states that "Since all female noctules (*Nyctalus noctula*) and pipistrelles (*Pipistrellus pipistrellus*) taken from hibernation are inseminated, it would appear that in these species the burden of sperm storage falls on the female. In other species, the storage of spermatozoa destined to fertilise eggs is shared by both sexes".

Female sperm storage, or the storage of exogenous sperm in hermaphrodites, has been demonstrated in a wide variety of animal groups including mammals (Racey, 1979; Bernard *et al.*, 1997; Wessels and van der Merwe, 1997; Shimmin *et al.*, 1999; Töpfer-Petersen *et al.*, 2002), reptiles (Fox, 1956; Fox and Dessauer, 1962; Cuellar, 1966; Gist and Jones, 1989; Sever and Hamlett, 2002), amphibians (Sever and Brizzi, 1998; Sever *et al.*, 2001; Sever, 2002), birds (Hatch, 1983; Shugart, 1988; Birkhead and Møller, 1992), fish – both chondrichthys (Pratt, 1993; Hamlett *et al.*, 2002a; Hamlett *et al.*, 2002b) and osteichthys (Winge, 1927; Hogarth and Sursham, 1972; Warner and Harlan, 1982; Koya *et al.*, 2002; Muñoz *et al.*, 2002), insects (Parker, 1970; Walker, 1980) and most other invertebrates (Adiyodi and Adiyodi, 1983a; 1983b; 1988; 1990). Sperm stored in the female reproductive tract are used up relatively quickly in some cases, whereas in others the sperm may be stored for much longer periods of time; the maximum duration of storage of viable sperm varying

from a number of hours, in some mammal species, to several years, in some reptile species (see Table 1, Birkhead and Møller, 1993). In some insect species, sperm received from one or several matings may be stored in a viable condition in specialised storage organs throughout the entire egg-laying life of the recipient female (Parker, 1970; Walker, 1980; Page and Metcalf, 1982). Once sperm have been stored by the female their usage can be a remarkably efficient process, with up to 100% of stored sperm being used to produce zygotes (Lefevre and Jonsson, 1962; Ward and Carrel, 1979).

The ability of a female to store sperm imparts several potential advantages. The presence of sperm from more than one male in the reproductive tract of a female leads to conditions in which sperm competition may occur (competition between sperm from two or more males for fertilisation of the ova within a single female: Parker, 1970). Sperm competition may lead to the sperm from healthier, fitter males being used to fertilise the eggs of the female. In their study of Swedish adders, which store sperm for months before ovulation, Madsen *et al.*, (1992) showed that there was a reduced proportion of stillborn offspring in multiply mated females relative to singly mated females. Sperm competition may be enhanced by sperm storage as it increases the length of time that sperm from the different males overlap within the female reproductive tract. Females themselves may be able to exert some control over the use of stored sperm, a form of cryptic female choice (Thornhill, 1983; Eberhard, 1991; 1996). King (1962) and Werren (1980) provided evidence to support this theory in their studies of haplodiploid hymenoptera. In these studies they have shown that females can choose whether to produce male or female offspring and can therefore choose whether or not to use the stored sperm to fertilise their eggs. More recently, Bloch Qazi (2003) has shown that in the red flour beetle (*Tribolium castaneum*) “the opportunity exists for cryptic female choice by actively limiting sperm transfer”.

The long term storage of significant quantities of viable sperm by a female extends the period over which she can lay fertile eggs without having to copulate repeatedly. Copulation can be a very time consuming process, lasting for over 24

hours in some species of animal (e.g. some insects and snakes: Parker, 1984; Olsson and Madsen, 1998), and carries with it many costs that may be incurred by the female. Such costs associated with copulation include: loss of time which could otherwise be spent nest building or feeding; increased risk of predation due to reduced vigilance and motility or increased conspicuousness; increased risk of disease or parasite transmission; and finally an increased risk of being injured by the male (Daly, 1978; Hunter *et al.*, 1993). Therefore, sperm storage may help females to minimize the costs associated with copulation. In birds, each egg laid in a clutch is fertilised separately and the ability to store sperm may have initially evolved to obviate the need for copulation to occur prior to the ovulation of each egg (Birkhead, 1998).

Another benefit of sperm storage is that it allows the temporal separation of the processes of copulation and fertilisation (Hatch, 1983; Bernard *et al.*, 1997). The banana bat (*Pipistrellus nanus*), from southern Malawi, mates in the middle of the cool, dry season between mid-June and early July, but then stores the sperm received until ovulation occurs in August (Bernard *et al.*, 1997). In the northern fulmar (*Fulmarus glacialis*) the ability of the female to store sperm allows the separation of the sexes over pelagic water for several weeks prior to egg laying (Hatch, 1983). This extended foraging period allows the female to build the reserves she requires in order to produce the single large egg which is laid on her return, and allows the male to build the reserves required to fast during the first shift of incubation (Lack, 1966). The departure of males and females on this pre-laying exodus is rarely synchronised and therefore it is unlikely that the pair associate at sea (Hatch, 1983). On her return to the nest the female usually lays within the first 24 hours, a period during which her mate may or may not be present and, therefore, it is essential that copulation occurs before the exodus to ensure the fertility of the egg (Hatch, 1983). The ability to store sperm and, therefore, allow the separation of copulation and fertilisation is also beneficial to those animals that live in submarginal habitats where females may only rarely encounter males (Fox, 1963). It may also allow the colonization of insular habitats by a single female instead of a mating pair (Conner and Crews, 1980).

Finally, in the marine environment many invertebrates release sperm into the water current that are subsequently captured by the acting female and used to fertilise eggs internally (Holland, 1976; Daly and Golding, 1977; Picard, 1980; Miller, 1982; Kahmann, 1984; Bishop and Ryland, 1991; Todd *et al.*, 1997), a reproductive strategy referred to as spermcast mating (Pemberton *et al.*, 2003). Sperm that are released into the water will become progressively diluted as they move further away from the source, leading to reduced rates of fertilisation (Pennington, 1985; Levitan, 1991; Levitan *et al.*, 1992). The ability of the acting female to store exogenous sperm may help to increase fertilisation success as the sperm can be collected over time and re-concentrated at the site of storage (Temkin, 1996; Bishop, 1998), obviating the problem of dilution.

Sperm storage has been recorded in a variety of hermaphroditic invertebrates (Ghiselin, 1969; Adiyodi and Adiyodi, 1988; 1990), and in many cases the stored sperm is known to be of an exogenous origin (Beeman, 1970; Daly and Golding, 1977; Ward and Carrel, 1979; Picard, 1980; Clark, 1981; Bishop and Ryland, 1991; Todd *et al.*, 1997). The ability of *C. hyalina* to store sperm was first discussed by Cancino (1983), who argued that the production of larvae by colonies in reproductive isolation could only be accounted for by one of three possible explanations, sperm storage, parthenogenesis or self-fertilisation. Evidence suggesting that what was observed by Cancino was in fact sperm storage was subsequently obtained by Hoare *et al.* (1999) and then by Manríquez (1999). Both these authors reported the production of outcrossed progeny by colonies that had been returned to reproductive isolation for longer than the 3-4 week brooding time typical of this species. Moreover, Manríquez (1999) showed that even immature colonies, as small as three zooids in size, were able to capture and store sperm ready to use it once they had matured, possibly providing a mechanism of preventing self-fertilisation (Hunter and Hughes, 1993a).

In previous studies on Bryozoan species with zooidal polymorphism, sperm have been found to occur in all three zooid types and in the funicular system (Marcus, 1938), a fluid-filled mesenchymal strand connecting the stomach wall to the other parts of the zooid (Gordon *et al.*, 1987) and believed to act as a pathway for the transport of metabolites to the non-feeding polymorphic zooids (Best and Thorpe, 1985). This suggests that, possibly facilitated by chemotaxis (Miller, 1982), stored sperm may be able to move between zooids within a colony. Indirect evidence of intra-colonial sperm movement in *C. hyalina* was obtained by Manríquez (1999). Colonies were exposed to allosperm for a period of 1-2 hours, and then placed into reproductive isolation. Intra-colonial movement of allosperm was shown by the “production of oocytes and brooding of embryos in female zooids situated beyond the original growing edge of the colony”. Manríquez (1999) also found that the association of females with recycling autozooids may have a deleterious effect on brooding success in *C. hyalina*. Therefore, it is possible that in *C. hyalina* stored sperm moves around within the colony to areas where it has a better chance of being successfully used – i.e. where female zooids are surrounded by nutritive autozooids.

In order to fully understand the reproductive process in any sperm storing animal it is essential that the process and site of sperm storage are characterised. This study aims to use sperm radiolabelling and light-microscope autoradiography to identify the site of sperm storage in *C. hyalina*, a similar approach to that used by Bishop and Sommerfeldt (1996) to study the uptake and storage of exogenous sperm in the compound ascidian *Diplosoma listerianum*.

2.2. Materials and Methods.

2.2.1. Biological material

The two genotypes of *C. hyalina* used in the present study (Q_1 and E_1) were established from founder larvae released by colonies encrusting fronds of *Fucus serratus* and *Laminaria saccharina* collected from the Menai Strait at Beaumaris, Anglesey, U.K (53°16'N; 4°05'W). These colonies were collected during low spring tides in April 1996 by R.N. Hughes to establish genotypes for use in studies of mate choice in *C. hyalina* (Manríquez, 1999). Clones of these genotypes had been maintained in reproductive isolation in the laboratory at Bangor, using protocols described by Manríquez (1999), until their use in the current study. Mating compatability between the two genotypes had already been proven by earlier experimental work (Manríquez, 1999). Each ramet used was between 1.5 and 2 cm in diameter at the start of the experiment and was sexually mature, having both male and female zooids present. Genotype Q_1 was chosen as the labelled sperm donor and E_1 as the labelled sperm recipient.

2.2.2. Culture conditions

Ramets of the two genotypes used in the current study were all grown on pieces of acetate (ca. 38 x 75 mm) placed in slide racks in sets of 12 and housed in 2 litre plastic drinks bottles. Each bottle was furnished with a lid with a hole cut in its centre through which an airline was passed to provide gentle aeration via an aquarium airstone. 100 ml of the cryptophyte alga *Rhinomonas reticulata*, at a concentration of approximately 700 cells μl^{-1} , was added daily to each container as a food source. The ramets were maintained at 15°C in a controlled temperature room with a 12:12 dark/light photoperiod.

2.2.3. Radiolabelling

Sperm radiolabelling was attempted using thymidine-methyl- ^3H at a specific activity of 5 Ci mmol^{-1} (Sigma Chemical Company, Poole, Dorset, England). Two sets of 12 ramets of genotype Q_1 were each placed into 1 litre of 0.2 μm filtered, UV-sterilised seawater (FSW) containing 0.18 $\mu\text{Ci ml}^{-1}$ radiotracer.

After 5 d, the ramets were removed from the original solution and placed into 1 litre of a freshly made up solution for a further 5 d, giving a total of 10 d exposure to radiotracer. At the end of the second 5 d period the ramets were removed from the radiotracer solution and washed with FSW for 2-3 minutes before being placed into 1 litre of FSW without radiotracer. After 24 hours the ramets were subjected to a further water change in an attempt to ensure that any radiotracer not taken up by the colonies had been removed.

2.2.4. Establishment of crosses to investigate sperm storage

In order to investigate sperm storage, crosses were performed by placing together 12 ramets of sperm donor genotype Q_1 and 12 ramets of sperm recipient genotype E_1 in the same container with 1 litre of FSW (without radiotracer) for 7 d. At the end of the 7 d period, the ramets of the donor genotype Q_1 were placed into Bouins fixative for 24 hours at 15°C before being transferred to 70% ethanol. The ramets of the recipient genotype E_1 were placed back into reproductive isolation and cultured for a further 2 wk before being fixed and preserved in the same manner as ramets of Q_1 . The crosses were paralleled by control crosses using unlabelled ramets of the genotype Q_1 .

2.2.5. Production of autoradiographs

Before being sectioned, preserved specimens were decalcified for 2 weeks in 5% formic acid in 5% formaldehyde. After decalcification, specimens of both genotypes were embedded in wax and sectioned using a rotary microtome. The sections were mounted on cleaned and gelatinized glass microscope slides. Prior to being dipped (see below) the sections were thoroughly dewaxed in xylene and rehydrated in distilled water (before being rehydrated the slides were passed through a decreasing alcohol series to remove all traces of xylene). In a darkroom, the slides were dipped into molten Kodak NTB2 emulsion at 43°C in a water bath, drained and placed into drying racks for 2 h. The emulsion was diluted 10 to 15% with distilled water (a procedure that had previously been reported as helping to achieve a thin and even coverage of the slides: Bishop and Sommerfeldt, 1996). After drying, the slides were arranged into 1 of 4 plastic

staining racks each of which was placed inside a light-tight black plastic box along with silica-gel desiccant. Each box was held tightly shut using a rubber band, sealed inside a black plastic bag and placed in a freezer at -20°C for 4, 6, 8 or 10 wk. Each box contained a series of sections from a ramet of genotype E_1 that had received labelled sperm, a ramet of genotype E_1 that had received unlabelled sperm and a ramet of the donor genotype Q_1 that had been exposed to radiotracer.

After 4, 6, 8, or 10 weeks in the freezer, the slides were removed from the boxes in a darkroom and developed using Kodak Dektol developer at 20°C for 4 min, fixed in Kodak 3000 for 6 min and washed. The sections were then lightly stained with Ehrlich's haematoxylin for 2 min followed by eosin for 5 min, dehydrated through an alcohol series, dipped in xylene and then covered with a coverslip using DPX mountant.

2.3. Results and Discussion.

A total of 464 histological sections were produced during the course of this investigation, 72 of these being sections through sperm donor colonies. No labelled sperm were found in any of the sections produced from either sperm donor colonies or sperm recipient colonies. (Plate 2.1 is a section through a typical male zooid from a sperm donor colony, showing an abundance of unlabelled spermatozoa). Although spermatogenesis has been studied in *C. hyalina* (Hughes, 1987), no information is given about the duration of this process. However, Silén (1966) noted in *Electra posidoniae* that “the time required by the male cells to develop from the first stage perceivable under the preparation microscope to the stage of free motility within the zooid was never found to surpass 9 days”. Assuming that in *C. hyalina* the process of spermatogenesis is similar in duration to that in *E. posidoniae*, then the 10 d exposure to radiotracer, followed by 2 d in FSW without radiotracer, should have been long enough for mature spermatozoa to be labelled, and ready for release, during the 7 d period in which the crosses were performed.

There are two possible explanations to account for the apparent lack of labelled sperm in the male zooids of sperm donor colonies: failure of the colonies to take up the radiotracer and incorporate it into the spermatozoa, or too short an exposure time prior to development of the slides. The four batches of slides produced in this experiment were given exposure times of 4, 6, 8, or 10 weeks in the freezer. (The zooid shown in Plate 2.1, is from a batch that were given an exposure time of 8 weeks). Each of these batches were given exposure times longer than the three weeks used by Bishop and Sommerfeldt (1996) in their very similar study on the compound ascidian *Diplosoma listerianum*. Other autoradiographic studies on marine invertebrates, in which methyl-tritiated thymidine was used to label sperm, have included exposure times the same as, or similar to, those used by Bishop and Sommerfeldt (1996). Picard (1980) in his study of spermatogenesis and sperm-spermatheca relations in *Spirorbis spirorbis* used an exposure time of three weeks. Bishop (1996) again used an exposure

time of three weeks in his investigation of sperm movements in the female reproductive tract of *D. listerianum*. Also, Beeman (1970) in his study of sperm exchange and storage in the sea hare *Phyllaplysia taylori*, used exposure times of fifteen to eighty two days. The exposure times used in the present investigation seem to have been long enough for the successful production of autoradiographs and, therefore, too short an exposure time cannot account for the lack of labelled sperm seen in the sections produced. Therefore, it seems likely that the main reason for the observed lack of labelled sperm in the histological sections produced is the failure of the colonies to take up the radiotracer. In their study on *D. listerianum* (in which sperm labelling was achieved by culturing the experimental ramets in a seawater medium containing radiotracer) Bishop and Sommerfeldt (1996) make no suggestion as to how the radiotracer was taken up by the animal. However, due to the fact that the methods used in the current study are very similar to those used by Bishop and Sommerfeldt (1996) in their study, the lack of labelled sperm may be due to biological differences between the experimental animals used. Most ascidians are suspension feeders (Armsworthy *et al.*, 2001). The beating of the cilia on the branchial basket creates a water current that draws water in through the inhalant siphon (Armsworthy *et al.*, 2001). This current of water passes through the branchial basket, into the atrium and exits through the exhalant siphon, suspended particles being removed by mucus sheets that line the inside of the basket (Armsworthy *et al.*, 2001). Most tunicates (such as ascidians) feed continuously (Bone *et al.*, 2003) and can filter an enormous quantity of water (a specimen of *Phallusia* can filter 173 litres in 24 hours – Barnes, 1963). This means that internal surfaces of the ramets of *D. listerianum* used by Bishop and Sommerfeldt (1996) would have been exposed to seawater containing radiotracer almost continually during the period of radiolabelling. Bryozoans, however, feed using a structure called a lophophore (see Chapter 1) which is everted into the water column. Beating of the cilia on the tentacles of the lophophore creates a water current that enters the top of the lophophore and passes out between the tentacles forming a central stream directed towards the mouth (Ryland, 1970). Any particles contained in this water current are directed towards the mouth where the pharynx acts as a

suction pump drawing them in (Riisgård and Manríquez, 1997). Therefore, very little, if any water enters the inside of the zooid during feeding, except for the small amount required to fill the ascus during lophophore eversion (see Chapter 1). Another feature of bryozoan feeding is that in the laboratory colonies do not feed continually (pers. obs.), and undisturbed colonies are frequently seen to have only a few lophophores everted, or none at all. This may be due to the addition of relatively high concentrations of algae to the culture vessels, resulting in a zooid being able to completely fill its stomach with algal cells in a very short space of time. Therefore, in bryozoans the only parts of an individual that are exposed to the seawater medium that contains the radiotracer are the tentacles of the lophophore and the inside of the ascus, and this exposure may be for very short periods of time as opposed to the almost constant exposure suffered by the ascidians. The other feature of ascophoran cheilostomes, such as *C. hyalina*, that may have resulted in the failure to take up the radiotracer is the fact that each individual zooid is enclosed by a calcified outer wall. Therefore, it is very unlikely that the radiotracer could have entered the zooid through this outer layer. Previous studies on another marine bryozoan, *Flustrellidra hispida*, (Best and Thorpe, 1991) have shown that this species is able to absorb dissolved organic matter in the form of ^{14}C -glucose and ^{14}C -glycine. However, *F. hispida* belongs to the order Ctenostomata whose members have uncalcified body walls, and therefore no external barrier to absorption.

If the site of sperm storage in *C. hyalina* is to be revealed using autoradiographical techniques then a different method of labelling must be used. Previous studies involving radiolabelling of sperm in marine invertebrates have employed similar techniques to the current study, either culturing or incubating the experimental animals in seawater containing the chosen radiolabel (Picard, 1980; Bishop, 1996; Bishop and Sommerfeldt, 1996). However, Beeman (1970), working with the sea hare *P. taylori*, used the more invasive technique of injecting his sperm donor animals with a seawater thymidine solution. Although the use of a micromanipulator may be employed to help with the procedure of trying to get an injection into something as small as an individual zooid, the

process is likely to badly damage the zooid leading to any solution injected easily escaping before it can be translocated to other zooids. The feeding of radiolabelled algal food to bryozoans has successfully been used to study the transport of metabolites within colonies (Best and Thorpe, 1985; Miles *et al.*, 1995; Best and Thorpe, 2002), the funicular system being suggested as a pathway for transport, allowing metabolites to pass from the feeding autozooids to non-feeding polymorphic zooids (Best and Thorpe, 1985). Therefore, the feeding of radiolabelled algae to the donor colonies may be a suitable technique for labelling spermatozoa. If enough time is allowed for the radioisotope to reach the male zooids and be incorporated into the spermatozoa before the establishment of the crosses between donor colonies and recipient colonies, the site of storage of exogenous sperm in *C. hyalina* may be established using this technique.

Another possible technique that could be used to establish the site of sperm storage in *C. hyalina* involves using a confocal laser scanning microscope to find the position of fluorescence stained sperm in recipient colonies. Ramets of a donor genotype could be induced to release sperm by maintaining them in the dark for 12 h and then exposing them to a bright light source for 15 min (dark/light reaction – Manríquez, 1999). The sperm released could then be collected onto a filter paper and resuspended into a known volume of seawater by backwashing them off the filter paper. A fluorescent dye, such as fluorescein isothiocyanate, could then be added to the sperm suspension, resulting in fluorescence stained sperm. The stained sperm could then be placed in with the sperm recipient colonies, which would be cultured for a couple of days to allow the sperm to reach the storage sites within the colonies before being viewed under the confocal laser scanning microscope.

The site of sperm storage in *C. hyalina* is most likely to be found within the autozooids. The evidence to suggest this comes from Manríquez (1999) who discovered that sexually immature colonies of Welsh *C. hyalina*, as small as three autozooids in size, were able to capture and store allosperm until they had reached sexual maturity and were ready to use it. Hunter and Hughes (1993a)

suggested that if *C. hyalina* was capable of storing sperm before the onset of sexual maturity, and subsequently using this sperm to fertilise ova, then sperm storage “theoretically could provide a mechanism of preventing self-fertilisation, and would also increase the genetic diversity of the progeny”. However, data in chapter 3 provides evidence to suggest that individual colonies from the majority of populations studied are incapable of selfing, and therefore it is unlikely that the ability to store sperm has evolved as a mechanism to prevent selfing in *C. hyalina*. It is possible, however, that being capable of obtaining and storing sperm from a very early age prior to being able to reproduce does lead to sperm from a variety of sources being obtained, helping to ensure a good level of genetic diversity amongst the offspring. The ability to store sperm may, therefore, lead to conditions in which sperm competition can occur, and may present the colony with the opportunity for sperm selection, a form of cryptic female choice (Thornhill, 1983; Eberhard, 1991; 1996). Evidence to suggest that storage of allosperm has not evolved as a mechanism to enable cryptic female choice in *C. hyalina* was obtained by Manríquez (1999). In an experiment with Welsh individuals he showed that the progeny of colonies exposed to a cocktail of allosperm occurred in the same proportions as the allosperm in the cocktail. Another study on Welsh colonies of *C. hyalina* has demonstrated that early allocation to female zooids (gynozoids), but not male zooids (androzooids), is retarded pending reception of allosperm (Hughes *et al.*, 2002). Therefore, in the absence of allosperm the colony can allocate more resources to somatic growth and maximise reproductive success through the male function. The acquisition of allosperm leads to an enhancement in the allocation to female zooids (Hughes *et al.*, 2002). In order for this sperm to be utilised by the new female zooids it must be stored within the colony until the female zooids become functional. Therefore, the ability to store allosperm is strongly linked to the modulation of female allocation in *C. hyalina*.

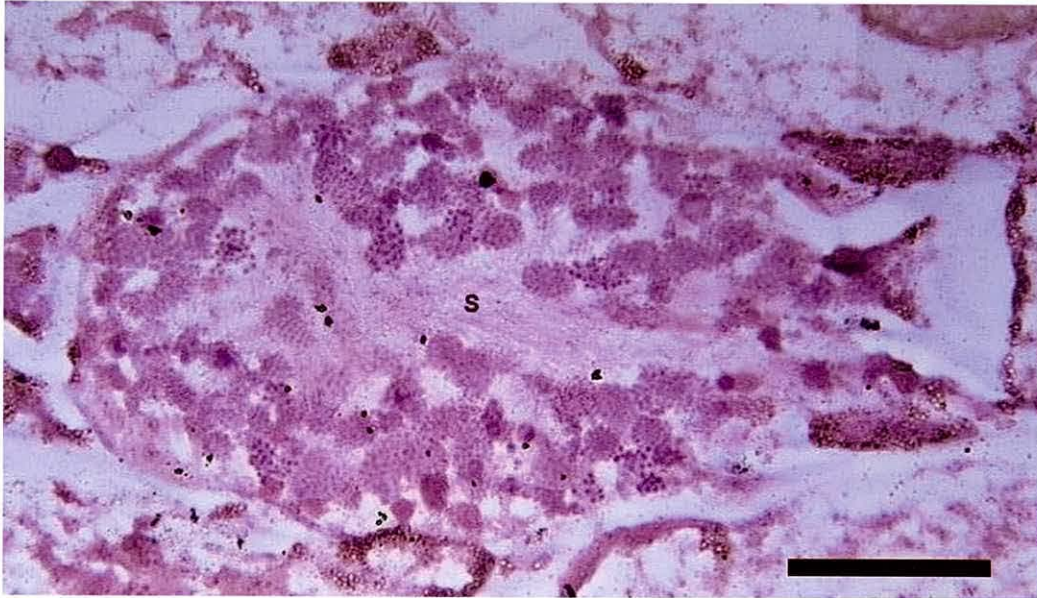


Plate 2.1. Section through a male zooid from a sperm donor colony showing unlabelled mature spermatozoa ready for release (S).

Scale bar = 100 μ m.

Chapter 3.

Self-fertilization in *Celleporella hyalina*.

3.1. Introduction.

Simultaneous hermaphroditism, the condition in which both male and female reproductive systems are present in a single genetic entity at the same time, affords the potential for self-fertilisation (Darwin, 1876; Stebbins, 1950). In plants, hermaphroditism is a very common phenomenon, and although in nearly every major subdivision of the plant kingdom some species occur that require cross-fertilisation, self-fertilising species are by no means uncommon (Stebbins, 1950; Jarne and Charlesworth, 1993). In most animal species cross-fertilisation is necessary (Stebbins, 1950). However, simultaneous hermaphroditism has been shown to occur in many animal phyla (Ghiselin, 1969), and is indeed a common feature of many sessile invertebrates, affording the potential for self-fertilisation in these groups.

Close inbreeding, of which self-fertilisation is the most extreme form, has long been known to have harmful effects on the fitness of the resulting offspring (Darwin, 1868; 1876; Crampe, 1883 and Ritzema-Bos, 1894: both cited by Wright, 1977). This detrimental result, now referred to as inbreeding depression, is caused primarily by an increase in levels of homozygosity within the breeding population, resulting in the unmasking of the effects of deleterious recessive alleles and preventing heterozygote overdominance (Charlesworth and Charlesworth, 1987). Maynard-Smith (1978) suggested that when maintained in the long-term, complete selfing would lead to a species suffering the same long-term disadvantages as a parthenogenetic population, these being a lack of genetic variability and therefore, a lack of evolutionary potential.

However, inbreeding, particularly the ability to self-fertilise, also carries with it certain advantages. The ability of a genetic individual to self-fertilise conveys the advantage of being able to ensure sexual reproduction in the absence of conspecifics (reproductive assurance), and should be favoured even when strong

inbreeding depression will result (Jarne and Charlesworth, 1993). This is particularly advantageous when an individual becomes isolated, such as when colonising new habitat space, as it may allow the founding of a new population by sexual means (Ryland and Bishop, 1990). Many sessile invertebrates are broadcast spawners, a reproductive method exposing gametes to the problems of sperm dilution (Pennington, 1985; Levitan, 1991; Levitan *et al.*, 1992). Selfing ability may provide sessile organisms with an efficient means of reducing the costs associated with such a reproductive strategy (Jarne and Charlesworth, 1993). Hsieh (1997) suggested that due to the osmotic shock and dilution problems experienced by gametes broadcast spawned in an estuarine environment, selfing may be an advantageous fertilisation alternative in the sabellid polychaete *Laonome albicingillum*. Inbreeding of any form in a sessile organism may allow the preservation of genotypes that are adapted to local stable environmental conditions (Antonovics, 1968). In such a population outcrossing may lead to the dilution or disruption of these genotypes, potentially leading to outbreeding depression (Shields, 1982). Finally, continued inbreeding can have the effect of purging deleterious recessive alleles from a population (Shields, 1982), often leading to a fitness rebound after an early bout of inbreeding depression (e.g., Saccheri *et al.*, 1996).

Although self-fertilisation is reported as being frequently suppressed in hermaphroditic animals exposed to the possibilities of outcrossing (Williams, 1975; Heath, 1977; Maynard-Smith, 1978), it still appears to be a common phenomenon among invertebrate animals, both in natural populations and under laboratory conditions, having been reported as occurring in freshwater snails (Colton and Pennypacker, 1934; Njiokou *et al.*, 2000), terrestrial slugs (McCracken and Selander, 1980) and snails (Mulvey and Vrijenhoek, 1981), barnacles (Barnes and Crisp, 1956), polychaetes (Gee and Williams, 1965; Hsieh, 1997), tunicates (Sabbadin, 1971; Berrill, 1975), sea-anemones (Cain, 1974), ctenophores (Pianka, 1974), nematodes (Ward and Carrel, 1979), corals (Heyward and Babcock, 1986; Kojis and Quinn, 1981; Bassim *et al.*, 2002), marine molluscs (Beaumont and Budd, 1983; Ibarra *et al.*, 1995; Winkler and

Estévez, 2003) and cestodes (Lüscher and Milinski, 2003) (see also Ghiselin, 1969; Clark, 1978; Bell, 1982; Knowlton and Jackson, 1993 for reviews). Amongst the bryozoa, early reports of possible self-fertilisation in both freshwater (Allman, 1856; Braem, 1897; Marcus, 1934: all cited by Hunter, 1991), and marine (for references see Silén, 1966) representatives led to the widely held belief that this was a common method of reproduction in bryozoans. Moreover, amongst the Gymnolaemata, the commonly accepted view was that members of this class of bryozoans reproduced sexually by self-fertilisation alone (Silén, 1966). In more recent times, however, opinions have changed and it is now believed that most bryozoans are at least potentially outbreeding (Silén, 1972; Ryland, 1976). This opinion was originally based on observations of sperm release from the two dorsomedial tentacles of zooids of the gymnolaemate bryozoan *Electra posidoniae* (Silén, 1966), the first clear evidence of the process. Silén (1966) reports that earlier supposed observations of sperm release by bryozoans (Joliet, 1877; Hincks, 1880) were generally doubted by other authors. Since this first recognised report of sperm release in *E. posidoniae*, several other species of gymnolaemate bryozoa have also been reported as disseminating sperm through the terminal pores of tentacles (Bullivant, 1967; Silén, 1972; Temkin, 1994), thus suggesting the widespread occurrence of outcrossing in this group. Genetic evidence also exists to support the idea that bryozoan colonies are outbreeding in wild populations. Gooch and Schopf (1970) showed that genotype frequencies at several loci in populations of two species of bryozoans, *Schizoporella unicornis* (now *S. errata*) and *Bugula stolonifera*, from the vicinity of Woods Hole, Massachusetts, largely conform to the Hardy-Weinberg equilibrium, suggesting the occurrence of outbreeding in these populations. However, although these observations point to the occurrence of outcrossing in bryozoan species, they by no means rule out the possibility that some species may have the ability to self-fertilise, since self-sterility mechanisms, such as those reported for some ascidians (Scofield *et al.*, 1982; Byrd and Lambert, 2000), are yet to be reported for any bryozoan (Ryland, 1976; Bell, 1982). Also Maturo (1991) has reported the production of larvae by colonies of *Bowerbankia gracilis*, *Buskia* sp., *Bugula neritina*, *Antropora*

leucocypha, *Hippoporina verrilli*, and *Schizoporella pungens* that had been raised from ancestrulae cultured in isolation.

Celleporella hyalina is a simultaneous hermaphrodite, each colony consisting of separate male, female, and sterile feeding zooids and therefore, has the potential to self-fertilise. Hoare *et al.*, (1999) and Manríquez (1999) have observed sperm being released into the surrounding seawater from protruded male lophophores, and colonies are known to be protandrous, indicating that at least some outcrossing occurs in this species. Hoare *et al.*, (1999) also found that in two populations of British *C. hyalina* (Wales and Plymouth) all loci investigated were found to be in Hardy-Weinberg equilibrium, and Hoare and Hughes (2001) reported severe inbreeding depression resulting from full-sib mating in the Welsh population, results that are to be expected in a population that is normally outbreeding. In a study carried out on a Chilean population of *C. hyalina*, Cancino *et al.*, (1991a) reported that colonies reared in isolation failed to produce any female zooids in more than a year of observations and suggest that self-fertilisation is not possible in this species. However, other studies have reported differing results. Hunter and Hughes (1993a), working with a Welsh population of *C. hyalina*, reported that in isolation autozooids and sexual zooids were produced as normal, moreover 19 out of the 21 isolated colonies produced embryos. However, they also found that abortion of selfed embryos was common and settlement of the few larvae produced was never observed. Yund and McCartney (1994), working with a population from Maine, on the NE coast of America, reported that self-fertilisation rates increase as availability of allosperm decreases, and suggest that this population of *C. hyalina* is capable of self-fertilisation under field conditions.

The evidence so far assembled seems to suggest widespread variation in the occurrence of self-fertilisation between geographically isolated populations of *C. hyalina*. The current study aims to investigate the possibility of self-fertilisation in isolated colonies of *C. hyalina* from a wide range of geographically isolated

populations, using a common garden rearing environment as a control for reproductive competence through outbreeding.

3.2. Materials and Methods.

3.2.1. Biological material

Wild colonies growing on the fronds of various macroalgal species were collected from populations in the following locations (for full details on the exact location of each population and the macroalgal substrate they were growing on see Appendix 1); North Wales (Menai Strait, Amlwch Harbour, Holyhead Harbour and Porth Neigwl); Ireland (Lough Hyne, Achill Sound and Dublin Harbour); Scotland (Oban); England (Plymouth); Spain (Ría de Ferrol – this population consists of *Celleporella hyalina* var. normal and *C. hyalina* var. reticulata (Fernández Pulpeiro and Reverter Gil, 1992), both treated separately in this study); Iceland (Reykjavík); Norway (Tromsø); Sweden (Tjärnö); Russia (White Sea); Svalbard (Kapp Linné); America (Maine, Woods Hole, California and Alaska); and Canada (Nova Scotia and Victoria Island). Populations of *Celleporella angusta* Alvarez, 1991, (Spain, Foz) and *Celleporella carolinensis* Ryland, 1979, (America, Woods Hole) were also included in the study as no information on self-fertilisation capability are recorded for either of these species. The Porth Neigwl population and 3 of the 7 colonies from the Achill Sound population (Table 3.1) were later identified as being *C. angusta* (see chapter 5) and are referred to as such from now on. The Californian and Victoria Island samples were posted to the Bangor laboratory by colleagues, the algal fronds, bearing colonies, being packed in layers of damp tissue paper in a sealed plastic bag. Algal fronds, bearing colonies, from both of the Spanish locations were transported to the laboratory in plastic containers. Algal fronds, bearing colonies, from each of the other locations were transported to the laboratory in thermos flasks to help maintain a constant temperature for the duration of the journey.

3.2.2. Larval Settlement

Once in the laboratory the algal fronds were placed in 2 litre plastic drinks bottles each containing 1 litre of 2 µm-filtered, U.V.-irradiated sea water. Each population was maintained separately and fronds from each population were split into 3-4 separate bottles (depending on how many there were) to ensure that the

ancestrulae collected were not all released by the same colony. 100 ml of the cryptophyte alga *Rhinomonas reticulata* was added daily at a concentration of approximately 700 cells μl^{-1} as a food source. Each bottle was furnished with a lid with a hole cut in its centre through which an airline was passed to provide light aeration via an aquarium airstone. An acetate sheet, which had been conditioned in running seawater for 1 week, was wrapped around the interior of each bottle to act as a substrate for larval settlement. Water changes were carried out three times each week, during which the acetate sheets were checked for the presence of newly formed ancestrulae. Newly formed ancestrulae were immediately isolated from the settlement sheet by cutting out a surrounding 10x10 mm square of acetate, which was subsequently glued with cyanoacrylate onto another piece of acetate 38x75 mm, the size of a large microscope slide. The above procedure was carried out before the ancestrula had started feeding (determined by the presence or absence of red algal cells in the stomach) to prevent the precocious uptake and storage of allosperm (Hughes *et al.*, 2002). The Kapp Linné population was maintained at 5°C, the Alaska population was maintained at 10°C and all other populations were maintained at 15°C, in controlled temperature rooms, for the duration of the experiment. These temperatures were based on the temperatures recorded at the collection sites.

3.2.3. Experimental procedure

To test self-fertilising capability, a number of ancestrulae from each population (Table 3.1) were placed in isolation in 300 ml glass jars containing 225 ml of 2 μm -filtered, U.V.-irradiated seawater. Each jar was fitted with a lid, furnished with aeration and food ports, specially designed to reduce the risk of accidental allosperm transfer (Manríquez *et al.*, 2001). The water in the jars was changed twice weekly and 20 ml of the algal diet was added daily. To test for outcrossing capability, a number of ancestrulae from each population (Table 3.1) were placed in a slide rack (held in place by glass slides), which in turn was placed in a 2 litre plastic drinks bottle containing 1 litre of 2 μm -filtered, U.V.-irradiated sea water. These bottles were fitted with lids, as described for the settlement bottles above, and 100 ml of the algal diet was again added daily, however this time the water was changed twice weekly. Both isolated colonies, and colonies maintained in the common garden environment, were grown on for 3 months and inspected

weekly for the presence of embryos and newly settled larvae. After 3 months of observations, the isolated colonies were each cloned (see Appendix 2) and the resulting ramets placed in slide racks and housed in 2 litre plastic drinks bottles as described above. Each genotype was maintained in reproductive isolation ready for use in the mating trials (Chapter 4). During the mating trial experiments many of the genotypes maintained in reproductive isolation in this experiment were given the opportunity to outcross. The colonies grown in a common garden environment were removed from their containers after the 3 month observation period and dried out ready for use in the morphological study (Chapter 5).

3.3. Results.

3.3.1. *Celleporella hyalina*

Individuals from all populations, except Tromsø, Tjärnö and the White Sea, grew normally, forming compact, circular colonies and producing abundant frontal male and female zooids whether grown in isolation or in a common garden environment. Individuals from Tromsø and Tjärnö formed compact, circular colonies but failed to produce, or produced very few, frontal male and female zooids under either treatment. Individuals from the White Sea population formed irregularly shaped colonies and again failed to produce, or produced very few, frontal male and female zooids under either treatment. None of the colonies from any of these three populations, whether grown in isolation or in a common garden environment, was observed to brood any embryos in the few ovicells that were produced (Table 3.2). Colonies from one other population, Victoria Island, also failed to produce a single embryo, whether grown in isolation or in a common garden environment, during the three month observation period (Table 3.2). However, all individuals from this population were observed to produce compact, circular colonies and abundant frontal male and female zooids under both treatments.

Colonies from ten of the populations studied (Menai Strait, Holyhead Harbour, Dublin Harbour, Plymouth, Ría de Ferrol (both varieties), Reykjavík, Maine, California and Alaska) were found to brood embryos (Table 3.2) and produce viable larvae (Table 3.3) in a common garden environment, while the isolated colonies from the same populations remained free of embryos (Table 3.2). Colonies from nine of these populations produced abundant, normal larvae and colonies from one population (California) produced occasional, normal larvae when grown in a common garden environment (Table 3.3). The production of only occasional larvae by just six of the ten common garden grown Californian colonies may have been as a result of them being sibs or half sibs. This is due to only a few wild colonies being received at the Bangor laboratory, none of which were brooding embryos on their arrival. They were therefore all placed together in a single settlement bottle to try to encourage reproduction. It is possible that as

a result of this, all of the ancestrulae separated from the settlement sheet were released by just one or two reproducing colonies. At the end of the three month observation period colonies from seven of these populations (Plymouth, Ría de Ferrol (both varieties), Reykjavík, Maine, California and Alaska) that were grown in isolation were cloned (see Appendix 2), ready for use in the mating trials study (Chapter 4). The ramets that were produced were placed in slide racks and housed in 2 litre plastic drinks bottles as described for the common garden grown colonies. Each bottle contained the ramets produced from only one genotype therefore maintaining reproductive isolation. Genotypes from all of these populations were maintained, through regular cloning, in this environment for the following three years and most were never seen to brood embryos in isolation. Moreover, ramets of each of the genotypes from all seven of these populations were found to produce viable larvae when given the opportunity for outcrossing (Chapter 4). Three of the Ría de Ferrol var. normal genotypes and two of the Ría de Ferrol var. *reticulata* genotypes were later observed to brood embryos when maintained in reproductive isolation in controls testing for self-fertilisation activity (Chapter 4). Reasons as to why these genotypes were observed to brood embryos at this point are discussed in this chapter (Chapter 4).

For three of the populations used in this study (Achill Sound, Oban and Kapp Linné) only four of the ancestrulae collected from the settlement sheets that were placed in with the wild colonies on their arrival in the laboratory founded viable colonies. Therefore, all four of the colonies, from each population, were grown in reproductive isolation with no colonies grown in a common garden environment for comparison (Table 3.1). Due to this shortfall in numbers it was not possible to show, during the course of this study, that colonies from these three populations were capable of producing viable larvae under laboratory conditions when given the opportunity for outcrossing. However, at the end of the three month observation period the colonies that were grown in isolation were cloned (see Appendix 2) and some of the ramets that were produced could be given the opportunity to outcross. A single ramet from all four of the genotypes from the Achill Sound population were placed together in a jar for two weeks. They were then separated and placed in isolation in their own jars. The same procedure was carried out on two of the four genotypes from the Kapp

Linné population. All four genotypes from the Achill Sound population and both the genotypes from the Kapp Linné population produced abundant, normal progeny within four weeks of being placed together, demonstrating the ability of these genotypes to produce viable larvae under laboratory conditions when given the opportunity for outcrossing. Two of the four genotypes from the Oban population failed to survive the cloning procedure, however, the remaining two genotypes were found to produce viable larvae when given the opportunity for outcrossing in the mating trials study (Chapter 4).

Evidence of embryo production by isolated colonies was found in only four of the twenty one populations studied (Table 3.2), and in all but one of these populations the abundance and/or viability of the offspring produced, was reduced when compared to the offspring produced by colonies grown in a common garden environment (Table 3.3). Every colony that was observed to brood embryos in isolation brooded large numbers of embryos, with 90-100% of ovicells occupied by an embryo at any one time. One of the four isolated colonies from both the Amlwch Harbour and Lough Hyne populations was observed to produce embryos (Table 3.2), however, in both cases the colony released very few progeny, although any larvae released that did settle went on to produce a normal colony (Table 3.3). Both these genotypes, when used in the mating trials study (Chapter 4), were found to increase their larval output when given the opportunity for outcrossing. In both cases the colony that produced embryos was observed to be noticeably smaller at the end of the three month observation period than the other isolated colonies that failed to produce embryos. Two of the four isolated colonies from the Nova Scotia population were observed to produce embryos (Table 3.2), however, both these colonies only produced occasional progeny and these were of impaired viability (Table 3.3). As with the Amlwch Harbour and Lough Hyne populations, the colonies that were observed to produce embryos in isolation were noticeably smaller at the end of the three month observation period than the other isolated colonies that failed to produce embryos. The Woods Hole population was unique in that embryo production (Table 3.2), and abundance and viability of progeny (Table 3.3) was comparable between colonies that were isolated and those that were grown in a common garden environment.

3.3.2. *Celleporella angusta*

Individuals from all three populations grew normally, forming compact, circular colonies and producing abundant frontal male and female zooids whether grown in isolation or in a common garden environment. The Achill Sound population was the only population for which there were not enough ancestrulae collected from the settlement sheets to house colonies in both treatments, and therefore all colonies from this population were grown in isolation (Table 3.1). All colonies, whether grown in isolation or in a common garden environment, produced embryos (Table 3.2) and released abundant progeny of normal viability (Table 3.3).

3.3.3. *Celleporella carolinensis*

All individuals grown in a common garden environment formed compact, circular colonies and produced abundant male and female zooids in the basal layer (this species is unilaminar – Ryland, 1979). The four colonies grown in isolation also formed compact, circular colonies but were not observed to produce any female zooids during the three month observation period, male zooids were produced as normal. The nine colonies that were grown in a common garden environment were found to produce both embryos (Table 3.2) and occasional progeny of normal viability (Table 3.3). As with the Californian population, the reason why the common garden grown colonies produced only occasional larvae may have been due to them being sibs or half sibs. This is due to there being very few wild colonies on any of the fronds of *Sargassum filipendula* that were collected.

Table 3.1. Number of colonies grown in reproductive isolation and in a common garden environment from each location.

Population	Isolated colonies	Common garden colonies
<i>Celleporella hyalina</i>		
Menai Strait	4	10
Amlwch Harbour	4	10
Holyhead Harbour	4	10
Lough Hyne	4	10
Achill Sound	4	0
Dublin Harbour	4	10
Oban	4	0
Plymouth	4	10
Ría de Ferrol – var. normal	4	10
Ría de Ferrol – var. reticulata	4	3
Reykjavik	4	10
Tromsø	4	2
Tjärnö	4	10
White Sea	4	10
Kapp Linné	4	0
Maine	10	10
Woods Hole	4	10
California	4	10
Alaska	4	10
Nova Scotia	4	10
Victoria Island	4	10
<i>Celleporella angusta</i>		
Foz	4	10
Porth Neigwl	4	3
Achill Sound	3	0
<i>Celleporella carolinensis</i>		
Woods Hole	4	9

Table 3.2. Embryo production by isolated and common garden grown colonies. Data are the fraction of colonies in each treatment that produced embryos.

Population	Embryo production	
	Isolated colonies	Common garden colonies
<i>Celleporella hyalina</i>		
Menai Strait	0/4	10/10
Amlwch Harbour	1/4	10/10
Holyhead Harbour	0/4	10/10
Lough Hyne	1/4	10/10
Achill Sound	0/4	
Dublin Harbour	0/4	10/10
Oban	0/4	
Plymouth	0/4	10/10
Ría de Ferrol – var. normal	0/4	10/10
Ría de Ferrol – var. reticulata	0/4	3/3
Reykjavík	0/4	10/10
Tromsø	0/4	0/2
Tjärnö	0/4	0/10
White Sea	0/4	0/10
Kapp Linné	0/4	
Maine	0/10	10/10
Woods Hole	4/4	10/10
California	0/4	6/10
Alaska	0/4	10/10
Nova Scotia	2/4	10/10
Victoria Island	0/4	0/10
<i>Celleporella angusta</i>		
Foz	4/4	10/10
Porth Neigwl	4/4	3/3
Achill Sound	3/3	
<i>Celleporella carolinensis</i>		
Woods Hole	0/4	9/9

Table 3.3. Nominal abundance (abundant = >2000, occasional = <100, rare = <50) and viability (development either normal or impaired) of progeny released by colonies that brooded embryos. (Normal development = compact circular colony formed producing frontal male and female zooids; impaired development = irregularly shaped colony often failing to produce frontal male and female zooids).

Population	Progeny abundance and viability	
	Isolated colonies	Common garden colonies
<i>Celleporella hyalina</i>		
Menai Strait		Abundant - normal
Amlwch Harbour	Rare - normal	Abundant - normal
Holyhead Harbour		Abundant - normal
Lough Hyne	Rare - normal	Abundant - normal
Achill Sound		
Dublin Harbour		Abundant - normal
Oban		
Plymouth		Abundant - normal
Ría de Ferrol – var. normal		Abundant - normal
Ría de Ferrol – var. reticulata		Abundant - normal
Reykjavík		Abundant - normal
Tromsø		
Tjärnö		
White Sea		
Kapp Linné		
Maine		Abundant - normal
Woods Hole	Abundant - normal	Abundant - normal
California		Occasional - normal
Alaska		Abundant - normal
Nova Scotia	Occasional - impaired	Abundant - normal
Victoria Island		
<i>Celleporella angusta</i>		
Foz	Abundant - normal	Abundant - normal
Porth Neigwl	Abundant - normal	Abundant - normal
Achill Sound	Abundant - normal	

Table 3.3. (continued).

<i>Celleporella carolinensis</i>	
Woods Hole	Occasional - normal

3.4. Discussion.

3.4.1. *Celleporella hyalina*

Self-fertilisation in gymnolaemate bryozoans has been suggested as being possible by one of two methods, intra-colonial sperm migration (Marcus, 1938; Hughes, 1987) and re-entry of liberated sperm (Bell, 1982). Intracolony sperm migration was first suggested as being possible by Marcus (1938) who found sperm in all three zooid types and in the funicular system of *C. hyalina*, and has more recently been proven to occur by Manríquez (1999). Sperm release in *C. hyalina* occurs via the male lophophore (Hoare *et al.*, 1999; Manríquez, 1999), and liberated sperm is captured by the feeding current generated by the lophophores of feeding autozooids (Manríquez, 1999). Therefore, it is quite possible that sperm could be captured by autozooids that are feeding at the same time that sperm are released by males of the same colony. However, sperm release is generally accepted as a method by which cross-fertilisation can be achieved.

C. hyalina is found in dense monospecific stands growing on the fronds of *Laminaria saccharina* (Cancino, 1986), and other marine macroalgae (pers. obs.). This form of aggregation has been suggested as a mechanism of increasing the probability of cross-fertilisation in sessile hermaphrodites such as tunicates, barnacles and bryozoans (Ryland, 1972; Ryland, 1973; Hayward and Ryland, 1975; Ryland, 1976; Crisp, 1979; Schmidt, 1982; Cancino, 1986; Cancino *et al.*, 1991a). Hoare *et al.* (1999) showed that sib larvae of *C. hyalina* settle randomly with respect to each other, not exhibiting the kin-recognition that larvae of the marine bryozoan *Bugula neritina* have been shown to exhibit (Keough, 1984). Cancino *et al.* (1991b) showed that larval release in colonies of *C. hyalina* is induced by daylight, commencing shortly after sunrise and peaking 1.5 hours later. Such synchronous release of larvae, coupled with the lack of kin-recognition at settlement, will lead to the dense monospecific stands of *C. hyalina* being made up of a genetically mixed population, further increasing the probability of outcrossing in this species. Opportunity for outcrossing in *C. hyalina* is further promoted by the relatively prolonged fertility of water borne

sperm (Manríquez *et al.*, 2001), the efficient capture of sperm by active lophophores (Temkin, 1996; Manríquez, 1999), the ability to use stored sperm to fertilise ova (see Chapter 2) and the precocious capture and storage of allosperm by newly formed, sexually immature colonies (Hughes *et al.*, 2002). Although self-sterility mechanisms are yet to be reported for any bryozoan species (Ryland, 1976; Bell, 1982), several experiments have shown that colony fusion is possible between two growing edges of the same colony, or between very closely related colonies (siblings) (Chaney, 1983; Manríquez, 1999; Craig and Wasson, 2000). Craig and Wasson (2000) demonstrated that fusion occurred far more frequently between self/self contacts than between self/non-self contacts in the bryozoan *Hippodiplosia insculpta*, and Chaney (1983) and Manríquez (1999) demonstrated that fusibility was dependent on colonial relatedness in the bryozoans *Thalamoporella californica* and *Celleporella hyalina* respectively. This suggests that bryozoan colonies are able to tell self from non-self and therefore, may well be able to discern between self and non-self sperm providing the possibility of a mechanism to prevent self-fertilisation.

From the experiments above, no conclusions can be drawn regarding the ability of individuals from the Tromsø, Tjärnö, White Sea and Victoria Island populations to self-fertilise. The Tromsø, Tjärnö and White Sea populations are all genetically very similar, occupying the same phylogenetic clade based on mitochondrial DNA sequencing (Fennoscandia clade - A. Gomez pers. comm.). It is possible that the conditions under which the experiments were carried out were unsuitable for individuals from these three populations, and as a result of this, normal production of gonozooids, and therefore reproduction, was not possible. Some individuals from all three of these populations were later transferred to another controlled temperature room set at 10°C. This was found to have no effect on growth, production of gonozooids or the reproductive success of the individual colonies from any of the three populations. Another possibility is that the algal food provided daily to all colonies, *Rhinomonas reticulata*, was insufficient to provide individuals from these populations with the required nutrition to grow normally, produce gonozooids and reproduce successfully. Hunter and Hughes (1993b) have previously shown *R. reticulata* to be a satisfactory food source, allowing Welsh *C. hyalina* to form compact, circular

colonies in the laboratory showing a pattern of astogeny typical of wild specimens. However, individuals from the three populations of the Fennoscandia clade are quite genetically dissimilar to individuals from Welsh populations (A. Gomez pers. comm.) and so it is quite possible that the diet provided was insufficient for normal growth and reproduction to occur. Colonies from the Victoria Island population appeared to grow normally during the course of the experiment, forming compact, circular colonies and abundant gonozooids whether grown in isolation or in a common garden environment. The lack of reproductive activity from this population is therefore more difficult to explain, but it may be that the conditions and food supply were adequate for normal growth of zooids to occur but insufficient for the energy expensive process of gametogenesis to occur. Another observation that makes it difficult to explain what was happening with this population is that at the end of the 3 month observation period the four isolated colonies were all placed together in the same container, and were propagated by cloning (Appendix 2). This arrangement was maintained for the next three years. For the following two and a half years, no embryos were observed to be produced by any of the four genotypes despite regular observations. However, after this two and a half year period the four genotypes all suddenly started brooding embryos and releasing viable larvae, even though there were no changes made to either the culture conditions or food supply.

Although the number of colonies (genotypes) used in this study was insufficient to give a precise estimate to the frequency of self-fertilisation capability within each population, the results obtained suggest that, in the absence of opportunity for outcrossing, the majority of natural colonies (genotypes) from sixteen of the twenty one populations studied will be either incapable of selfing or able to produce selfed offspring only at a very low level. Moreover, in three of the four populations from which embryo production by isolated colonies was found, severe inbreeding depression was observed, as would be expected in populations that are normally outcrossing. This was shown by the very low levels of larval settlement in relation to the high numbers of embryos brooded by isolated colonies, and the impaired viability of some of the selfed progeny. This conclusion is in agreement with the results obtained by the authors of several

previous studies on the self-fertilisation capability of *C. hyalina*, who report either a complete lack of self-fertilisation (Cancino *et al.*, 1991a; Manríquez *et al.*, 2001) or very low levels of self-fertilisation, coupled with severe inbreeding depression (Hunter and Hughes, 1993a; Hoare *et al.*, 1999). Whether the very low numbers of settled larvae observed in the isolated Amlwch Harbour, Lough Hyne and Nova Scotia colonies that brooded embryos was due to abortion, as reported by Hunter and Hughes (1993a) and Hoare *et al.* (1999), or failure of released larvae to settle, as also reported by Hunter and Hughes (1993a), was not recorded. The results of the current study do, however, disagree with the findings of Yund and McCartney (1994) who reported that colonies of *C. hyalina* from the Maine population are capable of producing progeny by self-fertilisation both when reared in isolation in the laboratory and under field conditions. Of the ten colonies from this population grown in reproductive isolation in the current study none were observed to brood any embryos during the three months of observations (Table 3.2). Moreover, the four colonies from this population that were reared in isolation did not brood any embryos in a further 3 years of observations when maintained in reproductive isolation, but did produce abundant embryos and viable offspring when given the opportunity for outcrossing (Chapter 4).

Despite the apparent predominance of outcrossing in *C. hyalina*, in the Woods Hole population selfing capability appears to be universal, with no obvious signs of inbreeding depression detected. Due to the isolated colonies from this population releasing comparable numbers of viable larvae to the colonies grown in a common garden environment it is not possible to say whether the common garden grown colonies are actually outcrossing. Therefore, it remains possible that obligate inbreeding is the norm for individuals from this population in the natural environment. However, the native Woods Hole population occurs at a relatively high density as an epiphyte on the fronds of *Chondrus crispus* (R.N. Hughes pers. comm.), presenting ample opportunity for outcrossing. Whether this population is selfing or outcrossing in the natural environment must be investigated using molecular methods to assign paternity to F1 colonies with a known maternal background. That this population of *C. hyalina* is the only population in the study in which selfing and resistance to inbreeding depression

appear to be universal, may represent a phylogenetic trait with a deep history, since this population is cladistically distinct within the *C. hyalina* complex (A. Gomez pers. comm.).

Cancino *et al.* (1991a) were the first to report a total absence of self-fertilisation in *C. hyalina*, the results of their study showing a complete lack of female investment by isolated colonies. Hughes *et al.* (2002) reported that female production was severely retarded, although not ultimately suppressed, in colonies from a Welsh population grown in the absence of allosperm. Colonies from all populations in the current study (except the three mentioned above) were observed to produce abundant numbers of both male and female zooids when grown in isolation, however, the numbers produced in isolation were not compared with the numbers produced by colonies in a common garden environment and so it is impossible to know whether the absence of allosperm had any effect on the numbers of male and female zooids produced. Sex ratio among colonies of *C. hyalina* is known to vary within populations, the extremes ranging from all-male (Hunter and Hughes, 1995; McCartney, 1997) but apparently never to all female. Due to the differences already shown in female investment by isolated colonies from Chilean and Welsh populations, and the fact that sex ratio has been shown to vary amongst colonies within populations, sex allocation warrants further study, looking especially at the differences seen in gonozooid investment between populations for which different levels of self-fertilisation have been observed.

3.4.2. *Celleporella angusta*

As with *C. hyalina*, the number of colonies (genotypes) used in this study was insufficient to give a precise estimate to the frequency of self-fertilisation capability in this species. However, from the experiments above it would appear that in *C. angusta* selfing capability appears to be universal, with no obvious signs of inbreeding depression detected. As with the Woods Hole *C. hyalina* population however, it is not possible to discern from this experiment whether the common garden grown colonies are actually outcrossing, and again this must be investigated using molecular methods. This species has only previously been reported as occurring along the coasts of Spain (Alvarez, 1991; Fernández

Pulpeiro and Reverter Gil, 1992) and France (Reverter Gil *et al.*, 1995) and it is possible that the ability to self-fertilise has assisted it in establishing new populations at Porth Neigwl (but see Chapter 6) and Achill Sound. This would appear to be the first study into the reproductive biology of this species.

3.4.3. *Celleporella carolinensis*

With this species, as with the other two species reported above, the number of colonies (genotypes) used in the present study was insufficient to give a precise estimate of the frequency of self-fertilisation capability. However, the results obtained would indicate that in the absence of opportunity for outcrossing, the majority of natural colonies (genotypes) from this population will be incapable of selfing. The total lack of female investment observed in this species when grown in reproductive isolation concurs with Cancino *et al.* (1991a) in their study of a Chilean population of *C. hyalina*.

Chapter 4.

Reproductive compatibility of geographically isolated populations of *C. hyalina*.

4.1. Introduction.

There are many popular concepts of species in use today (Mayden (1997) lists 22), such as the various formulations of the phylogenetic species concept (e.g., Nelson and Platnick, 1981; Cracraft, 1983; Donoghue, 1985; Nixon and Wheeler, 1990), the recognition species concept (Paterson, 1985) and the cohesion species concept (Templeton, 1989) (see also Coyne, 1994; Table 1; Mayden, 1997; Table 19.1). However, perhaps the most influential of these concepts, at least amongst evolutionists (Coyne, 1994), is the biological species concept (BSC). This concept has at its heart the notion of reproductive isolation and is stated as follows: a species is a group of individuals fully fertile inter se, but barred from interbreeding with other similar groups by its physiological properties (producing either incompatibility of parents, or sterility of the hybrids or both) (Dobzhansky, 1937); or, species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups (Mayr, 1942). Mayr (1963) refined his definition of the BSC to remove the problematic words “potentially interbreeding”, emphasising that it is best applied to sympatric taxa. However, as Coyne (1994) states, “allopatric taxa that produce sterile or inviable hybrids in captivity are surely biological species”. The biological factors that prevent hybridisation between species, or isolating mechanisms (Dobzhansky, 1937), may act prior to fertilisation (prezygotic isolation) or after fertilisation has occurred (postzygotic isolation) (Coyne and Orr, 1998). Prezygotic isolating mechanisms include differences in ecology, behaviour, timing of reproduction, gametic incompatibility and mate choice, whereas postzygotic isolating mechanisms include hybrid inviability, hybrid breakdown and hybrid sterility (Coyne and Orr, 1998).

Several modes have been proposed to explain how new species originate (how the genes coding for reproductive isolation can become fixed in a natural population). Sympatric speciation is the term applied to the process of speciation

in a sexually reproducing species without effective geographical isolation. It involves the establishment of a stable polymorphism and the subsequent divergence of the two morphs to such an extent that reproductive isolation evolves between them (Maynard-Smith, 1966). However, the frequency of occurrence of sympatric speciation, and even whether this method of speciation occurs at all, is still a controversial issue (Futuyma and Mayer, 1980). Indeed Felsenstein (1981) doubts whether the genes affecting the isolating mechanism would be linked to the genes affecting adaptation to the different environments (the two morphs). Parapatric speciation, as suggested by Endler (1977), involves the formation of a cline with eventual reproductive isolation occurring at either end. This method of speciation is, however, also unlikely to occur for reasons discussed by Mayr and O'Hara (1986). The gradual acquisition of reproductive isolation between spatially isolated populations is referred to by the terms geographic or allopatric speciation (Mayr and Ashlock, 1991) and it is generally accepted that this is the most common process by which new species originate. Traditional allopatric speciation, or dichopatric speciation, occurs when the continuous range of a single population is divided by a newly arising barrier to gene flow (e.g., geological, geographical, vegetational), which leads to the splitting of the original population into two or more isolated groups of populations (Mayr and Ashlock, 1991). Peripatric speciation, on the other hand, occurs when a new population is founded by a single individual (fertilised female) or a small proportion of a much larger population outside the continuous species range. This new population must remain isolated for a long enough period to allow for the acquisition of the genetic basis for reproductive isolation (Mayr and Ashlock, 1991).

The "cosmopolitan" bryozoan *C. hyalina* is a larval brooder that releases short-lived, non-feeding, lecithotrophic larvae (Zimmer and Woolacott, 1977) which usually settle within four hours of release from the parent colony (Ryland, 1960; Cancino and Hughes, 1988). This short larval phase of the life-cycle, coupled with the fact that sperm from this species have a half-life of only 1-2 hours in the water column (Manríquez, 1999) leads to *C. hyalina* having a very low potential for dispersal, with low levels of gene flow between populations. This lack of gene flow between populations would be expected to lead to genetic

differentiation occurring over fairly short distances. Goldson (1998) showed that in *C. hyalina* populations in the Menai Strait, North Wales, genetic differentiation occurs over distances of only a few metres. Although *C. hyalina* has a very limited potential for dispersal it does occupy a circumglobal distribution amongst the polar and temperate waters of the northern and southern hemispheres (Marcus, 1938; Ryland and Gordon, 1977; Hastings, 1979; Ryland, 1979; Morris, 1980; Moyano, 1986; Fernández Pulpeiro and Reverter Gil, 1992). Long distance rafting on substrates such as wood, algae or pumice may be a very common form of long distance dispersal for clonal sessile invertebrates (Jackson, 1986). It may not be unlikely, therefore, that *C. hyalina* has achieved its circumglobal distribution via either this method or via transport on the hulls of ships, another mechanism thought to be very important in the dispersal of bryozoans (Watts *et al.*, 1998). Short or long distance rafting, or dispersal via fouling, may be two ways of maintaining sufficient gene flow between spatially isolated populations to prevent speciation from occurring. However, this type of dispersal also produces opportunities for a species to colonise new areas, and speciation will eventually occur if this founding population remains isolated for a long enough period of time to acquire the genetic basis for reproductive isolation, a form of peripatric speciation (Mayr and Ashlock, 1991).

C. hyalina occurs as a common bryozoan on the shores of Chile (Moyano, 1986). Previous studies have been carried out comparing different aspects of the life histories of Welsh and Chilean populations. Differences were found in the ability of larvae from the two populations to metamorphose after periods of enforced swimming (Orellana and Cancino, 1991; Orellana *et al.*, 1996), with the ability of Welsh larvae to metamorphose being impaired if forced to swim for over 4 hours, whereas the Chilean larvae were able to metamorphose after 28 hours of enforced swimming. Differences between the two populations when grown in isolation are also apparent, with Chilean colonies producing only male sexual zooids (androzooids) when reared in isolation (Cancino *et al.*, 1991a), and Welsh colonies producing both male (androzooids) and female (gynozooids) sexual zooids when reared under the same conditions of reproductive isolation (Hunter and Hughes, 1993a). Another difference between Welsh and Chilean populations is their choice of substratum, with Chilean colonies being found mainly in the

holdfasts of *Macrocystis integrifolia* (Cancino *et al.*, 1991a), whereas Welsh colonies are most often found on the fronds of *Laminaria saccharina* and *Fucus serratus* (Hayward and Ryland, 1979; Cancino, 1986). There is also a minor morphological difference between the two populations with Chilean colonies having a greater number of tubular pore chambers than Welsh colonies (Goldson, 1998). Hoare *et al.* (2001) investigated the phylogeography of *C. hyalina* collected from six different sites, four from around the Atlantic basin and two from Chile. The results obtained by Hoare *et al.* (2001) indicated that there are three subgroups of *C. hyalina* from within the six sites collected from, with the Chilean populations forming a distinct clade.

Field or experimental data on reproductive compatibility are crucial in recognising biological species (although reproductive isolation can be inferred from genetic studies alone: e.g., Solé-Cava *et al.*, 1985). However, breeding tests, or mating trials, are uncommon and studies of F2s are even more limited. This is especially so in the marine realm due to the problems associated with rearing marine organisms to sexual maturity (Knowlton, 1993). To date breeding tests have successfully been undertaken on copepods (Battaglia and Volkmann-Rocco, 1973; Carrillo *et al.*, 1974), hydroids (Buss and Yund, 1989) and polychaetes (Rice, 1991; Marsden, 1992; Kruse and Reise, 2003) and in vitro fertilisation tests have been performed using sea urchin gametes (Lessios and Cunningham, 1990; Palumbi and Metz, 1991). Goldson (1998) attempted to carry out mating trials between Welsh and Chilean populations of *C. hyalina*. However, although Goldson's results did indicate that the two populations were reproductively incompatible, due to a lack of larval settlement in the Chilean controls the results of these mating trials are inconclusive. The aim of the current study is to investigate the levels of reproductive compatibility between *C. hyalina* colonies from several geographically isolated populations, to test the null hypothesis that this bryozoan is a single cosmopolitan biological species.

4.2. Materials and Methods.

4.2.1. Biological Material

The genotypes used in this study were the same as those grown in isolation in 300 ml glass jars for use in the study of self-fertilising capability (Chapter 3). After 3 months of observations in the previous study the colonies that had been grown in isolation were each cloned (see Appendix 2) and the resulting ramets placed in slide racks (held in place by glass slides) and housed in 2 litre plastic drinks bottles (stock bottles), each furnished with a lid and aquarium airstone to provide aeration. The water in the bottles was changed twice weekly and 100 ml of the algal food source was added daily. Each genotype was maintained in reproductive isolation after cloning, ensuring that all the ramets that were used in mating trials were virgin. As ramets were assigned to different mating trials the cloning process was repeated, thus ensuring a constant supply of ramets for subsequent use. All populations were maintained at the same temperatures at which they had been grown during the self-fertilisation study.

4.2.2. Selection of populations

During the cloning procedure a section of each genotype from all populations was placed into 100% ethanol and sent to the molecular laboratory at Hull University, where Dr. A. Gomez constructed a phylogeny based on mitochondrial DNA sequencing (see Appendix 3). The resulting phylogenetic tree was then used to select which populations were to be involved in the mating trials. The mating trials were split into two groups, within-clade mating trials and between-clades mating trials. For the within-clade mating trials, six populations from the NE Atlantic clade were chosen (Table 4.1). This clade contained the most populations and it also contained the most local populations from around the British isles that had already been shown to respond well to the culture conditions used. For the between-clades mating trials, one population from each of seven clades was chosen (Table 4.2). The Amlwch Harbour population (North Wales) was chosen to represent the NE Atlantic clade for the between-clades mating trials. However, the Reykjavík population (Iceland) was also included in the between-clades mating trials after failing to successfully reproduce with

members of four other populations from the NE Atlantic clade (see results). Fig. 4.1 shows a list of populations for which mating trials were undertaken in this study. The Tjärnö population (Sweden) has been left out of Fig. 4.1 since the four genotypes from this population were not healthy enough to be used in any mating trial (see results).

4.2.3. Experimental procedure

Potential partners, previously maintained only with clone mates (see above), were placed together in a 300 ml glass jar, set up and maintained as described in Chapter 3, for a period of two weeks. Each colony was, therefore, able to act as both a male (sperm donor) and a female (sperm recipient) in each mating. Each partner was checked weekly for the presence of embryos, which can clearly be seen in both the body and in the brood chamber (ovicell) of the female zooid under a bottom lit light microscope, and those that were found to be brooding embryos were removed to a separate glass jar in which they were cultured in isolation for a further 2 - 3 months. Separating the partners before the release of larvae enabled maternal and paternal genotypes to be ascertained with certainty for each larva released. The culture jars had a strip of acetate sheet, which had been conditioned in running seawater for 1 week, wrapped around the interior to act as a substrate for larval settlement. The number of larvae released by each ramet was recorded (for any ramet that released over 100 larvae, the number was recorded as >100). To investigate the levels of fitness of the offspring, the first 100 larvae released were grown on until they either died or reached sexual maturity (determined by the presence of both male (androzooids) and female (gynozooids) zooids within the colony). In order to follow the growth of the first 100 larvae, any subsequently released larvae were scraped off the acetate sheet. If any ramet released less than 100 larvae, all of the offspring were grown on. After the growth of these 100 larvae had been followed, 10 that had successfully reached sexual maturity were preserved in 100% ethanol and sent to the molecular laboratory at Hull university where Dr. A. Gomez subjected them to paternity analysis to ensure that they were the product of successful cross-fertilisation. Each mating trial was set up using four replicate genotypes from each of the two populations whose reproductive compatibility was being investigated, and these were used to form four mating pairs (Fig. 4.2). However,

only three genotypes from the Ría de Ferrol population (*C. hyalina* var. *reticulata*) survived long enough to be used in this study. Likewise, only two genotypes from each of the Woods Hole and Oban populations survived long enough to be used in mating trials.

Each mating trial performed was complemented by a set of controls to ensure that all of the genotypes being used in the trial were reproductively active at the time that the trial took place. A ramet of every genotype from each of the two populations that were being used in a mating trial was placed in a 300 ml glass jar with another genotype from its own population. The glass jars were again set up and maintained as described in Chapter 3. The results of any particular mating trial were only trusted when the genotypes involved had been seen to be reproductively active in the relevant control matings. As well as being involved in these control matings, other ramets of each genotype were maintained in reproductive isolation in the stock bottles. These ramets were checked on a weekly basis during the course of each mating trial to observe whether or not those genotypes that were currently being used in mating trials could produce embryos by self-fertilisation.

For each successful mating trial one newly settled ancestrula (F1) released by each maternal colony, was removed from the settlement jar by excision of the acetate substrate (as described in Chapter 3), glued onto another piece of acetate (38x75 mm), and placed in isolation in another glass jar set up and maintained as described in Chapter 3. This procedure was carried out before first feeding to prevent the precocious uptake and storage of allosperm (Hughes *et al.*, 2002). The resulting colony was grown on to such a size whereby it could be cloned (see Appendix 2) to produce two ramets. One of these ramets was used to perform a backcross mating to a genotype from the maternal population (not the maternal genotype) and the other ramet was used in a mating with another F1 genotype released by a colony from the paternal population, but not the paternal genotype (Fig. 4.2), to ascertain viability of the offspring produced by each successful mating. During the cloning procedure a small part of each F1 colony was preserved in 100% ethanol and sent to the molecular laboratory at Hull

university for paternity analysis to ensure that they were the product of successful cross-fertilisation.

As all but one of the populations used in this study were maintained at 15°C (see Chapter 3) the majority of the mating trials were carried out at this temperature. However, the population from Alaska was maintained at 10°C. Attempts to grow ramets from the genotypes in this population at 15°C were unsuccessful. Therefore, ramets from each of the other populations that were used in mating trials with the Alaskan population were placed in glass jars in a water bath and the temperature was slowly lowered, by 1°C a day, down to 10°C and all of these mating trials were carried out at this temperature. Each of the relevant controls was also carried out at this reduced temperature.

4.3. Results.

The population from Tjämnö (Sweden) chosen to represent the Fennoscandia clade (A. Gomez pers. comm.) in the mating trials was unable to be used. This was due to ramets of all four genotypes failing to produce, or producing very few, frontal male or female zooids during the course of the experiment. Moreover, those ramets that did produce a few frontal sexual zooids were found to be unreplicative when given the opportunity to outcross with a ramet of another genotype from the same population. (Suggestions as to why members of this population failed to produce large numbers of sexual zooids and reproduce, and details of the effect of changing the ambient temperature are given in Chapter 3).

None of the eighteen between-clade mating trials produced cross-fertilised progeny (Table 4.4; Appendix 4, Tables A.1-A.54). Larval release was observed in only four of these eighteen crosses, and in each case the larvae were always released by genotypes from the Woods Hole population (Appendix 4, Tables A.16, A.31, A.49 and A.52). However, in each of these mating trials with Woods Hole it was also observed that none of the genotypes from the four partner populations brooded embryos or released larvae (Appendix 4, Tables A.16, A.31, A.49 and A.52), but always brooded embryos and released larvae in the control matings (Appendix 4, Tables A.17, A.32, A.50 and A.53). Moreover, both Woods Hole genotypes were always observed to brood embryos and release abundant larvae when maintained in reproductive isolation as controls to test for self-fertilisation activity (Appendix 4, Tables A.18, A.33, A.51 and A.54). There were only three other observations of embryos being brooded by a genotype that was paired with one from another population, each case involving the AML₄ genotype. This genotype was observed to brood embryos whether paired with a genotype from another population, another genotype from its own population or when maintained in reproductive isolation, but was only observed to release larvae when paired with a genotype from its own population (Appendix 4, Tables A.13-A.15, A.28-A.30 and A.40-A.42).

Only four of the twelve within-clade mating trials were found to be unsuccessful, and each of these crosses involved the Reykjavík (Icelandic) population (Table 4.3; Appendix 4, Tables A.55-A.66). Although in each of these four crosses at least one genotype was observed to brood embryos when paired with a genotype from the other population (Appendix 4, Tables A.55, A.58, A.61 and A.64), the same genotype (or genotypes) were always observed to brood embryos when kept in reproductive isolation in the controls to test for self-fertilisation activity (Appendix 4, Tables A.57, A.60, A.63 and A.66). Moreover, the only situation in which any genotype in these four crosses was observed to release larvae was when paired with another genotype from its own population (Appendix 4, Tables A.56, A.59, A.62 and A.65).

Of the eight successful within-clade mating trials, five (AML * IRL, AML * PLY, AML * SPN, IRL * PLY and PLY * SPN) showed almost 100% reproductive compatibility between genotypes from different locations (only the AML₄ genotype in the AML * SPN cross failed to release large numbers of larvae), with a high percentage of settled larvae reaching sexual maturity (often 100%) and outcrossing confirmed by paternity analysis (A. Gomez pers. comm.) in each cross performed (Appendix 4, Tables A.67, A.72, A.77, A.85 and A.95). For each of these five crosses the control matings showed that all genotypes involved were reproductively active at the time that the crosses took place, with very low levels of self-fertilisation activity detected in only two of the genotypes involved (AML₄ and IRL₂) (Appendix 4, Tables A.68 & A.69, A.73 & A.74, A.78 & A.79, A.86 & A.87 and A.96 & A.97). The backcross matings, and F1 * F1 matings involving the progeny of the five successful crosses mentioned above all resulted in large numbers of larvae being produced, again all with a high percentage of settled larvae reaching sexual maturity (Appendix 4, Tables A.70 & A.71, A.75 & A.76, A.80 & A.81, A.88 & A.89 and A.98 & A.99). Only three F1 genotypes failed to brood embryos or release larvae in these matings (AML₄IRL₄, PLY₁SPN₁ and SPN₁PLY₁), and in each case the genotype that they were paired with successfully brooded and released high numbers of larvae with a high percentage reaching sexual maturity (Appendix 4, Tables A.70 & A.71 and A.98 & A.99).

Of the three remaining within-clade mating trials (IRL * SPN, SPN * RET and AML * RET), outcrossed larvae were detected in each case, with a high percentage of those larvae released reaching sexual maturity, however, each trial appeared to be somewhat asymmetrical (Appendix 4, Tables A.82, A.90 and A.100). In the IRL * SPN mating trial all of the IRL genotypes brooded embryos and released abundant outcrossed larvae, while only one of the SPN genotypes achieved the same (Appendix 4, Table A.90). Of the remaining three SPN genotypes, one failed to brood any embryos, one was observed to brood embryos but any larvae released failed to settle and one was observed to brood embryos and release larvae, however, only seven larvae were released and none survived through to sexual maturity (Appendix 4, Table A.90). The controls for this mating trial indicated that all of the genotypes involved were reproductively active at the time that the trial took place, with very low levels of self-fertilisation activity detected in only one of the genotypes involved (IRL₂) (Appendix 4, Tables A.91 and A.92). The backcross matings and F1 * F1 matings involving progeny of the IRL * SPN crosses all resulted in large numbers of larvae being released, with a high percentage reaching sexual maturity (Appendix 4, Tables A.93 and A.94). The only F1 genotype to release low numbers of larvae, none of which reached sexual maturity, was IRL₂SPN₂ in the backcross mating, however, the partner genotype (IRL₃) did release high numbers of larvae with a high percentage reaching sexual maturity (Appendix 4, Table A.93). The IRL₂SPN₂ genotype moreover, released high numbers of larvae, with 100% surviving to reach sexual maturity, in the F1 * F1 cross (Appendix 4, Table A.94).

In the SPN * RET mating trial all of the RET genotypes brooded embryos and released abundant larvae, with 100% of the larvae released by all three genotypes reaching sexual maturity (although only larvae released by two of the genotypes could be confirmed as being outcrossed) (Appendix 4, Table A.100). Of the SPN genotypes, two brooded embryos and released larvae, however, none of the few larvae released by the SPN₁ genotype reached sexual maturity and only 70% of the larvae released by the SPN₄ genotype reached sexual maturity (this was the lowest number of settled larvae surviving to sexual maturity in any successful mating trial), with none confirmed as being outcrossed (Appendix 4, Table

A.100). The controls for this mating trial indicated that all of the genotypes involved were reproductively active at the time that the trial took place, with no self-fertilisation detected in any genotype (Appendix 4, Tables A.101 and A.102). Of the four F1 genotypes that were isolated for use in the backcross matings and F1 * F1 crosses, only two survived long enough to be used, and both of these genotypes appeared to be in a bad condition at the time that these crosses took place. When these four F1 genotypes reached sexual maturity there was a sudden, huge increase in the numbers of female zooids, to the extent that not only were these zooids produced in the frontal layer of the colony, they were also produced all around the growing edge of the colony (Plate 4.1). The result of this was that each F1 colony could not increase its size, since no new autozooids (or only very few autozooids) were budded at the colony margin, and the autozooids that were already present seemed to be smothered by the huge numbers of new female zooids (gynozooids) produced. In the first of the two backcross matings that could be performed, the RET₁SPN₁ genotype released abundant larvae, with 100% reaching sexual maturity, but the genotype it was paired with (RET₄) did not brood embryos or release larvae. In the other backcross mating the RET₄.SPN₄ genotype did not brood embryos or release larvae, but the genotype it was paired with (RET₁) released abundant larvae, with 100% reaching sexual maturity (Appendix 4, Table A.103).

In the AML * RET mating trial a similar pattern to that seen in the SPN * RET mating trial was observed. All of the RET genotypes brooded embryos and released abundant larvae, with 100% of the larvae released by all three genotypes reaching sexual maturity and outcrossing confirmed in each case. Only one of the AML genotypes, however, brooded embryos and released larvae, again with 100% of the larvae released reaching sexual maturity and outcrossing confirmed (Appendix 4, Table A.82). The controls for this mating trial indicated that all of the genotypes involved were reproductively active at the time that the trial took place with no self-fertilisation activity detected in any of the genotypes involved (Appendix 4, Tables A.83 and A.84). The two AML genotypes that failed to brood embryos or release larvae (AML₁ and AML₂) were paired with the same RET genotypes (RET₁ and RET₂ respectively) as the two SPN genotypes that also failed to brood embryos and release viable larvae (SPN₁ and SPN₃

respectively) (Appendix 4, Tables A.82 and A.100). This mating trial did not include backcross matings and F1 * F1 crosses, since it was performed primarily to see whether the same asymmetrical pattern of reproductive success would be obtained as in the SPN * RET mating trials.

Table 4.1. Populations chosen for use in the within-clade mating trials. (See Appendix 3 for phylogenetic tree).

Phylogenetic clade	Populations used	Three letter code used to identify population
NE Atlantic clade	Amlwch Harbour (North Wales)	AML
	Lough Hyne (Ireland)	IRL
	Ría de Ferrol (Spain – <i>C. hyalina</i> var. normal)	SPN
	Ría de Ferrol (Spain – <i>C. hyalina</i> var. reticulata)	RET
	Plymouth (England)	PLY
	Reykjavík (Iceland)	ICE

Table 4.2. Populations chosen for use in the between-clades mating trials. (See Appendix 3 for phylogenetic tree).

Phylogenetic clade	Populations used	Three letter code used to identify population
NE Atlantic clade	Amlwch Harbour (North Wales)	AML
	Reykjavík (Iceland)	ICE
NW Atlantic clade	Maine (America)	MAN
Fennoscandia clade	Tjärnö (Sweden)	SWE
Californian clade	California (America)	CAL
NE Pacific clade	Alaska (America)	ALA
Scottish 1 clade	Oban (Scotland)	OBN
Woods Hole clade	Woods Hole (America)	WHE

Table 4.3. Summary of the results of the within-clade mating trials. Successful = production of embryos, unsuccessful = no embryos produced.

Mating Trial		Result
Amlwch Harbour	* Lough Hyne	Successful
Amlwch Harbour	* Plymouth	Successful
Amlwch Harbour	* Iceland	Unsuccessful
Amlwch Harbour	* Ría de Ferrol (var. normal)	Successful
Amlwch Harbour	* Ría de Ferrol (var. reticulata)	Successful
Lough Hyne	* Plymouth	Successful
Lough Hyne	* Iceland	Unsuccessful
Lough Hyne	* Ría de Ferrol (var. normal)	Successful
Iceland	* Ría de Ferrol (var. normal)	Unsuccessful
Iceland	* Ría de Ferrol (var. reticulata)	Unsuccessful
Plymouth	* Ría de Ferrol (var. normal)	Successful
Ría de Ferrol (var. normal)	* Ría de Ferrol (var. reticulata)	Successful

Table 4.4. Summary of the results of the between-clades mating trials.

Mating Trial		Result
Maine	* California	Unsuccessful
Maine	* Alaska	Unsuccessful
Maine	* Oban	Unsuccessful
Maine	* Iceland	Unsuccessful
Maine	* Amlwch Harbour	Unsuccessful
Maine	* Woods Hole	Unsuccessful
California	* Alaska	Unsuccessful
California	* Oban	Unsuccessful
California	* Iceland	Unsuccessful
California	* Amlwch Harbour	Unsuccessful
California	* Woods Hole	Unsuccessful
Alaska	* Oban	Unsuccessful
Alaska	* Iceland	Unsuccessful
Alaska	* Amlwch Harbour	Unsuccessful
Oban	* Iceland	Unsuccessful

Table 4.4. (continued).

Oban	* Amlwch Harbour	Unsuccessful
Woods Hole	* Iceland	Unsuccessful
Woods Hole	* Amlwch Harbour	Unsuccessful

Fig. 4.1. List of within- and between-clade mating trials.

Within-clade mating trials

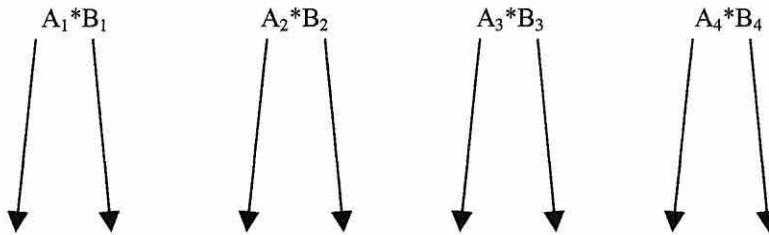
Amlwch Harbour * Lough Hyne
 Amlwch Harbour * Plymouth
 Amlwch Harbour * Iceland
 Amlwch Harbour * Ría de Ferrol (var. normal)
 Amlwch Harbour * Ría de Ferrol (var. reticulata)
 Lough Hyne * Plymouth
 Lough Hyne * Iceland
 Lough Hyne * Ría de Ferrol (var. normal)
 Iceland * Ría de Ferrol (var. normal)
 Iceland * Ría de Ferrol (var. reticulata)
 Plymouth * Ría de Ferrol (var. normal)
 Ría de Ferrol (var. normal) * Ría de Ferrol (var. reticulata)

Between-clades mating trials

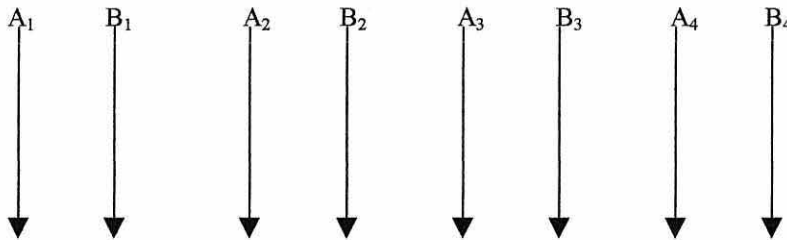
Maine * California
 Maine * Alaska
 Maine * Oban
 Maine * Iceland
 Maine * Amlwch Harbour
 Maine * Woods Hole
 California * Alaska
 California * Oban
 California * Iceland
 California * Amlwch Harbour
 California * Woods Hole
 Alaska * Oban
 Alaska * Iceland
 Alaska * Amlwch Harbour
 Oban * Iceland
 Oban * Amlwch Harbour
 Woods Hole * Iceland
 Woods Hole * Amlwch Harbour

Fig. 4.2. Diagrammatic representation of the system used to perform mating trials.

Four replicate genotypes from two populations (A and B) are used to form four mating pairs.



After two weeks together in the same jar the mating pairs are split up and each genotype is placed into its own jar.



The larvae released are counted and allowed to form colonies so that offspring fitness can be assessed.

$$A_1B_1 \quad A'_1B'_1 \quad A_2B_2 \quad A'_2B'_2 \quad A_3B_3 \quad A'_3B'_3 \quad A_4B_4 \quad A'_4B'_4$$

To assess offspring viability one colony is removed from the settlement jar and grown in isolation. When big enough it is cloned to form two ramets, one of which is used to perform a backcross to a genotype from the maternal population (but not including the maternal genotype) and one is used to perform a mating with another F1 genotype released by a colony from the paternal population (but not including the paternal genotype).

Backcross matings.

$$A_4*A_1B_1 \quad B_4*A'_1B'_1 \quad A_3*A_2B_2 \quad B_3*A'_2B'_2 \quad A_2*A_3B_3 \quad B_2*A'_3B'_3 \quad A_1*A_4B_4 \quad B_1*A'_4B'_4$$

F1 matings.

$$A_1B_1*A'_4B'_4 \quad A_2B_2*A'_3B'_3 \quad A_3B_3*A'_2B'_2 \quad A_4B_4*A'_1B'_1$$

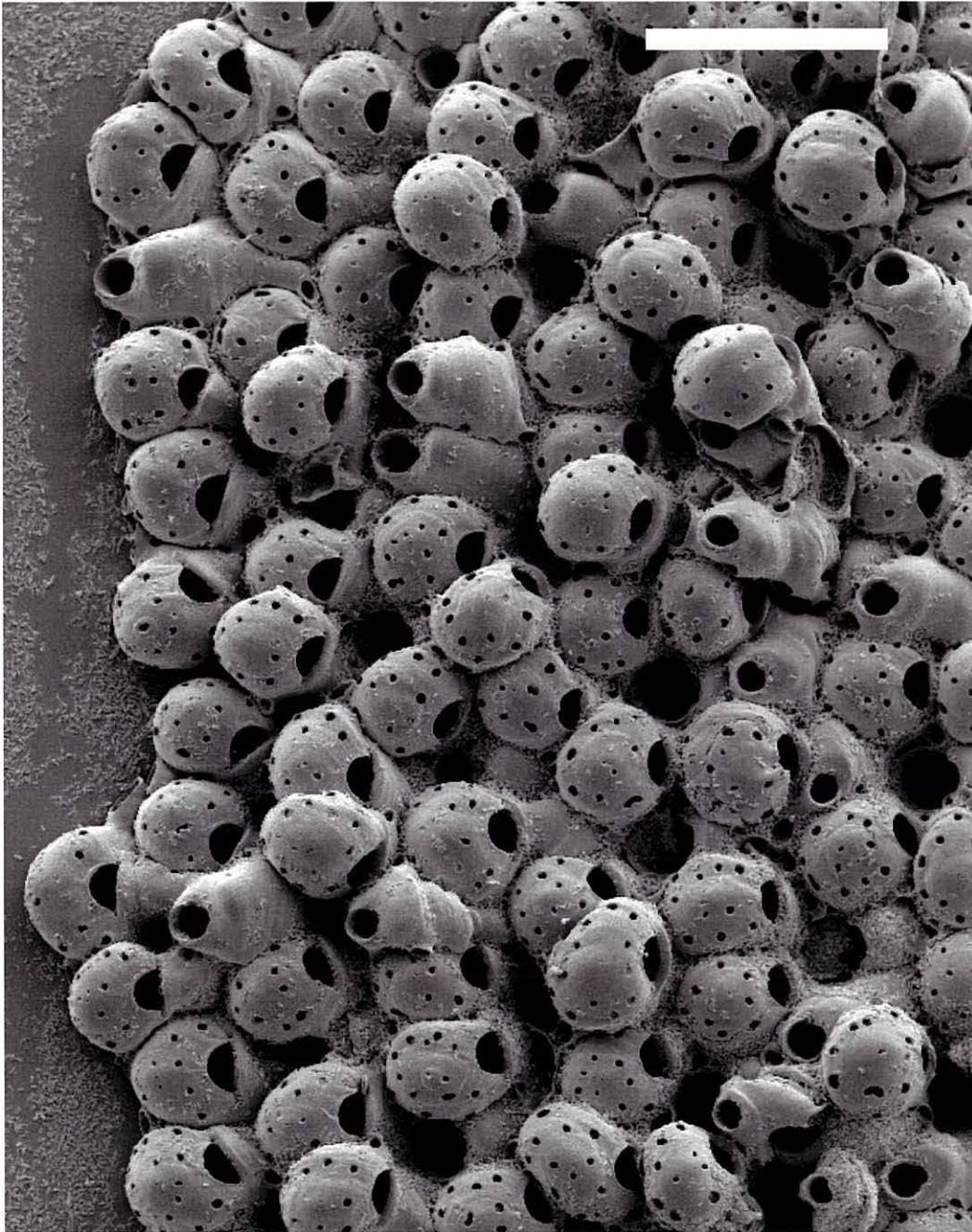


Plate 4.1. Colony margin of F1 hybrid produced in the SPN * RET mating trial showing proliferation of female zooids (gynozoids).

Scale bar = 500 μ m.

4.4. Discussion.

The failure of individuals from the Tjärnö population to produce sexual zooids unfortunately resulted in no information being obtained on the reproductive compatibility of this population with populations from the same clade (Fennoscandia clade: A. Gomez pers. comm.) or from other clades. As has already been discussed in chapter 3, it would seem that the conditions in which the colonies were maintained were unsuitable for individuals from this population, or other populations from the same clade, to grow and reproduce as normal. The wild colonies collected from Tjärnö were already mature when brought into the laboratory at Bangor and started releasing larvae within 3 weeks of their arrival. Observations on Welsh colonies of *C. hyalina* have shown that this species broods embryos for 3-4 weeks prior to the release of larvae (Hughes, 1987; Cancino and Hughes, 1988) which suggests that the larvae released by the Tjärnö colonies in the laboratory were already being brooded on their arrival in Bangor. It is possible that these wild colonies had enough energy reserves to complete the brooding of these embryos even in the apparently unfavourable culture conditions in the laboratory. Failure of the resulting colonies to mature properly and, in those colonies that did produce a few sexual zooids, brood embryos when given the opportunity to outcross, suggests that individuals from the Tjärnö population differ physiologically from individuals in the other populations used in this study. This physiological difference may itself act as a potential barrier to reproduction. Colonies from Tjärnö arriving at another geographically isolated population, via either rafting (Jackson, 1986) or transportation via fouling (Watts *et al.*, 1998), may find that the environmental conditions at the new location are unsuitable for them to reproduce successfully either amongst themselves or with members of the recipient population.

The results obtained from the eighteen between-clades mating trials suggest complete reproductive incompatibility between individuals from geographically isolated populations belonging to separate phylogenetic clades (Appendix 4, Tables A.1-A.54). This is in agreement with a study on three isolated populations from along the coast of Chile that belong to separate phylogenetic clades, the

results of which indicate reproductive incompatibility among the colonies from the three locations studied (A. Navarrete pers. comm.). The brooding of embryos and release of larvae by the Woods Hole genotypes in all of the crosses in which they were involved can be attributed to selfing. The controls to test for self-fertilisation activity in each of the four mating trials involving the Woods Hole genotypes showed that both were producing abundant viable larvae in isolation at the time that each mating trial was performed (Appendix 4, Tables A.18, A.33, A.51 and A.54). Moreover, in the study of self-fertilising capability of *C. hyalina* (Chapter 3), which included the two genotypes used in the present study, selfing capability appeared to be universal within the Woods Hole population. The brooding of embryos by the AML₄ genotype in all of the between-clades mating trials in which it was involved can also be attributed to selfing. In the controls to test for self-fertilisation activity (Appendix 4, Tables A.15, A.30 and A.42), as well as in all the crosses, this genotype was always observed to brood embryos, but never to release any viable larvae unless crossed with another genotype from its own population. This is in agreement with the results obtained in the study of self-fertilising capability of *C. hyalina* (Chapter 3), in which this genotype was observed to brood embryos in isolation but only release very few viable larvae.

The complete lack of embryos observed in crosses between colonies from different populations in the between-clades mating trials (except for those produced by the Woods Hole and AML₄ genotypes discussed above) suggests that strong prezygotic barriers to reproduction exist between populations from the different clades. *C. hyalina* is a spermcast spawner (Pemberton *et al.*, 2003) with the released sperm being taken up by the recipient colony (probably being entrained by the feeding currents of extended autozooid lophophores: e.g., Silén, 1966; Temkin, 1994). The lack of courtship or direct copulation in this species rules out any behavioural barrier to reproduction and suggests that the most important interaction is between the gametes themselves within the recipient colony. Lack of recognition between gametes from individuals belonging to different populations could be due to one of several factors, such as failure of a sperm chemoattractant, differences in gamete morphology or differences in gamete recognition proteins. It has been suggested that in *C. hyalina* the entrained sperm access the female zooids and, therefore eggs, by intra-colonial

sperm migration along the funicular system (Hughes, 1987). This intra-colonial sperm migration may be facilitated by sperm chemotaxis (induced sperm movement toward the egg as the result of the production of a gradient of an attractive substance by the egg: Miller, 1982). Species specificity of sperm chemotaxis has been shown to exist in marine invertebrates (e.g., Ward *et al.*, 1985; Miller, 1997), indicating that sperm chemotaxis could have an important role in gamete recognition.

Sperm morphology has been studied in detail in nine species of marine polychaetes (Eckelbarger and Grassle, 1987). This study included five sibling species of *Capitella*, three species in the related genus *Capistomastus* and one species in the genus *Capitellides*, and revealed that, “the sperm of all nine species differ significantly in the lengths of their middle pieces, acrosomes and especially in their nuclear lengths”. Such divergence in gamete morphology can be expected to accompany reproductive isolation and speciation (Eckelbarger and Grassle, 1987). Differences in sperm morphology have been suspected as causing reproductive breakdown at the fertilisation stage between two sympatric species of the marine polychaete genus *Scoloplos* (Kruse and Reise, 2003). The possibility that differences in sperm morphology may be the cause of the reproductive incompatibility seen in the between clades mating trials with *C. hyalina* populations should be easy to investigate further, due to the ease with which colonies can be induced to release sperm in the laboratory and the ease with which the released sperm can be collected (Manríquez, 1999).

Two other gamete recognition systems that have been well studied among marine invertebrates involve the action of gamete recognition proteins expressed in the spermatozoa. Abalone sperm penetrate the egg vitelline envelope through the non-enzymatic action of an acrosomal protein called lysine (Vacquier *et al.*, 1990). Experiments on two species of abalone from the Pacific Coast of North America, *Haliotis corrugata* and *H. rufescens*, show that the action of this protein is species specific (Vacquier *et al.*, 1990). In sea urchins, a sperm protein called “bindin” is believed to attach the sperm to the egg by interacting with glycoprotein receptors on the egg vitelline layer (Glabe and Vacquier, 1977; 1978), and this interaction is also believed to be primarily species specific (Glabe

and Vacquier, 1977; 1978; Glabe and Lennarz, 1979). Therefore, differences in any potential gamete recognition proteins present in *C. hyalina* sperm may play an important role in preventing reproduction between geographically isolated populations belonging to separate phylogenetic clades. Whether or not any of the factors discussed above play any role in the prezygotic isolation seen among the different populations in the between-clades mating trials warrants further investigation, as the discovery of the cause of the reproductive isolation will help further our understanding of the process of speciation.

The within-clade mating trials, using geographically isolated populations from the NE Atlantic clade, gave a variety of results ranging from complete reproductive isolation, to complete reproductive compatibility between populations (Appendix 4, Tables A.55-A.103). The results obtained from the four within-clade mating trials involving the Icelandic population suggest that members of this population are reproductively isolated from members of other populations in the NE Atlantic clade. In each of these four mating trials embryos brooded in the experimental crosses can be attributed to selfing. In the AML * ICE and IRL * ICE mating trials, the only genotypes observed to brood embryos in the experimental crosses were the AML₄ and IRL₂ genotypes. These genotypes also brooded embryos when kept in reproductive isolation in the controls testing for self-fertilisation activity (Appendix 4, Tables A.57 and A.60). Moreover, both of these two genotypes also brooded embryos when kept in isolation in the previous study of self-fertilising capability of *C. hyalina* (Chapter 3). In the ICE * SPN and ICE * RET mating trials all of the four genotypes that brooded embryos in the experimental crosses also brooded embryos in the controls testing for self-fertilisation activity (Appendix 4, Tables A.63 and A.66). However, none of these four genotypes, or any other genotypes from these two populations, brooded embryos in the previous study of self-fertilising capability of *C. hyalina* (Chapter 3). Just prior to use in these mating trials, all of the genotypes from the SPN and RET populations were cloned (see Appendix 2). It is possible that the selfing observed in ramets of certain genotypes from the SPN and RET populations during these mating trials was a stress response to the cloning procedure. This would not have been observed in the previous study of self-fertilising capability of *C. hyalina* as no cloning was carried out on any

genotype until after that study was completed. Since the only embryos that were produced in the experimental crosses involving the Icelandic population can be attributed to selfing, it would appear that strong prezygotic barriers to reproduction exist between the Icelandic population and other populations in the NE Atlantic clade. Factors that may play a role in the lack of recognition between gametes have already been discussed above.

The AML * IRL, AML * PLY, AML * SPN, IRL * PLY and PLY * SPN experimental crosses demonstrated a complete absence of reproductive barriers between the paired populations. In each of these five trials outcrossing was confirmed by two observations. First, only two of the genotypes involved produced embryos, or larvae, in the controls testing for self-fertilisation activity. The genotypes (AML₄ and IRL₂) showing signs of selfing have been discussed previously. Second, paternity analyses confirmed that outcrossed offspring had been produced by all but one of the genotypes involved in these five trials. The exception was AML₄ in the AML * SPN and AML * IRL mating trials. In the AML * SPN mating trial none of the four larvae released by this genotype reached sexual maturity and, unfortunately, none could be sent for paternity analysis, having settled on the sides of the glass jar rather than the acetate sheet. In the AML * IRL mating trial, 10 of the colonies resulting from larvae released by AML₄ were sent for paternity analysis. However, this analysis was unsuccessful due to the IRL alleles failing to amplify (A. Gomez pers. comm.). In all five of these mating trials, most individuals released high numbers of larvae, with between 85 and 100% of the settled larvae reaching sexual maturity. Very high levels of colony survivorship have also been reported for wild *C. hyalina* colonies. Cancino (1986) reported that on *Laminaria saccharina* fronds in the Menai Strait, North Wales, "individually identified colonies showed close to 100% survival until they were about to reach the frond tip". The high level of colony survivorship observed in this study suggests a high level of fitness of the hybrid offspring. In two of the crosses, AML * PLY and AML * IRL, the lower numbers of larvae released by some of the AML genotypes can be explained by a lack of female zooids present on the colony at the time of the trial. This is also the reason why the PLY₁ genotype frequently released comparatively few numbers of larvae in the trials in which it was involved. However, each of these

genotypes showed close to 100% occupancy of ovicells by embryos during the course of the trial. Further evidence of the complete absence of reproductive barriers between the two populations in each of these five mating trials was the high level of reproductive activity found among the F1 colonies in the F1 * F1 and backcross matings. The majority of F1 genotypes used in these matings released high numbers of larvae, between 85 and 100% of which generated colonies that reached sexual maturity. This rules out any form of postzygotic barrier to reproduction such as hybrid inviability, hybrid breakdown or hybrid sterility.

Perhaps the most interesting result of the within-clade mating trials was the apparent asymmetry observed in the IRL * SPN, SPN * RET and AML * RET experimental crosses. In each of these three crosses reproductive isolation was observed in only one direction of the hybridisation. In the AML * RET mating trial two of the three AML genotypes failed to brood any embryos in the experimental crosses. This lack of embryos would seem to suggest that the reproductive isolation observed in this cross was entirely prezygotic. However, care must be taken when interpreting the results of this cross as no further observations were made on the F1 colonies after they had reached sexual maturity. Therefore, although 100% of the F1s released in this mating trial were observed to reach sexual maturity, it is not known whether or not these hybrids are capable of reproducing, or if any type of hybrid breakdown would occur, as was observed in the hybrids resulting from the SPN * RET crosses (see below).

In both the IRL * SPN and SPN * RET crosses, evidence of postzygotic reproductive isolation was apparent. In the IRL * SPN experimental crosses, three of the SPN genotypes were observed to brood embryos but only one (SPN₄) released viable larvae. Of the other two genotypes one brooded embryos and released seven larvae, none of which survived to sexual maturity, and one brooded embryos but either aborted them or released unviable larvae that were unable to settle and metamorphose successfully. Neither of these two genotypes was observed to brood embryos in the controls testing for self-fertilisation activity, and so it is most likely that the brooded embryos were the result of fertilisation by sperm from IRL genotypes. All of the larvae that successfully

settled and reached sexual maturity in this mating trial appeared to be completely viable, as shown by the success of the F1 * F1 and backcross matings. In the SPN * RET experimental crosses, two of the SPN genotypes were observed to brood embryos and release larvae. However, the SPN₁ genotype only released nine larvae and none of these survived to reach sexual maturity. The lack of selfing recorded in the controls testing for self-fertilisation activity would suggest that the unviable larvae released by the SPN₁ genotype were the result of fertilisation by sperm from the RET₁ genotype. In this mating trial, further evidence of postzygotic reproductive isolation was apparent in the F1 colonies. Although a high percentage (70-100%) of the larvae released by the genotypes in this mating trial reached sexual maturity, evidence suggesting hybrid breakdown was observed in the four genotypes that were grown on for use in the F1 * F1 and backcross matings. This was shown by a proliferation of female zooids (gynozoids) on these genotypes (Plate 4.1) leading to the loss of two of the genotypes before they could be used in the F1 mating trials. Also the two genotypes that were able to be used in the F1 mating trials were not in a good condition at the time that these trials took place (although they were shown to be reproductive in the backcross matings). The F1 hybrids that were kept alive in order to assess the percentage of settled larvae reaching sexual maturity in this mating trial were killed off as soon as they had produced both male and female sexual zooids. If these colonies had been kept alive for longer it is possible that the proliferation of female zooids observed in the four genotypes that were maintained for use in the F1 mating trials would have been seen to be universal. This observed hybrid breakdown suggests a lack of fitness among the offspring resulting from crosses between individuals from the SPN and RET populations. The F1 hybrids produced in the laboratory were grown in conditions assuming very little competition for space and an excess of food available at all times. In the wild colonies would be expected to be in competition for both space and food. Whether or not these “unfit” offspring would be capable of surviving in the wild cannot be determined by laboratory based experiments.

The mating trials performed in this study were “no choice” mating trials, each genotype only had access to sperm released by a single genotype from another population. It would be interesting to repeat the three mating trials in which

mating asymmetry was observed, but this time give each genotype a choice between sperm from another genotype from its own population (conspecific sperm) and sperm from a genotype from the other population (heterospecific sperm). *C. hyalina* colonies obtained from Welsh populations have been shown to be capable of sperm storage (Hoare *et al.*, 1999; Manríquez, 1999; see also Chapter 2). The ability of colonies to store sperm may present the colony with the opportunity for sperm selection, a form of cryptic female choice (Thornhill, 1983; Eberhard, 1991; 1996). Previous studies on grasshoppers (Bella *et al.*, 1992), crickets (Gregory and Howard, 1994), flour beetles (Wade *et al.*, 1994) and *Drosophila* (Price, 1997) have demonstrated the preferential use of conspecific sperm by females when inseminated with both conspecific sperm and heterospecific sperm. This bias towards using conspecific sperm may be due to male-male sperm competition within the female or cryptic female choice. The SPN and RET populations used in this study are sympatric and, therefore, offer a chance to further study this mating asymmetry in wild populations. Recently Dr. Helen Hughes (pers. comm.) has perfected a technique for removing embryos from the ovicells in which they are being brooded. This technique could be used to obtain embryos from wild colonies to enable them to be subjected to genetic analysis to try to confirm whether or not hybridisation is occurring in the natural populations, and if so whether or not the asymmetry observed in the laboratory is occurring in the field.

Asymmetrical sexual isolation appears to be a common phenomenon, having previously been demonstrated in *Drosophila* (Ahearn *et al.*, 1974; Kaneshiro, 1976; Watanabe and Kawanishi, 1979), crickets (Shaw and Lugo, 2001), salamanders (Arnold *et al.*, 1996), pocket gophers (Bradley *et al.*, 1991), hydroids (Buss and Yund, 1989), copepods (Battaglia and Volkmann-Rocco, 1973) and sea urchins (Strathmann, 1981; Lessios and Cunningham, 1990), and it has been suggested that it may be ubiquitous in other animals (Coyne and Orr, 1998). Kaneshiro (1976) and Watanabe and Kawanishi (1979) proposed models for determining the direction of evolution from asymmetry in sexual isolation. However, these authors reached completely opposite conclusions, and subsequent studies have suggested that the direction of evolution cannot be inferred from patterns of asymmetry alone (Wasserman and Koepfer, 1980;

Moodie, 1982; Arnold *et al.*, 1996). In order for speciation between two populations to be complete, reproductive isolation must be bi-directional (Lessios and Cunningham, 1990). Therefore, the observed asymmetry in the three within-clade mating trials described above suggests the presence of incipient species within the NE Atlantic clade.

Chapter 5.

Morphological differentiation between geographically isolated populations of *C. hyalina*.

5.1. Introduction.

The results of chapter 4 demonstrated that complete reproductive isolation has developed between most of the geographically isolated populations of *C. hyalina* that were studied. The genetic divergence, leading to the acquisition of reproductive isolation, that occurs between two or more isolated gene pools during a speciation event is frequently followed by a divergence in morphology, with the resulting morphological differences often being suitable for diagnostic purposes (Mayr and Ashlock, 1991). However, this is not always the case and frequently the genetic divergence of two or more gene pools results in reproductive isolating mechanisms developing without a subsequent divergence in morphological appearance. These morphologically identical, or morphologically very similar, reproductively isolated populations are referred to by the terms cryptic or sibling species (Mayr, 1942; 1948; 1963; Bickham, 1983; Mayr and Ashlock, 1991). Classical taxonomy based solely on morphology cannot recognise sibling species (Mayr, 1948), with the result that reproductively isolated, yet morphologically identical, or morphologically very similar, entities are assigned to single species. This problem is more acute in the sea because of the dominant role of chemical recognition systems among marine invertebrates (Knowlton, 1993; Palumbi, 1994; Palumbi *et al.*, 1997), whereby short-lived gametic interactions are often associated with reduced among-species morphological differentiation. In particular, morphological species with apparently cosmopolitan distributions may each be composed of localised sibling species (Knowlton, 1993; De Vargas *et al.*, 1999). Much of the work carried out in the marine realm has uncovered a large array of marine sibling species and to date they appear to be a common occurrence among marine invertebrates (Grassle and Grassle, 1976; Knowlton, 1993; Table 1; Manchenko and Kulikova, 1996; Geller *et al.*, 1997; Soong *et al.*, 1999; Manchenko *et al.*, 2000; Pearse and Francis, 2000; Bucklin *et al.*, 2001; Dawson and Jacobs, 2001; Larsen, 2001;

Chaparro *et al.*, 2002; Hohenlohe, 2002; Mathews *et al.*, 2002; Vilela-Silva *et al.*, 2002; Kruse and Reise, 2003), marine vertebrates (Davies, 1963; Smith *et al.*, 1979; Smith and Robertson, 1981; Grant, 1987; Bowen *et al.*, 1991; Lavery and Shaklee, 1991; McDonald *et al.*, 1992; Colborn *et al.*, 2001) and marine plants (Rice and Bird, 1990; Medlin, 1991; Bakker *et al.*, 1992; Maggs *et al.*, 1992; Lindstrom, 2001). Past studies on bryozoans have already provided several classical examples of sibling species within the genus *Alcyonidium* (Thorpe *et al.*, 1978a; Thorpe *et al.*, 1978b; Thorpe and Ryland, 1979), and more recently studies on the “cosmopolitan” bryozoan *Bugula neritina* have suggested the presence of sibling species based on genetic variation and bryostatin content (Davidson and Haygood, 1999). It has been suggested that the numbers of sibling species among bryozoans with widespread distributions that are morphologically variable may be alarmingly high (Lidgard and Buckley, 1994).

Bryozoan species, both living and fossil, have almost all been defined solely on the basis of their morphological appearance (McKinney and Jackson, 1989), with the majority of descriptions focusing on the size and shape of zooidal features, such as primary and secondary orifices, and the presence or absence of structures such as avicularia (e.g., Larwood, 1962; Cheetham, 1966; 1968; Soule and Soule, 1973; Ryland and Hayward, 1977; Hayward and Ryland, 1979; 1985). Jackson and Cheetham (1990) explored the problem of whether morphospecies correspond to biological species by studying three distantly related genera of Panamanian cheilostomes. They reported that “evidence from breeding experiments and protein electrophoresis shows that morphospecific identity of cheilostomes is heritable and that morphospecies are genetically distinct with no indication of morphologically cryptic species”. Previous work carried out on the cheilostome *Parasmittina nitida* showed that there was a genetic basis for the differences found between two sympatric morphotypes (Humphries, 1975), and that the two morphotypes were clearly separate species. However, doubts have arisen about whether morphological characteristics alone can be used confidently to distinguish species (Levington *et al.*, 1991). It is also accepted that many cheilostome species, including *C. hyalina* (Morris, 1980), are morphologically variable, and a recent paper on population structure of *Membranipora*

membranacea found that “sympatric morphs varying in their spination and spine inducibility were genetically indistinguishable” (Schwaninger, 1999).

Recently, Hoare *et al.* (2001), in a paper investigating the phylogeography of *C. hyalina* collected from six different sites, suggested that the life history differences found between Welsh and Chilean populations, coupled with the degree of differentiation in mitochondrial sequences and at nuclear loci are enough to “strongly suggest cryptic speciation within *C. hyalina*”. This species has generally been regarded as being a “cosmopolitan” bryozoan, and Ryland and Gordon (1977) listed five features said to be distinctive of *C. hyalina*. These are: (1) a schizoporelloid ancestrula; (2) unilateral initial budding pattern; (3) sexual zooids usually frontally budded and smaller than the autozooids; (4) almost orbicular orifice (of auto- and male zooids) with a broad shallow sinus; and (5) numerous frontal pores in the ovicell. As well as the five features listed above many authors tend to report the measurements of various zooidal parameters, such as lengths and widths of zooids and zooidal apertures and numbers of tentacles, when describing this species and also when comparing it to new species (e.g. Pinter, 1973; Ryland and Gordon, 1977; Ryland, 1979; Morris, 1980; Fernández Pulpeiro and Reverter Gil, 1992). However, to date there appears to have been no attempt to perform anything other than the most basic statistical analysis on any of these data. The aim of this chapter is to investigate the morphological variability among the different populations studied, in order to assess the likelihood of cryptic speciation within *C. hyalina* *sensu lato*.

5.2. Materials and Methods.

The morphological investigations were split into two separate studies: 1) early astogeny and gross colonial morphology; and 2) a multivariate analysis of various linear and meristic data.

5.2.1. Early Astogeny and Gross Colony Morphology

The early astogeny of *C. hyalina* was studied in colonies from the following locations (for full details on the exact location of each population see Appendix 1); North Wales (Menai Strait, Amlwch Harbour, Holyhead Harbour and Porth Neigwl); Ireland (Lough Hyne, Achill Sound and Dublin Harbour); Scotland (Oban); England (Plymouth); Spain (Ría de Ferrol – this population consists of *C. hyalina* var. normal and *C. hyalina* var. reticulata); Iceland (Reykjavík); Norway (Tromsø); Sweden (Tjärnö); Russia (White Sea); Svalbard (Kapp Linné); America (Maine, Woods Hole, California and Alaska); and Canada (Nova Scotia and Victoria Island). A population of *C. angusta* Alvarez, 1991, (Spain, Foz) was also included in this study. The number of colonies studied from each location is given in Table 5.1. The observations were made on colonies that were settled for use in the study of self-fertilisation capability of *C. hyalina* (Chapter 3). Each colony was viewed under a Leica MZ APO binocular microscope 3 times a week and the pattern of astogeny was noted. Observations were continued until each colony had reached circularity.

In addition to the general pattern of early astogeny, the angle between the ancestrula and the first daughter zooid, and the angle between the first and second daughter zooids (Plate 5.1) was measured in 200 colonies from the following locations: Amlwch Harbour, Lough Hyne, Plymouth, Ría de Ferrol (var. normal and var. reticulata), Reykjavík, Maine, Woods Hole, California, Alaska and Nova Scotia. In order to accurately measure these angles, young colonies that had not started to produce frontal sexual zooids were placed under a Leica MZ APO binocular microscope mounted with a JVC colour video camera and the images were recorded onto S-VHS video cassette. The images were then captured using Microsoft® VidCap and printed out on an Epson Stylus Color

880 printer. Once printed out, the angles were easily measured using a protractor. Angular data were analysed using Oriana for Windows, version 1.01, a program for analysing orientation and other circular data. For the Nova Scotia population, the 200 colonies measured were the product of the selfing in isolated colonies (Chapter 3). For all other populations, the 200 colonies measured were products of outcrossing from within-population control matings (Chapter 4).

Observations of gross colonial morphology were made on the same individuals used to study early astogeny (see above). Observations were made on mature colonies prior to cloning at the end of the study of self-fertilisation capability (Chapter 3). Colonies were viewed under a Leica MZ APO binocular microscope and any peculiarities in morphology were noted.

5.2.2. Multivariate Analysis of Linear and Meristic Data

Eleven linear and three meristic characters were selected for use in the multivariate analysis as follows.

Linear

- 1) Length of autozooids (μm)
- 2) Width of autozooids (μm)
- 3) Length of ovicells (μm)
- 4) Width of ovicells (μm)
- 5) Length of frontal males (μm)
- 6) Width of frontal males (μm)
- 7) Depth of autozoid orifice (μm) (Plate 5.2)
- 8) Width of autozoid orifice (μm) (Plate 5.2)
- 9) Distance between condyles of autozoid orifice (μm) (Plate 5.2)
- 10) Depth of sinus of autozoid orifice (μm) (Plate 5.2)
- 11) Width of sinus of autozoid orifice at 0.5 depth (μm) (Plate 5.2)

Meristic

- 1) Number of tentacles on lophophore
- 2) Number of pores on ovicell (Plate 5.3)
- 3) Number of tubular pore chambers surrounding autozooids (Plate 5.3)

Each character was measured on ten different zooids per colony and the colony average was used in the analysis. Linear characters 1-6 were measured on live colonies under a Leica MZ APO binocular microscope fitted with an eyepiece graticule. Characters 7-11 were measured on scanning electron microscope (SEM) images using UTHSCSA Image Tool for Windows, version 2.0. Meristic character 1 was counted on live colonies that were observed feeding under a Leica MZ APO binocular microscope. Each count for character 1 was repeated three times to ensure accuracy. Characters 2 and 3 were counted on colonies that were being viewed under the SEM while obtaining the images used to measure linear characters 7-11. All measurements taken from autozooids were taken from individuals situated at the colony edge of mature colonies. This is because early autozooids, in the vicinity of the ancestrula, are smaller than those produced later (Cancino, 1983; Cancino and Hughes, 1988). However, autozoid size eventually stops increasing, with subsequent zooids being of approximately equal size (Cancino, 1983; Cancino and Hughes, 1988). SEM images were obtained using a Hitachi S-520 scanning electron microscope. Ramets of the colonies that were to be viewed under the SEM were first soaked in sodium hypochlorite in order to dissolve the highly reflective outer cuticle. Live ramets were soaked for four hours and dried ramets for two hours. Next, each ramet was transferred to a petri dish containing distilled water for 24 hours before being mounted on an SEM stub. Once mounted, the ramets were allowed to dry at room temperature for 48 hours. Finally each ramet was coated with gold using a Polaron SEM coating unit E5000.

All eleven linear characters were collected from the following populations (for full details on the exact location of each population see Appendix 1); North Wales (Menai Strait, Amlwch Harbour, Holyhead Harbour and Porth Neigwl); Ireland (Lough Hyne, Achill Sound and Dublin Harbour); Scotland (Oban); England (Plymouth); Spain (Ría de Ferrol – this population consists of *C. hyalina* var. normal and *C. hyalina* var. reticulata); Iceland (Reykjavík); Sweden (Tjärnö); Svalbard (Kapp Linné); America (Maine, Woods Hole, California and Alaska); Canada (Nova Scotia and Victoria Island); and Chile (Las Cruces) (the Chilean colonies were sent by colleagues for use in the morphological analysis).

The population of *C. angusta* Alvarez, 1991, (Spain, Foz) was also included in this study. However, in order to include the remaining two populations (Norway (Tromsø); and Russia (White Sea)) characters 3-6 were dropped from the analysis. These four characters could not be measured in these two populations due to the colonies not bearing any frontal sexual zooids at the time that the measurements were taken. Therefore, only seven of the eleven linear characters were used in the analysis. Table 5.2 shows the number of colonies from each population that the seven linear characters used in the analysis were measured from.

All three meristic characters were collected from the following populations (for full details on the exact location of each population see Appendix 1); North Wales (Amlwch Harbour); Ireland (Lough Hyne); Scotland (Oban); England (Plymouth); Spain (Ría de Ferrol – this population consists of *C. hyalina* var. normal and *C. hyalina* var. reticulata); Iceland (Reykjavík); America (Maine, Woods Hole, California and Alaska); and Canada (Nova Scotia and Victoria Island). Table 5.3 shows the number of colonies from each population that the three meristic characters used in the analysis were measured from.

Both the linear and meristic characters were analysed using canonical variate analysis (CVA; discriminant function analysis), a multivariate technique used to ordinate *a priori* groups of individuals. Such groups may include specimens from a single locality, compound locality or many localities (Thorpe, 1976) although groups comprising of single localities are preferred (see below). This technique takes into account the within group covariation between characters and, therefore, eliminates redundant information in a character set so long as one group represents one locality (Thorpe, 1976). If a group represents more than one locality this advantage is lost as one is no longer dealing with intra-locality correlation (Thorpe, 1976). SPSS version 11 was used to perform this analysis. Prior to inclusion in the analysis, each linear and meristic character was subjected to a one-way analysis of variance (one-way ANOVA) to ensure that they showed significant variation among localities. As with the CVA, SPSS version 11 was used to perform this analysis.

The results of mitochondrial DNA sequencing, carried out by Dr. A. Gomez at Hull University, showed that the populations collected from Achill Sound and Kapp Linné both contained genotypes belonging to different phylogenetic clades. Gross colonial morphology (see above) also indicated differences among some of the genotypes within the Achill sound population (see results). Therefore, each of the above populations was split into two groups for the purposes of the multivariate analysis, each group comprising of genotypes occupying the same phylogenetic clade (A. Gomez pers. comm.).

5.3. Results.

5.3.1. Early Astogeny and Gross Colony Morphology

All colonies observed for the study of early astogeny exhibited the same distinctive early budding pattern. One disto-lateral bud (first daughter zooid) was produced that could arise on either the left or right hand side of the ancestrula. During the growth of this first daughter zooid, a second bud was produced (second daughter zooid) between the first daughter zooid and the ancestrula. Once the first daughter zooid had completed its growth and started feeding the ancestral polypide regressed and the second daughter zooid completed its growth. A third bud (third daughter zooid) was produced between the first and second daughter zooids during the growth of the second daughter zooid. Once the second daughter zooid had commenced feeding the third daughter zooid completed its growth. After these initial three zooids had completed their growth, one at a time, continual simultaneous budding from between zooids, in all directions, resulted in the formation of a compact circular colony. No basal or frontal sexual zooids were observed in any colony until after circularity had been attained. Other occasional budding patterns observed in colonies released in the within-population control matings (Chapter 4) will be discussed below.

Each of the eleven populations for which angular data were collected were compared using a Watson's F-Test. For the 54 comparisons made, Bonferroni correction gave a threshold significance value (α) of 0.000925. For the angle between the ancestrula and the first daughter zooid (θ_1 – Plate 5.1 A) 43 of the 54 pairs of populations were found to differ significantly (Table 5.4). For the angle between the first and second daughter zooids (θ_2 – Plate 5.1 B) 41 of the 54 pairs of populations were found to differ significantly (Table 5.5). Only two pairs of populations were found not to differ significantly in at least one of the angles measured (Tables 5.4 and 5.5).

During the video recording of colonies that were used to obtain the angular data, two other patterns of budding were observed among colonies from three of the populations studied. Occasional colonies from the Reykjavík, Californian and Woods Hole populations were observed to produce two disto-lateral buds

simultaneously (Plate 5.4). All of these colonies were observed to reach circularity and frontal sexual zooids were produced as normal. One colony from the Woods Hole population was observed to produce the first daughter zooid disto-medially. This first zooid, however, was a male zooid (androzooid) and the colony did not progress any further than the two zooid stage.

The majority of colonies in which gross colonial morphology was studied formed colonies consisting of two layers (bilaminar). The basal layer of each of these colonies consisted almost entirely of autozooids, although male zooids (androzooids) were observed in low numbers in the basal layer of all colonies. The frontal layer of each of these colonies consisted entirely of male and female zooids (gynozooids). All of the colonies from Foz (*C. angusta*) and Porth Neigwl, and three of the seven colonies from Achill Sound were found to be nodular in appearance (multilaminar) at the time that the observations were made. The basal layer of each of these colonies appeared to consist mainly of autozooids, with occasional male zooids present in all colonies. The frontal layers consisted entirely of male and female zooids. Each of the genotypes on which observations of gross colonial morphology were made were checked several times in the two weeks after they had been cloned. In all but two of the populations, there was an increase in the number of male zooids produced in the basal layer in the area of the colony that had been cut during the cloning procedure (see Appendix 2). In the other two populations (Ría de Ferrol – var. normal and Ría de Ferrol – var. *reticulata*) occasional female zooids were produced in the basal layer in the area of the colony that had been cut during the cloning procedure (see Appendix 2).

The large lacunae that occur between the autozooids of *C. hyalina* var. *reticulata* (and which are used to distinguish this variety from *C. hyalina* var. normal) were found to be much more obvious in young colonies than in older colonies. Indeed, in older colonies of *C. hyalina* var. *reticulata* the lacunae almost completely disappeared and the colony very much resembled a colony of *C. hyalina* var. normal in this respect. Moreover, the large lacunae that were present in the younger parts of the colony became obscured by the presence of frontal male and female zooids as the colony matured.

5.3.2. Multivariate Analysis of Linear and Meristic Data

All linear and meristic characters examined by one-way ANOVA were highly significant with respect to variation among groups (Tables 5.6 and 5.7).

Canonical variate analysis on all statistically significant linear characters revealed the presence of three discrete groups and one large, loosely defined group (Fig. 5.1). Two of the discrete groups consisted of single populations (Woods Hole and Kapp Linné 1) and the other consisted of three populations (*C. angusta*, Porth Neigwl and Achill Sound 2) (Fig. 5.1). Within the large loosely defined group there are three populations that did not overlap with any other population (Tjärnö, White Sea and Nova Scotia) (Fig. 5.1). High standardised first canonical discriminant function coefficients were most associated with autozoid length and distance between condyles (Table 5.8). High standardised second canonical discriminant function coefficients were most associated with width of sinus at 0.5 depth, distance between condyles and depth of sinus (Table 5.8). Removal of the three discrete groups from the analysis did not reveal any significant changes to the pattern of separation within the remaining loosely defined group (Fig. 5.2).

Canonical variate analysis on all statistically significant meristic characters revealed the presence of two discrete groups and one large loosely defined group (Fig. 5.3). The two discrete groups both consisted of single populations (Woods Hole and California) (Fig. 5.3). High standardised first canonical discriminant function coefficients were most associated with number of tentacles on the lophophore and number of tubular pore chambers surrounding autozooids (Table 5.9). High standardised second canonical discriminant function coefficients were most associated with number of tubular pore chambers surrounding each autozoid and number of pores on the ovicell (Table 5.9). Removal of the two discrete groups from the analysis revealed the presence of one further discrete group from within the large loosely defined group (Fig. 5.4). This group consisted of a single population (Reykjavík) (Fig. 5.4). In this second analysis, high first canonical discriminant function coefficients were most associated with number of tentacles on the lophophore and number of tubular pore chambers

surrounding each autozoid (Table 5.10). High second canonical discriminant function coefficients were most associated with number of pores on the ovicell and number of tubular pore chambers surrounding each autozoid (Table 5.10).

Table 5.1. Number of colonies studied for the early astogeny observations from each location.

Population	Number of colonies studied
Menai Strait	14
Amlwch Harbour	14
Holyhead Harbour	14
Porth Neigwl	7
Lough Hyne	14
Achill Sound	7
Dublin Harbour	14
Oban	4
Plymouth	14
Ría de Ferrol – var. normal	14
Ría de Ferrol – var. reticulata	7
Reykjavík	14
Tromsø	6
Tjärnö	14
White Sea	14
Kapp Linné	4
Maine	20
Woods Hole	14
California	14
Alaska	14
Nova Scotia	14
Victoria Island	14
Foz (<i>C. angusta</i>)	14

Table 5.2. Number of colonies from which the linear characters were measured from each location.

Population	Number of colonies from which measurements were taken
Menai Strait	10
Amlwch Harbour	10
Holyhead Harbour	10
Porth Neigwl	7
Lough Hyne	10
Achill Sound 1	3
Achill Sound 2	3
Dublin Harbour	10
Oban	4
Plymouth	10
Ría de Ferrol – var. normal	10
Ría de Ferrol – var. reticulata	7
Reykjavík	10
Tjörnö	10
Kapp Linné 1	3
Kapp Linné 2	1
Maine	10
Woods Hole	10
California	10
Alaska	8
Nova Scotia	10
Victoria Island	10
Las Cruces	6
Tromsø	6
White Sea	10
Foz (<i>C. angusta</i>)	10

Table 5.3. Number of colonies from which the meristic characters were measured from each location.

Population	Number of colonies from which measurements were taken
Amlwch Harbour	10
Lough Hyne	10
Oban	4
Plymouth	10
Ría de Ferrol – var. normal	10
Ría de Ferrol – var. reticulata	7
Reykjavík	10
Maine	10
Woods Hole	10
California	10
Alaska	5
Nova Scotia	10
Victoria Island	10

Table 5.4. Results of Watson's F-Test (Oriana, version 1.01) comparing the angle between the ancestrula and first daughter zooid (θ_1 – Plate 5.1 A). Data presented are F-values (F), with probabilities (P). * = significant values.

Populations compared	F	P
Woods Hole & Nova Scotia	347.68	0.00000*
Woods Hole & Maine	239.39	0.00000*
Woods Hole & Lough Hyne	269.73	0.00000*
Woods Hole and Amlwch Harbour	240.41	0.00000*
Woods Hole & Ría de Ferrol (var. normal)	130.91	0.00000*
Woods Hole & Alaska	130.91	0.00000*
Woods Hole & California	19.82	0.00001*
Woods Hole & Ría de Ferrol (var. reticulata)	94.29	0.00000*
Woods Hole & Reykjavík	49.56	0.00000*
Woods Hole & Plymouth	523.15	0.00000*
Nova Scotia & Maine	0.34	0.56054
Nova Scotia & Lough Hyne	1.03	0.30965
Nova Scotia & Amlwch Harbour	4.31	0.03837
Nova Scotia & Ría de Ferrol (var. normal)	37.38	0.00000*
Nova Scotia & Alaska	37.38	0.00000*
Nova Scotia & California	153.64	0.00000*
Nova Scotia & Ría de Ferrol (var. reticulata)	48.96	0.00000*
Nova Scotia & Reykjavík	89.58	0.00000*
Nova Scotia & Plymouth	30.30	0.00000*
Maine & Lough Hyne	1.99	0.15845
Maine & Amlwch Harbour	1.57	0.21052
Maine & Ría de Ferrol (var. normal)	22.83	0.00000*
Maine & Alaska	22.83	0.00000*
Maine & California	107.90	0.00000*
Maine & Ría de Ferrol (var. reticulata)	31.81	0.00000*
Maine & Reykjavík	61.59	0.00000*
Maine & Plymouth	28.70	0.00000*
Lough Hyne & Amlwch Harbour	7.39	0.00685
Lough Hyne & Ría de Ferrol (var. normal)	37.31	0.00000*
Lough Hyne & Alaska	37.31	0.00000*
Lough Hyne & California	131.96	0.00000*
Lough Hyne & Ría de Ferrol (var. reticulata)	47.60	0.00000*
Lough Hyne & Reykjavík	81.40	0.00000*
Lough Hyne & Plymouth	13.39	0.00029*
Amlwch Harbour & Ría de Ferrol (var. normal)	14.67	0.00015*

Table 5.4 (continued)

Amlwch Harbour & Alaska	14.67	0.00015*
Amlwch Harbour & California	98.24	0.00000*
Amlwch Harbour & Ría de Ferrol (var. reticulata)	22.93	0.00000*
Amlwch Harbour & Reykjavík	51.43	0.00000*
Amlwch Harbour & Plymouth	51.39	0.00000*
Ría de Ferrol (var. normal) & Alaska	0.00	1.00000
Ría de Ferrol (var. normal) & California	38.62	0.00000*
Ría de Ferrol (var. normal) & Ría de Ferrol (var. reticulata)	1.27	0.26101
Ría de Ferrol (var. normal) & Reykjavík	12.58	0.00044*
Ría de Ferrol (var. normal) & Plymouth	119.82	0.00000*
Alaska & California	38.62	0.00000*
Alaska & Ría de Ferrol (var. reticulata)	1.27	0.26101
Alaska & Reykjavík	12.58	0.00044*
Alaska & Plymouth	119.83	0.00000*
California & Ría de Ferrol (var. reticulata)	23.75	0.00000*
California & Reykjavík	6.10	0.01396
California & Plymouth	279.03	0.00000*
Ría de Ferrol (var. reticulata) & Reykjavík	5.46	0.01990*
Ría de Ferrol (var. reticulata) & Plymouth	134.16	0.00000*

Table 5.5. Results of Watson's F-Test (Oriana, version 1.01) comparing the angle between the first and second daughter zooids (θ_2 – Plate 5.1 B). Data presented are F-values (F), with probabilities (P). * = significant values.

Populations compared	F	P
Woods Hole & Nova Scotia	528.55	0.00000*
Woods Hole & Maine	57.07	0.00000*
Woods Hole & Lough Hyne	199.23	0.00000*
Woods Hole & Amlwch Harbour	202.09	0.00000*
Woods Hole & Ría de Ferrol (var. normal)	185.90	0.00000*
Woods Hole & Alaska	15.75	0.00009*
Woods Hole & California	129.68	0.00000*
Woods Hole & Ría de Ferrol (var. reticulata)	168.20	0.00000*
Woods Hole & Reykjavík	8.19	0.00444
Woods Hole & Plymouth	122.75	0.00000*
Nova Scotia & Maine	266.07	0.00000*
Nova Scotia & Lough Hyne	53.04	0.00000*
Nova Scotia & Amlwch Harbour	92.50	0.00000*
Nova Scotia & Ría de Ferrol (var. normal)	98.94	0.00000*
Nova Scotia & Alaska	438.27	0.00000*
Nova Scotia & California	148.84	0.00000*
Nova Scotia & Ría de Ferrol (var. reticulata)	97.99	0.00000*
Nova Scotia & Reykjavík	542.84	0.00000*
Nova Scotia & Plymouth	224.54	0.00000*
Maine & Lough Hyne	55.99	0.00000*
Maine & Amlwch Harbour	48.11	0.00000*
Maine & Ría de Ferrol (var. normal)	40.92	0.00000*
Maine & Alaska	16.16	0.00007*
Maine & California	16.47	0.00006*
Maine & Ría de Ferrol (var. reticulata)	34.77	0.00000*
Maine & Reykjavík	30.65	0.00000*
Maine & Plymouth	9.44	0.00226
Lough Hyne & Amlwch Harbour	1.64	0.20126
Lough Hyne & Ría de Ferrol (var. normal)	2.82	0.09382
Lough Hyne & Alaska	128.81	0.00000*
Lough Hyne & California	14.19	0.00019*
Lough Hyne & Ría de Ferrol (var. reticulata)	3.62	0.05775
Lough Hyne & Reykjavík	168.66	0.00000*
Lough Hyne & Plymouth	28.60	0.00000*
Amlwch Harbour & Ría de Ferrol (var. normal)	0.21	0.64427

Table 5.5 (continued)

Amlwch Harbour & Alaska	126.92	0.00000*
Amlwch Harbour & California	7.84	0.00537
Amlwch Harbour & Ría de Ferrol (var. reticulata)	0.56	0.45585
Amlwch Harbour & Reykjavík	173.25	0.00000*
Amlwch Harbour & Plymouth	20.58	0.00001*
Ría de Ferrol (var. normal) & Alaska	113.43	0.00000*
Ría de Ferrol (var. normal) & California	5.34	0.02133
Ría de Ferrol (var. normal) & Ría de Ferrol (var. reticulata)	0.09	0.76699
Ría de Ferrol (var. normal) & Reykjavík	155.88	0.00000*
Ría de Ferrol (var. normal) & Plymouth	15.73	0.00009*
Alaska & California	67.33	0.00000*
Alaska & Ría de Ferrol (var. reticulata)	99.75	0.00000*
Alaska & Reykjavík	1.92	0.16663
Alaska & Plymouth	58.33	0.00000*
California & Ría de Ferrol (var. reticulata)	3.77	0.05292
California & Reykjavík	98.21	0.00000*
California & Plymouth	1.96	0.16274
Ría de Ferrol (var. reticulata) & Reykjavík	137.17	0.00000*
Ría de Ferrol (var. reticulata) & Plymouth	12.16	0.00054*

Table 5.6. Results of one-way ANOVA (SPSS, version 11) showing which linear characters exhibit significant variation among groups. Data presented are F-values (F), with probabilities (P). * = significant values.

Character	F	P
Length of autozooids	161.85	0.000*
Width of autozooids	93.43	0.000*
Depth of autozoid orifice	66.01	0.000*
Width of autozoid orifice	110.06	0.000*
Distance between condyles of autozoid orifice	115.46	0.000*
Depth of sinus of autozoid orifice	38.76	0.000*
Width of sinus of autozoid orifice at 0.5 depth	92.03	0.000*

Table 5.7. Results of one-way ANOVA (SPSS, version 11) showing which meristic characters exhibit significant variation among groups. Data presented are F-values (F), with probabilities (P). * = significant values.

Character	F	P
Number of tentacles on lophophore	99.92	0.000*
Number of pores on ovicell	35.37	0.000*
Number of tubular pore chambers surrounding autozooids	51.82	0.000*

Table 5.8. Standardised canonical discriminant function coefficients from the analysis on all significantly variable linear characters performed on all populations. (See materials and methods for character descriptions).

Character	Function 1	Function 2
1	0.644	-0.400
2	0.237	0.603
7	0.324	-0.599
8	-0.024	0.372
9	0.509	1.174
10	-0.120	1.033
11	0.013	-1.808

Table 5.9. Standardised canonical discriminant function coefficients from the analysis on all significantly variable meristic characters performed on all populations from which data were gathered. (See materials and methods for character descriptions).

Character	Function 1	Function 2
1	0.976	-0.361
2	0.267	0.501
3	0.533	0.597

Table 5.10. Standardised canonical discriminant function coefficients from the analysis on all significantly variable meristic characters performed on all populations from which data were gathered except Woods Hole and California. (See materials and methods for character descriptions).

Character	Function 1	Function 2
1	0.982	0.095
2	-0.078	0.892
3	0.476	0.349

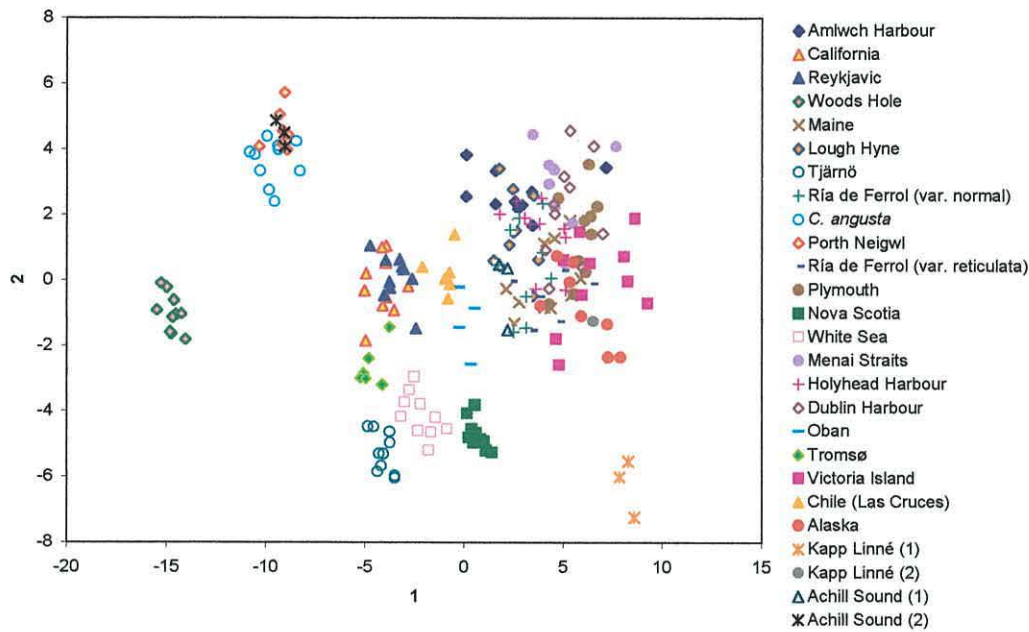


Fig 5.1. Ordination based on all significantly variable linear characters along the first two canonical variates. The first and second canonical variates summarise 71.2% and 14.5% of the total variance respectively.

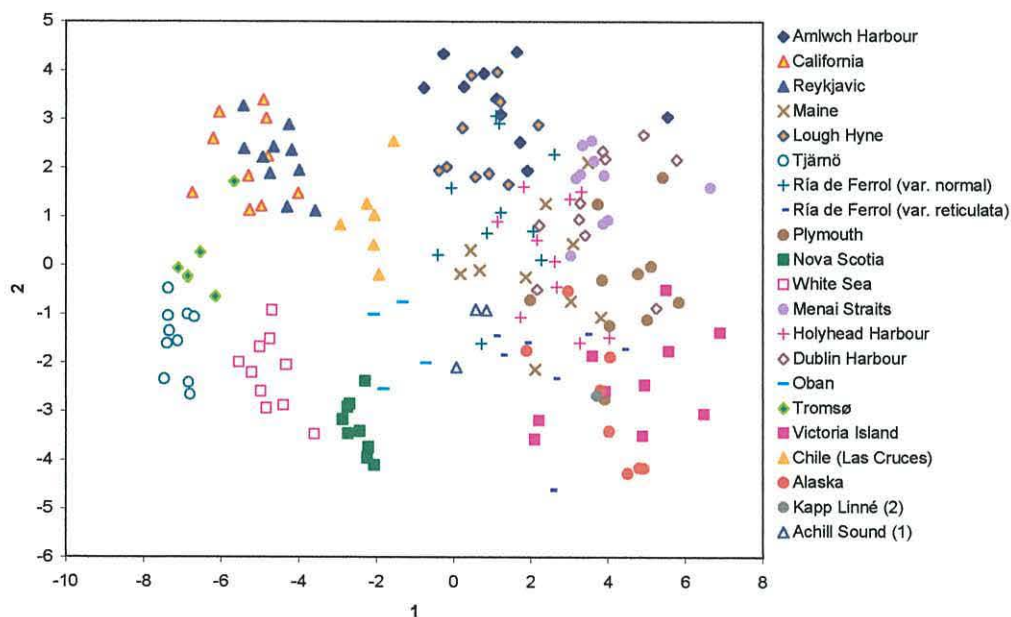


Fig 5.2. Ordination based on all significantly variable linear characters along the first two canonical variates after removal of the populations forming the three discrete groups revealed in the original analysis. The first and second canonical variates summarise 62.7% and 17.0% of the total variance respectively.

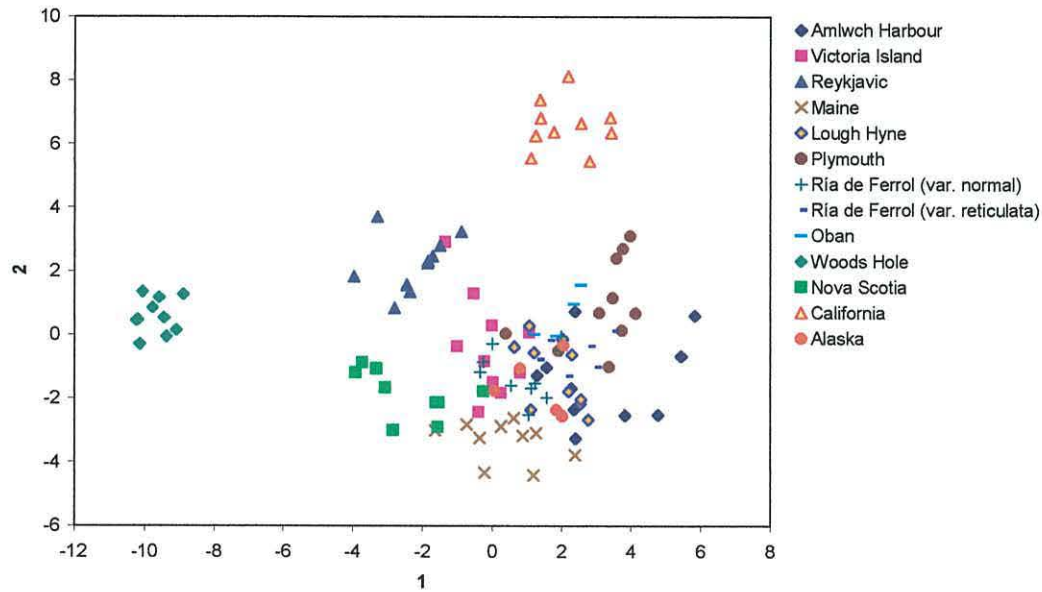


Fig 5.3. Ordination based on all significantly variable meristic characters along the first two canonical variates. The first and second canonical variates summarise 62.4% and 31.4% of the total variance respectively.

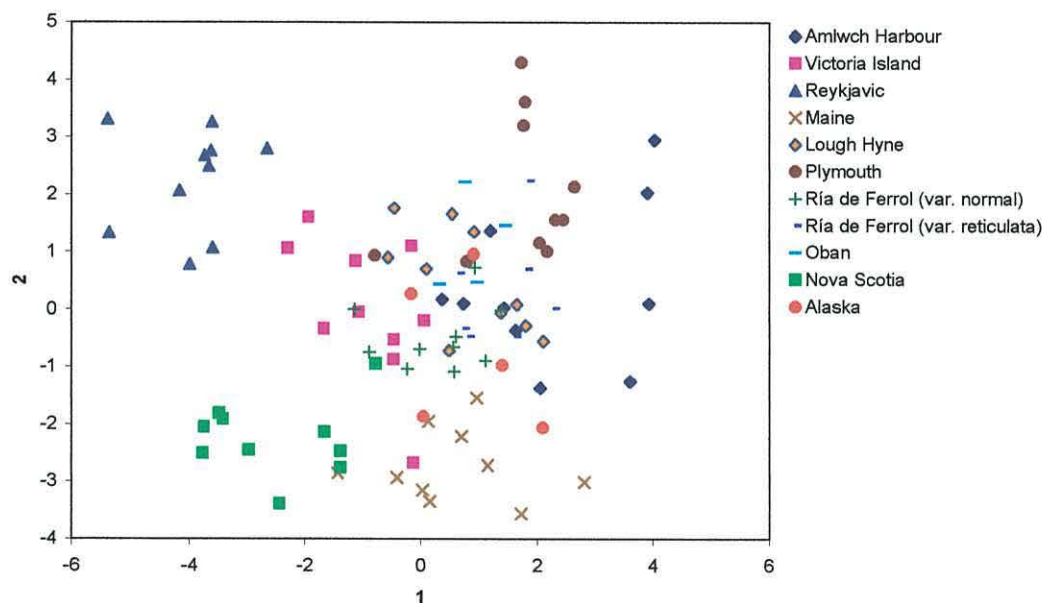


Fig 5.4. Ordination based on all significantly variable meristic characters along the first two canonical variates after removal of the populations forming the two discrete groups revealed in the original analysis. The first and second canonical variates summarise 55.7% and 37.9% of the total variance respectively.

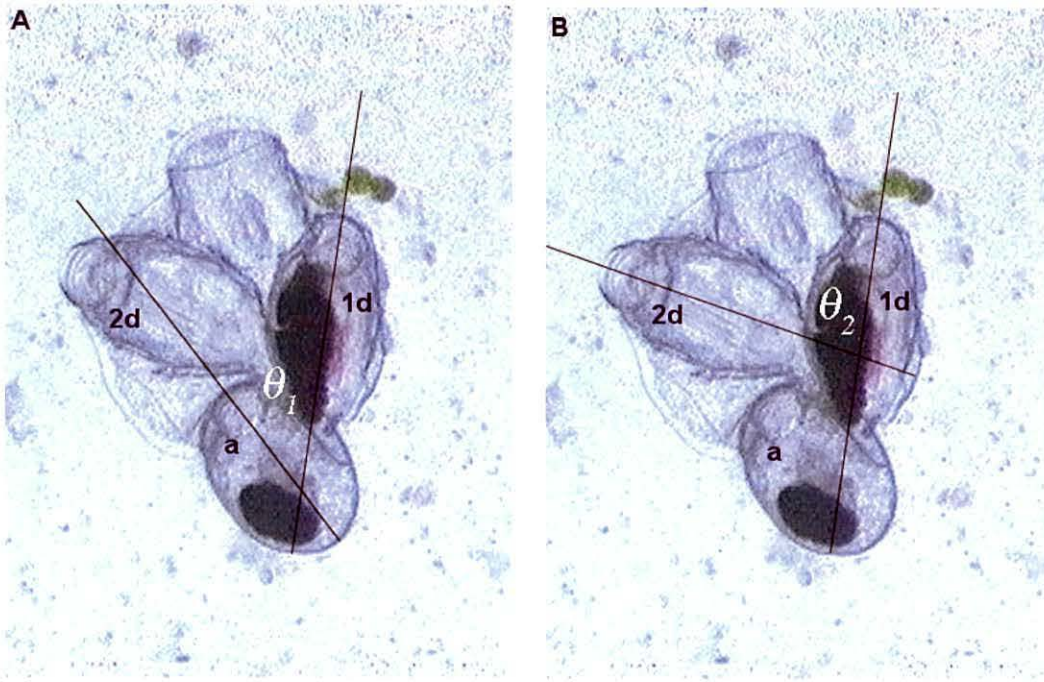


Plate 5.1. Video stills of an immature colony demonstrating (A) the angle between the ancestrula and first daughter zooid, θ_1 , and (B) the angle between the first and second daughter zooids, θ_2 . a = ancestrula, 1d = first daughter zooid and 2d = second daughter zooid.

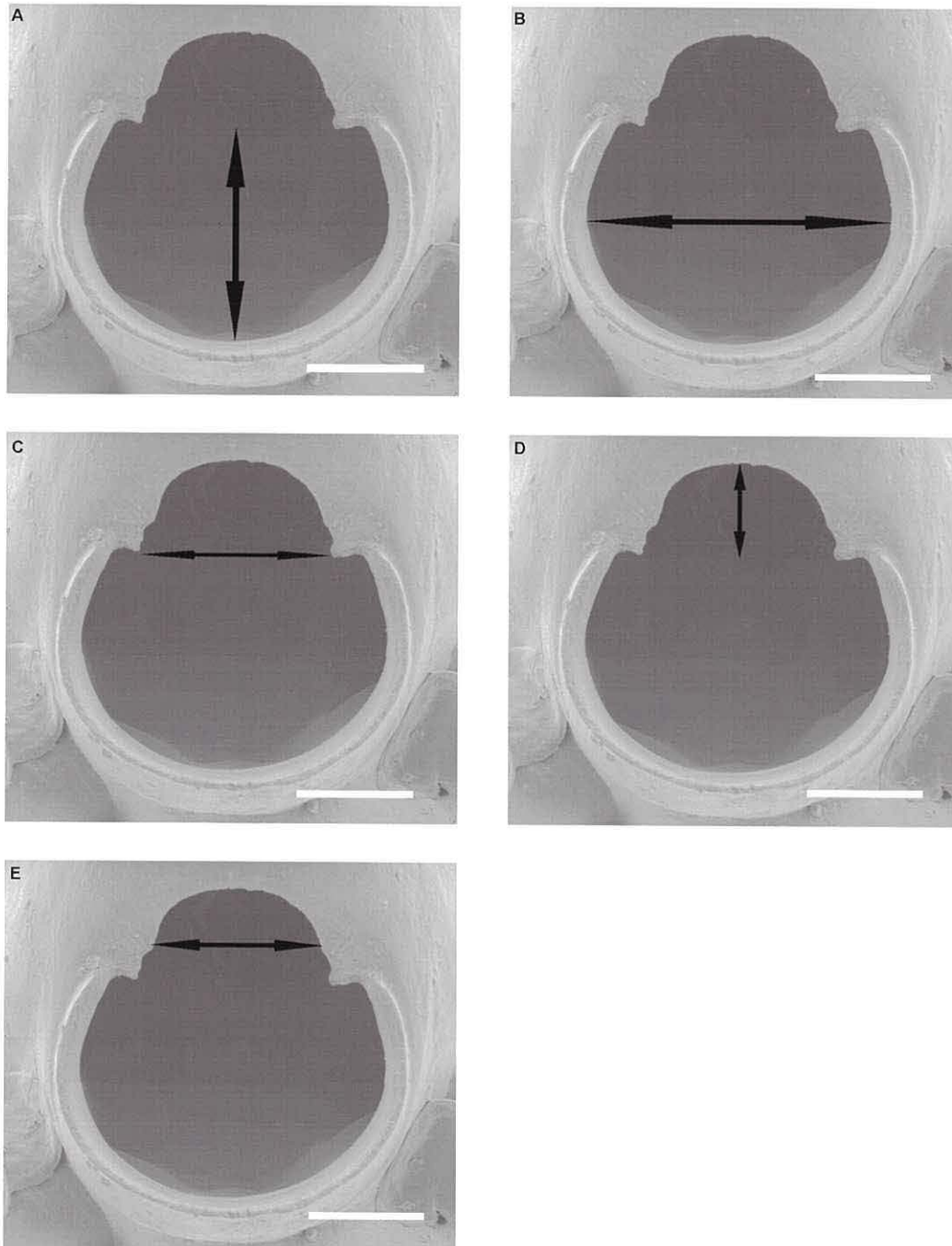


Plate 5.2. Scanning electron micrographs of an autozoid orifice from the Dublin Harbour population demonstrating the linear characters 7-11. (A) = depth of orifice, (B) = width of orifice, (C) = distance between condyles, (D) = depth of sinus and (E) = width of sinus at 0.5 depth.

All scale bars = 50 μ m.

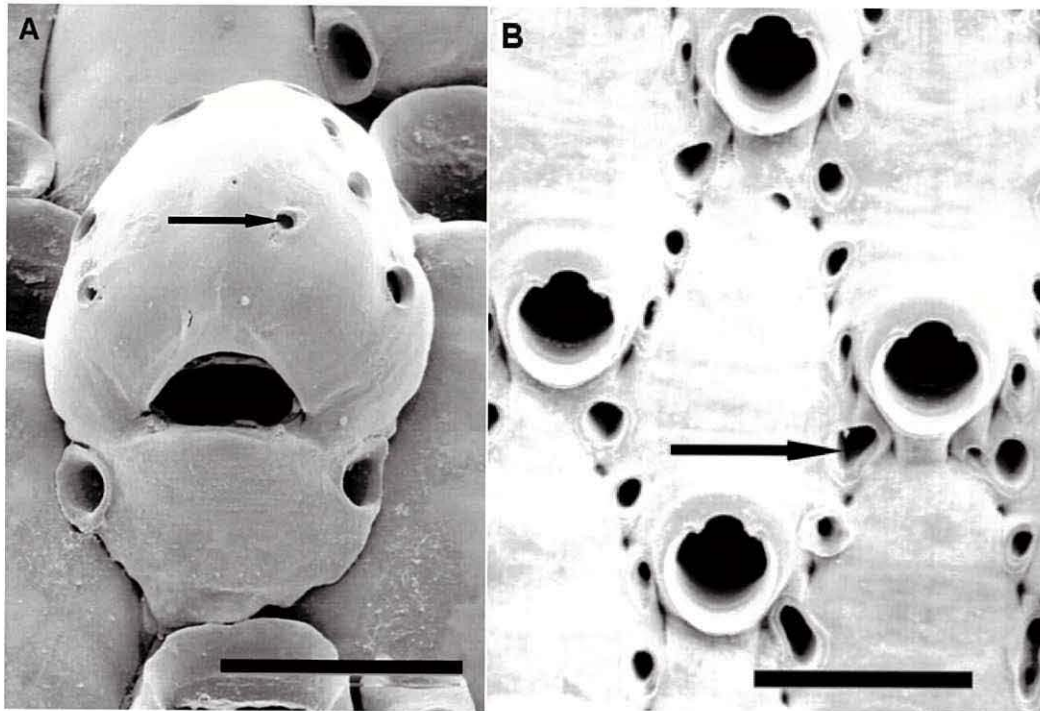


Plate 5.3. Scanning electron micrographs of a female zooid with ovicell from the Woods Hole population (A), and Californian autozooids (B). Arrows point to ovicell pore (A), and tubular pore chamber (B).

Scale bars: (A) = 100 μ m, (B) = 200 μ m.

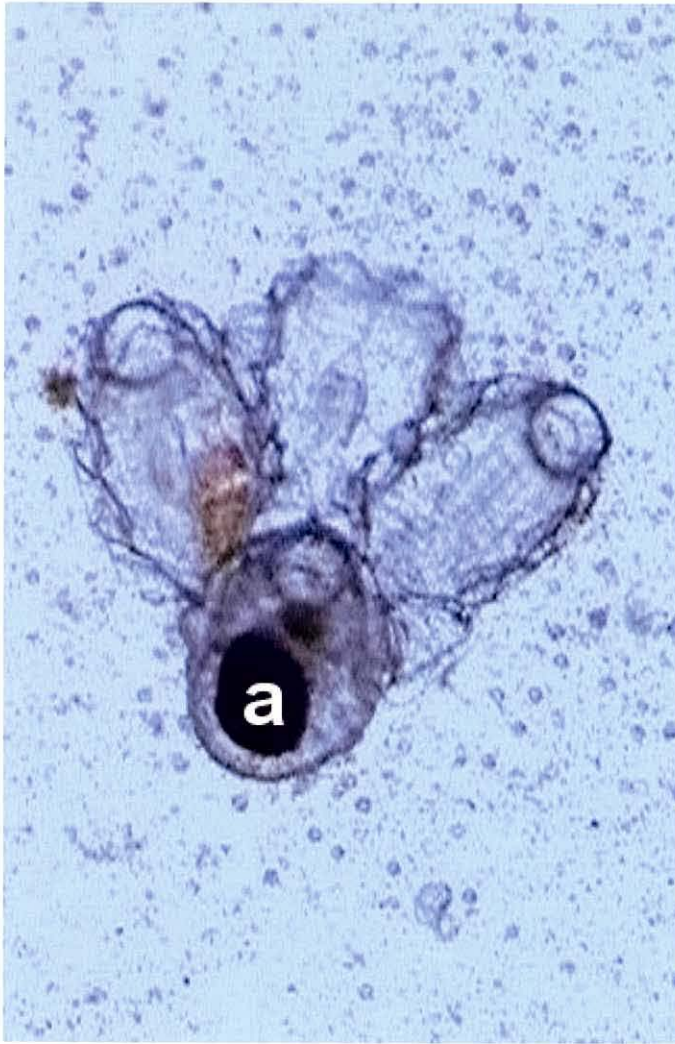


Plate 5.4. Video still of a Californian colony demonstrating the budding pattern in which two disto-lateral buds are produced simultaneously. a = ancestrula.

5.4. Discussion.

5.4.1. Early Astogeny and Gross Colony Morphology

Colonies from each of the populations studied predominantly exhibited the unilateral budding pattern and spiral early astogeny previously reported as being typical of *C. hyalina* colonies from Europe, Chile and California (Pinter, 1973; Ryland and Gordon, 1977; Hayward and Ryland, 1979; Moyano, 1986). Exceptions to this typical pattern were found in colonies from only three of the populations studied (Reykjavic, California and Woods Hole). Occasional colonies from all three of these populations were found to produce two disto-lateral buds from the ancestrula and exhibit a symmetrical early astogeny typical of other species of *Celleporella* such as *C. bathamae*, *C. delta*, *C. tongima*, *C. carolinensis*, *C. muricata*, *C. discreta*, *C. antarctica* and *C. bougainvillei* (Ryland and Gordon, 1977; Ryland, 1979; Moyano, 1986). All these eight species differ from *C. hyalina*, however, in being unilaminar throughout. Also, one colony from the Woods Hole population produced one disto-medial bud from the ancestrula. This type of budding pattern has not been reported as occurring in any other species of *Celleporella* possessing a schizoporelloid ancestrula (Cancino and Hughes, 1988). Both of these two alternative budding patterns have previously been reported as occurring occasionally among colonies of *C. hyalina* from the Menai Strait, North Wales (Cancino and Hughes, 1988). Although in both studies these two budding patterns were only infrequently observed, care must be taken when using this character for distinguishing immature *C. hyalina* from any of the eight species listed above before the frontal budding of sexual zooids has commenced.

The angle between the ancestrula and the first daughter zooid and/or the angle between the first and second daughter zooids were found to differ significantly in 52 of the 54 comparisons made between colonies from the eleven populations studied. This result is in agreement with a study on five Chilean populations in which significant differences in budding angles were found among the five localities studied (A. Navarrete pers. comm.). Colonies exhibiting larger budding angles will reach circularity sooner than colonies exhibiting smaller budding

angles, allowing them to grow outwards in all directions across the substrate sooner. Competition between encrusting organisms frequently involves the overgrowth of part or all of one of the organisms by the other (Jackson, 1979). When two cheilostome bryozoans encounter each other the contact may either occur between two growing edges or between the growing edge of one colony and the non-growing edge of the other colony (Jackson, 1979). When the growing edge of one colony meets a non-growing edge of another colony the colony which has its growing edge at the site of contact may gain an initial advantage (Jackson, 1979), possibly resulting in overgrowth of the other colony. The ability to utilise all of the two dimensional space available to them as soon as possible will enable colonies to be more competitive and more likely to survive encounters with other colonies.

Individuals from the majority of the populations studied formed colonies consisting of two layers (bilaminar), with the basal layer consisting predominantly of autozooids and the frontal layer consisting of sexual zooids as has been previously described for colonies of *C. hyalina* from Europe, Chile and North America (Ryland and Gordon, 1977; Ryland, 1979; Hayward and Ryland, 1979; Moyano, 1986; Cancino and Hughes, 1988). All colonies were also observed to produce small numbers of male zooids in the basal layer as well as in the frontal layer. This phenomenon has also been previously reported for colonies of *C. hyalina* (Ryland and Gordon, 1977; Cancino and Hughes, 1988; Hunter, 1991; Hunter and Hughes, 1993b; Goldson, 1998; Manríquez, 1999). However, the number of basal male zooids was observed to increase in the area of the colony that had been cut during the cloning procedure in colonies from all but two of the populations studied. Hunter and Hughes (1993b), Goldson (1998) and Manríquez (1999) have all reported an increase in the number of basal male zooids in colonies of *C. hyalina* during times of stress. Goldson (1998) suggested that the likelihood of perpetuation of the parental genes may be increased by the colony producing large numbers of relatively physiologically inexpensive male gametes prior to the demise of the colony. Cancino (1986) reported that the main mortality factor within epifaunal communities on the fronds of *Laminaria saccharina* is substratum abrasion leading to substratum disintegration. The process of cutting the colonies up during the cloning procedure is very similar to

what may be experienced by a colony as it nears the end of an algal frond. Therefore, the physical damage incurred by each colony during the cloning procedure may have led to the temporary switch from somatic growth to sexual growth in order to produce as many gametes as possible before the demise of the colony.

Colonies from the two populations that were collected at Ría de Ferrol in Spain (*C. hyalina* var. normal and *C. hyalina* var. reticulata) were observed to produce occasional female zooids in the basal layer of the colony in the area that had been cut during the cloning procedure. As with the colonies mentioned above, this was likely to have been as a response to the stress experienced during the cloning procedure, with the colonies temporarily investing more in sexual growth than in somatic growth. The presence of female zooids in the basal layer of colonies contradicts the findings of some previous authors who report finding female zooids only in the frontal layer of *C. hyalina* colonies (Pinter, 1973; Ryland and Gordon, 1977; Hayward and Ryland, 1979, Cancino, 1983). An earlier observation of female zooids occurring in the basal layer of Brazilian *C. hyalina* colonies (Marcus, 1938) has since been questioned, with Ryland and Gordon (1977) suggesting that Marcus “was misled by having two very similar species in his collection”, a suggestion later proved to be correct by Ryland (1979), who identified the specimen referred to by Ryland and Gordon (1977) as being the unilaminar species *C. carolinensis*. However, Hunter (1991) and Hunter and Hughes (1993b), have reported the very rare occurrence of female zooids in the basal layer of colonies from North Wales, and Wright and Hughes (2002), in a separate study using genotypes from the Woods Hole population, reported the presence of female zooids in the basal layer of these colonies.

The only colonies that formed more than two layers of zooids were the *C. angusta* colonies from Foz, the seven colonies from Porth Neigwl and three of the seven colonies from Achill Sound. Each of these colonies became nodular (multilaminar) with the majority of frontal sexual zooids forming around the edge of the colony so that they became doughnut shaped with a shallow centre. In all of these colonies the only sexual zooids found in the basal layer were male zooids, and only male and female zooids were found in the frontal layers. *C.*

angusta is described as differing from *C. hyalina* in the size of the zooids, the presence in both the autozooids and ancestrula of a pronounced sinus and the presence of a slightly pronounced peristome (Alvarez, 1991; Fernández Pulpeiro and Reverter Gil, 1992). No mention is made of the colonies being nodular (multilaminar). This would suggest that the original description of this species was made on relatively young colonies, before they had started to become nodular, or that the nodular appearance that they took on in this study was a laboratory artifact. Wild colonies of a South African species (as yet unrecognised), sent by colleagues from Cape Town, were also found to be very nodular (pers. obs.). These specimens were found to be very similar genetically to the *C. angusta* colonies used in the current study (A. Gomez pers. comm.). This would suggest that the nodular appearance of the *C. angusta* colonies observed in the current study, may be due to a genetic trait and not a laboratory artifact. The fact that the seven colonies from Porth Neigwl and the three colonies from Achill Sound shared this trait with the *C. angusta* colonies suggests that these may also be colonies of *C. angusta*. This suggestion is supported by several other observations. Canonical variate analysis on all statistically significant linear characters groups all of these populations together (see results and below). Also colonies from all of these populations share the trait of being capable of self-fertilisation (Chapter 3), and they all occupy the same phylogenetic clade based on mitochondrial DNA analysis (A. Gomez pers. comm.).

One final observation made while observing colonies for this study was the fact that the large lacunae, that have been described as being the most prominent feature with which to distinguish between *C. hyalina* var. normal and *C. hyalina* var. reticulata (Fernández Pulpeiro and Reverter Gil, 1992), become less apparent as the colony gets older. Also the younger parts of the colony, in which the large lacunae are most apparent, become obscured by frontally budded sexual zooids. Therefore, care must be taken when trying to differentiate between these two varieties when working with older specimens.

The observations made on the early astogeny and gross colony morphology have only revealed two possible useful characters for distinguishing between colonies

from each of the different populations, these being the angle between the ancestrula and the first daughter zooid and the angle between the first and second daughter zooids. The pattern of early astogeny did not vary among the different populations, and the gross colony morphology only provided another possible character for distinguishing between the already established species *C. angusta* and *C. hyalina*. However, the two angles measured (θ_1 and θ_2 – Plate 5.1 A and B) become obscured by the presence of frontally budded sexual zooids when the colony has reached sexual maturity. Moreover, a study on Welsh *C. hyalina* colonies reports that frontal budding begins in the older parts of the colony and progresses outwards as the colony grows (Cancino and Hughes, 1988). This would lead to these angles being obscured as soon as the colony started to produce sexual zooids. Therefore, the two angles measured would only be useful when working with sexually immature colonies .

5.4.2. Multivariate Analysis of Linear and Meristic Data

The results of the canonical variate analysis on all statistically significant linear and meristic characters appear to reveal the presence of at least four morphologically distinct populations within *C. hyalina* sensu lato (Woods Hole, Kapp Linné (1), California and Reykjavic) (Figs. 5.1 – 5.4). The Woods Hole and Kapp Linné (1) populations were revealed to be morphologically distinct in the analysis using just the linear characters. In this analysis, high standardised first canonical discriminant function coefficients are most associated with autozooid length and distance between condyles. High standardised second canonical discriminant function coefficients are most associated with width of sinus at 0.5 depth, distance between condyles and depth of sinus. This would suggest that both autozooid length and autozooid sinus shape will be taxonomically useful characters within *C. hyalina* sensu lato. Mean length of autozooids is a measurement frequently reported for wild *C. hyalina* populations, and considerable geographic variation in this character is apparent within the current literature (Pinter, 1973; Ryland and Gordon, 1977; Ryland, 1979; Morris, 1980; Hunter, 1991; Fernández Pulpeiro and Reverter Gil, 1992). Also, shape and size of autozooid sinus is known to vary considerably between different *Celleporella* species (Ryland and Gordon, 1977; Moyano, 1986; Fernández

Pulpeiro and Reverter Gil, 1992; César-Aldariz *et al.*, 1999). The one remaining distinct cluster (consisting of *C. angusta* (Foz), Porth Neigwl and Achill Sound (2)), further supports the discovery of the Porth Neigwl and Achill Sound (2) populations being *C. angusta*.

The Woods Hole and Californian populations were revealed to be morphologically distinct in the analysis using just the meristic characters. In this analysis, high standardised first canonical discriminant function coefficients are most associated with number of tentacles on the lophophore and number of tubular pore chambers surrounding each autozoid. High standardised second canonical discriminant function coefficients are most associated with number of tubular pore chambers surrounding each autozoid and number of pores on the ovicell. This would suggest that all three of the meristic characters will be taxonomically useful within *C. hyalina* sensu lato. After removal of these two discrete groups, further analysis revealed the Reykjavic population to be morphologically distinct also. The discovery that the number of pores on the ovicell may be a useful taxonomic character is in agreement with a recent study on colonies from five localities within Chile, that found differences in the number of pores per ovicell in wild colonies (A. Navarrete pers. comm.). Also, Fernández Pulpeiro and Reverter Gil (1992) report that *C. hyalina* var. *reticulata* has fewer pores per ovicell than *C. hyalina* var. *normal*. In the present study, however, it was not found possible to separate these two populations when using either the meristic or the linear characters. The tubular pore chambers that surround each autozoid (and give rise to frontally budded zooids) have previously been found to differ in structure, leading Gordon and Hastings (1979) to recognise three generic groupings within the family Hippothoidae. More recent observations by Goldson (1998) revealed differences in the number of tubular pore chambers surrounding the autozooids of Welsh and Chilean colonies of *C. hyalina*. Unfortunately, the colonies sent from Chile for use in the current study were not able to be included in the analysis of meristic characters due to them not being alive on their arrival, resulting in the tentacle number data not being obtainable. Finally, the number of tentacles on the polypide of the autozooids has previously been shown to vary among geographically isolated populations (Marcus, 1938; Hincks, 1880 – cited by Ryland and Gordon, 1977;

Waters, 1900 – cited by Ryland and Gordon, 1977). Also, Goldson (1998) reported variation in tentacle number was extremely limited in Welsh *C. hyalina* when restricting counts to autozooids from the periphery of the colony, as in the present study. This apparent lack of variation within a population makes tentacle number even more useful taxonomically.

The observations reported in this chapter were made on colonies that had been cultured under controlled environmental conditions. Naturally occurring populations are subjected to a range of different environmental conditions and temperatures depending on locality and time of year. Many of the characters used in this study have previously been shown to vary with environmental conditions within colonies of cheilostome bryozoans. Zooid size within colonies has been shown to vary inversely with temperature (Hunter and Hughes, 1994; O'Dea and Okamura, 1999; 2000; O'Dea and Jackson, 2002), tentacle number has been shown to vary due to environmental influences (Thorpe *et al.*, 1986) and position on the shore (Porter *et al.*, 2000), and ovicell pore number has been shown to vary with respect to oxygen concentration (Dr. S. Morley pers. comm.). A molecular and morphological analysis of the green seaweeds *Enteromorpha intestinalis* and *E. compressa* (Blomster *et al.*, 1998) reported that high levels of morphological plasticity led to these two species being very difficult to distinguish from each other morphologically. However, if environmental factors such as salinity are taken into account then the two species can be correctly identified based on morphology. Further investigations are required, using measurements taken from wild colonies, to discover whether morphological plasticity in naturally occurring populations of *C. hyalina* are likely to further hamper the correct identification of species within *C. hyalina sensu lato*.

The current study has demonstrated that some of the reproductively isolated populations identified in chapter 4 correspond to morphologically distinct populations. However, the morphological analysis has failed to separate some populations that are known to occupy separate phylogenetic clades based on mitochondrial DNA sequencing (A. Gomez pers. comm.), and be reproductively isolated from each other (Chapter 4). Therefore, it is not possible as yet to rule out the existence of morphologically cryptic species within *C. hyalina sensu lato*.

Chapter 6.

General Discussion.

6.1. Reproduction

The investigation into sperm storage in *C. hyalina* unfortunately failed to resolve the site of storage of exogenous sperm in this species. The failure of the sperm donor colonies to take up the radiotracer used in the experiment appears to be the most likely reason as to why this experiment failed to deliver any positive results. Further investigations are necessary if the site of storage of exogenous sperm is to be revealed in this species, using either radiolabelled food to ensure labelling of donor sperm or using fluorescence stained sperm in conjunction with a confocal laser scanning microscope. It is essential that the site of sperm storage in *C. hyalina* be discovered if the reproductive process in this species is to be fully understood.

Considerable variation in the ability to self-fertilise was observed among the geographically isolated populations studied in chapter 3. In total, colonies from nine of the populations studied were observed to brood embryos in isolation, three of these populations consisting of colonies of *C. angusta*. This appears to be the first report of selfing activity in *C. angusta*. Of the six populations of *C. hyalina* in which self-fertilisation was observed, four (Amlwch Harbour, Lough Hyne and Ría de Ferrol – both varieties) showed only very low levels of selfing ability. All of these four populations belong to the same phylogenetic clade based on mitochondrial DNA sequencing (A. Gomez pers. comm.), and most were found to be reproductively compatible (the only two populations between which mating trials were not carried out were Lough Hyne and Ría de Ferrol var. *reticulata*). Of the two remaining populations in which selfing was observed, some of the colonies from Nova Scotia released occasional selfed larvae whose development was impaired, while all the colonies from Woods Hole released abundant larvae with normal development. The differences observed between the populations used in this study, along with the observations of Cancino *et al* (1991a), who reported a total lack of female investment by isolated Chilean colonies, suggests that the behaviour of reproductively isolated colonies may be

a useful taxonomic character within *C. hyalina* sensu lato. Among the populations of both *C. hyalina* and *C. angusta* in which selfing was observed, no information was obtained on whether the genotypes observed to self-fertilise in isolation continued to do so when given the opportunity for outcrossing. Except for in the *C. angusta* and Woods Hole populations, larval production was seen to increase when colonies were given the opportunity for outcrossing. This indicates that colonies from these populations will preferentially outcross when the chance arises. For colonies from the *C. angusta* and Woods Hole populations, however, further investigations must be undertaken in order to discover whether or not genotypes of either species preferentially self-fertilise when given the opportunity to outcross. Further work is also necessary to discover whether the brooding of embryos and release of larvae observed in the Victoria Island population, after two and a half years without any reproductive activity being detected, was the result of outcrossing or whether this population is also capable of self-fertilisation.

The results of the mating trials carried out in chapter 4 indicate the presence of at least seven good biological species within *C. hyalina* sensu lato. This study, and a study run at the same time in Chile (A. Navarrete pers. comm.), appear to be the first to conclusively show that reproductive isolation has developed between some of the geographically isolated populations previously referred to as the single cosmopolitan species *C. hyalina* (e.g. Ryland and Gordon, 1977; Hastings, 1979; Ryland, 1979; Morris, 1980; Moyano, 1986; Fernández Pulpeiro and Reverter Gil, 1992). All of the mating trials between populations from separate phylogenetic clades (based on mitochondrial DNA sequencing) failed to produce a single embryo, except for those that could be attributed to selfing. In the within clade mating trials, for which populations from within the NE Atlantic clade were used (Appendix 3), the mating trials revealed the presence of at least two good biological species, and perhaps several incipient species. Two of the populations which appear to be incipient species, Ría de Ferrol var. normal and Ría de Ferrol var. reticulata, are sympatric in distribution and offer a good opportunity to further study the process of speciation within this group. Specifically, further work should be carried out on these two populations to investigate the phenomenon of asymmetrical sexual isolation. Further mating

trials are also necessary to investigate the reproductive compatibility of the geographically isolated populations of *C. angusta* (see below) that were discovered during the course of the current study.

6.2. Morphology

The morphological analysis, based on characters frequently referred to by previous authors when describing *C. hyalina* (e.g. Pinter, 1973; Ryland and Gordon, 1977; Ryland, 1979; Morris, 1980; Fernández Pulpeiro and Reverter Gil, 1992), appears to reveal the presence of at least four morphologically distinct populations within *C. hyalina* sensu lato. This emphasises the need for a rigorous statistical analysis of morphological characters when relying on morphology alone to discriminate between species in marine bryozoans. However, the fact that some of the biological species discovered in chapter 4 currently appear to be morphologically cryptic, also emphasises the fact that morphological studies alone may not be sufficient to recognise species, at least not in *C. hyalina* sensu lato. Although the identification of cryptic species may pose something of a problem, it is an essential task that must be carried out carefully. In her influential review, Knowlton (1993) states that “sibling species are rife in marine environments, and that failure to recognise them cripples evolutionary and ecological understanding of the sea”. Greater understanding of cryptic speciation and the effects of human transportation is necessary for both basic and applied research (Knowlton, 1993). For example, the marine pollution indicator *Capitella capitata*, once considered to be a cosmopolitan species, was actually found to be a complex of up to six cryptic species by Grassle and Grassle (1976) with very distinctive differences in life history. They stated that “the use of *Capitella capitata* as a pollution indicator will have to be modified – the large differences in life history suggest that each species may provide even more sensitive indications of previous patterns of disturbance”. Also, Knowlton *et al.*, (1992) discovered three cryptic species of *Montastrea annularis* on Panamanian and Venezuelan reefs. Two of these cryptic species were found to have highly significant differences in growth rate and oxygen isotopic ratios, both of which are parameters routinely used to estimate past climatic conditions. Knowlton *et al.* (1992) also suggest the unusual colouration of the third cryptic species may have confused research on coral bleaching. The impact of

nonindigenous species on environments around the world can have profound effects on the balance of predators and prey, and their competitive abilities within their environments, with the possibility of indigenous species suffering greatly as a result of these invasions. If cryptic species are not correctly identified then many of these invasions may go unnoticed, causing the number and impact of invasions to be badly underestimated (Geller *et al.*, 1997). Cryptic species will also present problems to population managers, and conservation measures developed for plants and animals whose continued existence in the wild is under threat will be jeopardised by a failure to recognise them (Bickham, 1983). Finally, the grouping together of morphologically very similar, or morphologically identical, reproductively isolated populations as a single species will lead to the serious under-estimation of global biodiversity (Heywood and Watson, 1995). Therefore, the use of a concordant suite of unrelated morphological, reproductive and molecular genetic characters may be necessary for the discrimination of species in bryozoan groups and in other comparable colonial invertebrates with similar biology and life histories.

6.3. *Celleporella angusta*

The results of chapters 3 and 5 suggest that the seven genotypes established from wild colonies collected from Porth Neigwl, and three of the seven genotypes established from wild colonies collected from Achill Sound, are specimens of *C. angusta*. Unfortunately, the colonies collected from Porth Neigwl were encrusting fronds of *Laminaria saccharina* that had become detached from the seabed and were floating freely in the water column. Rafting in this way is thought to be a common form of long distance dispersal for clonal sessile invertebrates (Jackson, 1986), and therefore, it is not possible to state where the population that these colonies originated from is situated. Further sampling in the Porth Neigwl area is required in order to resolve whether or not this species occurs as a permanent resident on the Welsh coastline. The founder colonies for the Achill Sound population, however, were all found growing on algae attached to the seabed. This population represents a new distribution for *C. angusta*, a species previously only reported as occurring on the Galician and Cantabrian coasts (Alvarez, 1991; Fernández Pulpeiro and Reverter Gil, 1992) and more recently in the north of Brittany and Arcachon (Reverter *et al.*, 1995). The results

in chapter 5 also suggest that this species may take on a nodular appearance as the colony ages, a feature not previously reported.

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Appendix 1.

Location of populations studied and substrate that colonies were growing on.

Population (<i>Celleporella hyalina</i>)	Coordinates	Substrate
North Wales		
Church Island, Menai Bridge. (Menai Strait).	53 13 27N 04 09 50W	<i>Laminaria digitata</i>
Amlwch Harbour.	53 25 11N 04 20 18W	<i>L. saccharina</i>
Holyhead Harbour.	53 18 44N 04 38 48W	<i>L. digitata</i>
Ireland		
Lough Hyne Rapids.	51 32N 09 15W	<i>L. saccharina</i>
Achill Sound, Corraun, County Mayo.	53 52N 09 56W	<i>Condrus crispus</i>
Dublin Harbour.	53 21N 06 12W	<i>Fucus serratus</i>
Scotland		
Port nan Cuilc, Gallanach Beg, Oban.	56 23 34N 05 30 54W	<i>L. digitata</i>
England		
Mayflower Marina, Plymouth.	50 15N 04 10W	<i>L. digitata</i>
Spain		
Ría de Ferrol, San Felipe (La Coruña province). (<i>C. hyalina</i> var. normal and var. reticulata).	43 28 06N 08 16 09W	<i>Laminaria</i> sp.
Iceland		
Alfsnes, Reykjavík.	64 09N 21 58W	<i>Laminaria</i> sp.
Norway		
Tromsø.	69 42N 19 00W	<i>L. saccharina</i>
Sweden		
Tjärnö.	58 52 54N 11 08 78W	<i>L. saccharina</i>
Russia		
Kandalaksha Guba, White Sea.	66 20N 33 39W	<i>L. saccharina</i>

Appendix 1. (cont.)

Svalbard		
Kapp Linné.	78 04N 13 14W	<i>F. distichus</i>
America		
Damariscotta Estuary, Maine.	43 56N 69 40W	<i>Laminaria sp.</i>
Nobska Point, Woods Hole, Massachusetts.	41 32N 70 40W	<i>C. crispus</i>
Pacific Grove, California.	36 35N 121 55W	<i>Rhodomenia sp.</i>
Floating Dock, Kachemak Bay, Cook Inlet, Alaska.	59 27N 151 43W	<i>Sparlingia sp.</i>
Canada		
Northwest Arm, Halifax, Nova Scotia.	44 40N 63 35W	<i>L. longicornis</i>
Victoria Island, Bamfield, British Columbia.	48 50N 125 10W	<i>Egregia sp.</i>
Chile		
El Tabo, Las Cruces, Lo Castilla.	33 29S 71 38W	Algae
Population (<i>Celleporella angusta</i>)		
Coordinates		
Substrate		
Spain		
Foz, Playa de peizás (Lugo province).	43 35 16N 07 16 10W	<i>Laminaria sp.</i>
North Wales		
Porth Neigwl (Hells Mouth), Lleyrn Peninsula.	52 48N 04 35W	<i>L. saccharina</i>
Ireland		
Achill Sound, Corraun, County Mayo.	53 52N 09 56W	<i>L. saccharina</i>

Appendix 1. (cont.)

Population (<i>Celleporella carolinensis</i>)	Coordinates	Substrate
America		
Nobska Point, Woods Hole, Massachusetts.	41 32N 70 40W	<i>Sargassum filipendula</i>

Appendix 2.

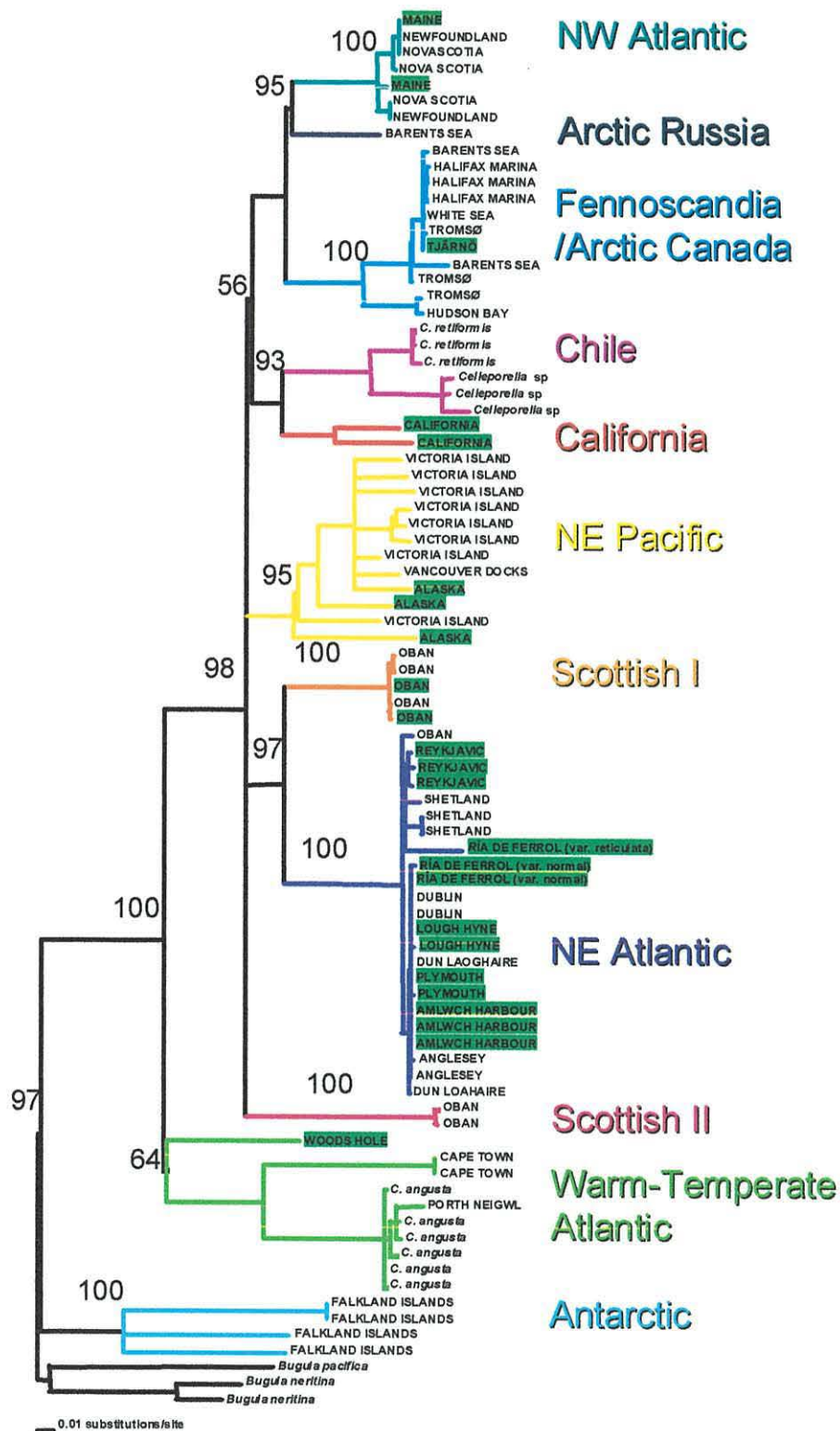
Cloning procedure.

Once each colony had reached approximately 2-3 cm in diameter they were subjected to a cloning procedure whereby they were cut into 5-10 smaller fragments. The acetate of each fragment was trimmed so that there was a margin of about 2mm beyond the growing edge of the colony to facilitate handling. Further trimming ensured that the majority of zooids present were healthy, with a functional polypide capable of feeding. Each small piece of acetate bearing a colony fragment was then carefully blotted dry using tissue paper and glued with cyanoacrylate onto a new piece of acetate of 38x75mm. This procedure was repeated whenever the new ramets reached the diameter shown above thus ensuring a constant supply of ramets for use in the various experiments.

Appendix 3.

Phylogeny produced by Dr. A. Gomez based on mitochondrial DNA sequencing.

Highlighted populations were used in mating trials.



Appendix 4.

Table A.1. Between-clades mating. Outcome of the mating between genotypes from the Maine and Californian populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
MAN ₁ * cal ₁	No	-	-	-	-
MAN ₂ * cal ₂	No	-	-	-	-
MAN ₃ * cal ₃	No	-	-	-	-
MAN ₄ * cal ₄	No	-	-	-	-
CAL ₁ * man ₁	No	-	-	-	-
CAL ₂ * man ₂	No	-	-	-	-
CAL ₃ * man ₃	No	-	-	-	-
CAL ₄ * man ₄	No	-	-	-	-

Table A.2. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Maine and Californian populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁ * man ₂	Yes	54
MAN ₂ * man ₁	Yes	>100
MAN ₃ * man ₄	Yes	>100
MAN ₄ * man ₃	Yes	>100
CAL ₁ * cal ₄	No	-
CAL ₄ * cal ₁	Yes	>100
CAL ₂ * cal ₃	Yes	>100
CAL ₃ * cal ₂	Yes	>100

Table A.3. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Maine and Californian populations.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁	No	-
MAN ₂	No	-
MAN ₃	No	-
MAN ₄	No	-
CAL ₁	No	-
CAL ₂	No	-
CAL ₃	No	-
CAL ₄	No	-

Table A.4. Between-clades mating. Outcome of the mating between genotypes from the Maine and Alaskan populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
MAN ₁ * ala ₁	No	-	-	-	-
MAN ₂ * ala ₂	No	-	-	-	-
MAN ₃ * ala ₃	No	-	-	-	-
MAN ₄ * ala ₄	No	-	-	-	-
ALA ₁ * man ₁	No	-	-	-	-
ALA ₂ * man ₂	No	-	-	-	-
ALA ₃ * man ₃	No	-	-	-	-
ALA ₄ * man ₄	No	-	-	-	-

Table A.5. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Maine and Alaskan populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁ * man ₂	Yes	50
MAN ₂ * man ₁	Yes	>100
MAN ₃ * man ₄	Yes	>100
MAN ₄ * man ₃	Yes	>100
ALA ₁ * ala ₄	Yes	>100
ALA ₄ * ala ₁	Yes	>100
ALA ₂ * ala ₃	Yes	>100
ALA ₃ * ala ₂	Yes	>100

Table A.6. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Maine and Alaskan populations.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁	No	-
MAN ₂	No	-
MAN ₃	No	-
MAN ₄	No	-
ALA ₁	No	-
ALA ₂	No	-
ALA ₃	No	-
ALA ₄	No	-

Table A.7. Between-clades mating. Outcome of the mating between genotypes from the Maine and Oban populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
MAN ₃ * obn ₁	No	-	-	-	-
MAN ₄ * obn ₄	No	-	-	-	-
OBN ₁ * man ₃	No	-	-	-	-
OBN ₄ * man ₄	No	-	-	-	-

Table A.8. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Maine and Oban populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
MAN ₃ * man ₄	Yes	>100
MAN ₄ * man ₃	Yes	>100
OBN ₁ * obn ₄	Yes	>100
OBN ₄ * obn ₁	Yes	>100

Table A.9. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Maine and Oban populations.

Genotype	Were embryos produced?	No. of larvae released
MAN ₃	No	-
MAN ₄	No	-
OBN ₁	No	-
OBN ₄	No	-

Table A.10. Between-clades mating. Outcome of the mating between genotypes from the Maine and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
MAN ₁ * ice ₁	No	-	-	-	-
MAN ₂ * ice ₂	No	-	-	-	-
MAN ₃ * ice ₃	No	-	-	-	-
MAN ₄ * ice ₄	No	-	-	-	-
ICE ₁ * man ₁	No	-	-	-	-
ICE ₂ * man ₂	No	-	-	-	-
ICE ₃ * man ₃	No	-	-	-	-
ICE ₄ * man ₄	No	-	-	-	-

Table A.11. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Maine and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁ * man ₂	Yes	50
MAN ₂ * man ₁	Yes	>100
MAN ₃ * man ₄	Yes	>100
MAN ₄ * man ₃	Yes	>100
ICE ₁ * ice ₂	No	-
ICE ₂ * ice ₁	No	-
ICE ₃ * ice ₄	Yes	>100
ICE ₄ * ice ₃	Yes	>100

Table A.12. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Maine and Icelandic populations.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁	No	-
MAN ₂	No	-
MAN ₃	No	-
MAN ₄	No	-
ICE ₁	No	-
ICE ₂	No	-
ICE ₃	No	-
ICE ₄	No	-

Table A.13. Between-clades mating. Outcome of the mating between genotypes from the Maine and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
MAN ₁ * aml ₁	No	-	-	-	-
MAN ₂ * aml ₂	No	-	-	-	-
MAN ₃ * aml ₃	No	-	-	-	-
MAN ₄ * aml ₄	No	-	-	-	-
AML ₁ * man ₁	No	-	-	-	-
AML ₂ * man ₂	No	-	-	-	-
AML ₃ * man ₃	No	-	-	-	-
AML ₄ * man ₄	Yes	0	-	-	-

Table A.14. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Maine and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁ * man ₂	No	-
MAN ₂ * man ₁	Yes	>100
MAN ₃ * man ₄	No	-
MAN ₄ * man ₃	Yes	>100
AML ₁ * aml ₂	Yes	>100
AML ₂ * aml ₁	Yes	>100
AML ₃ * aml ₄	Yes	>100
AML ₄ * aml ₃	Yes	>100

Table A.15. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Maine and Amlwch Harbour populations.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁	No	-
MAN ₂	No	-
MAN ₃	No	-
MAN ₄	No	-
AML ₁	No	-
AML ₂	No	-
AML ₃	No	-
AML ₄	Yes	0

Table A.16. Between-clades mating. Outcome of the mating between genotypes from the Maine and Woods Hole populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
MAN ₁ * whe ₂	No	-	-	-	-
MAN ₄ * whe ₃	No	-	-	-	-
WHE ₂ * man ₁	Yes	>100	100	10	-
WHE ₃ * man ₄	Yes	>100	100	10	-

Table A.17. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Maine and Woods Hole populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁ * man ₄	Yes	>100
MAN ₄ * man ₁	Yes	>100
WHE ₂ * whe ₃	Yes	>100
WHE ₃ * whe ₂	Yes	>100

Table A.18. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Maine and Woods Hole populations.

Genotype	Were embryos produced?	No. of larvae released
MAN ₁	No	-
MAN ₄	No	-
WHE ₂	Yes	>100
WHE ₃	Yes	>100

Table A.19. Between-clades mating. Outcome of the mating between genotypes from the Californian and Alaskan populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
CAL ₁ * ala ₁	No	-	-	-	-
CAL ₂ * ala ₂	No	-	-	-	-
CAL ₃ * ala ₃	No	-	-	-	-
CAL ₄ * ala ₄	No	-	-	-	-
ALA ₁ * cal ₁	No	-	-	-	-
ALA ₂ * cal ₂	No	-	-	-	-
ALA ₃ * cal ₃	No	-	-	-	-
ALA ₄ * cal ₄	No	-	-	-	-

Table A.20. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Californian and Alaskan populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
CAL ₁ * cal ₂	Yes	>100
CAL ₂ * cal ₁	Yes	>100
CAL ₃ * cal ₄	Yes	58
CAL ₄ * cal ₃	Yes	43
ALA ₁ * ala ₄	Yes	68
ALA ₄ * ala ₁	Yes	63
ALA ₂ * ala ₃	Yes	5
ALA ₃ * ala ₂	Yes	23

Table A.21. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Californian and Alaskan populations.

Genotype	Were embryos produced?	No. of larvae released
CAL ₁	No	-
CAL ₂	No	-
CAL ₃	No	-
CAL ₄	No	-
ALA ₁	No	-
ALA ₂	No	-
ALA ₃	No	-
ALA ₄	No	-

Table A.22. Between-clades mating. Outcome of the mating between genotypes from the Californian and Oban populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
CAL ₂ * obn ₁	No	-	-	-	-
CAL ₃ * obn ₄	No	-	-	-	-
OBN ₁ * cal ₂	No	-	-	-	-
OBN ₄ * cal ₃	No	-	-	-	-

Table A.23. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Californian and Oban populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
CAL ₂ * cal ₃	Yes	>100
CAL ₃ * cal ₂	Yes	>100
OBN ₁ * obn ₄	Yes	>100
OBN ₄ * obn ₁	Yes	>100

Table A.24. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Californian and Oban populations.

Genotype	Were embryos produced?	No. of larvae released
CAL ₂	No	-
CAL ₃	No	-
OBN ₁	No	-
OBN ₄	No	-

Table A.25. Between-clades mating. Outcome of the mating between genotypes from the Californian and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
CAL ₄ * ice ₁	No	-	-	-	-
CAL ₂ * ice ₂	No	-	-	-	-
CAL ₁ * ice ₃	No	-	-	-	-
CAL ₃ * ice ₄	No	-	-	-	-
ICE ₁ * cal ₄	No	-	-	-	-
ICE ₂ * cal ₂	No	-	-	-	-
ICE ₃ * cal ₁	No	-	-	-	-
ICE ₄ * cal ₃	No	-	-	-	-

Table A.26. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Californian and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
CAL ₁ * cal ₄	No	-
CAL ₄ * cal ₁	Yes	>100
CAL ₂ * cal ₃	Yes	>100
CAL ₃ * cal ₂	Yes	>100
ICE ₁ * ice ₄	Yes	20
ICE ₄ * ice ₁	Yes	>100
ICE ₂ * ice ₃	Yes	>100
ICE ₃ * ice ₂	Yes	>100

Table A.27. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Californian and Icelandic populations.

Genotype	Were embryos produced?	No. of larvae released
CAL ₁	No	-
CAL ₂	No	-
CAL ₃	No	-
CAL ₄	No	-
ICE ₁	No	-
ICE ₂	No	-
ICE ₃	No	-
ICE ₄	No	-

Table A.28. Between-clades mating. Outcome of the mating between genotypes from the Californian and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
CAL ₁ * aml ₁	No	-	-	-	-
CAL ₂ * aml ₂	No	-	-	-	-
CAL ₃ * aml ₃	No	-	-	-	-
CAL ₄ * aml ₄	No	-	-	-	-
AML ₁ * cal ₁	No	-	-	-	-
AML ₂ * cal ₂	No	-	-	-	-
AML ₃ * cal ₃	No	-	-	-	-
AML ₄ * cal ₄	Yes	0	-	-	-

Table A.29. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Californian and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
CAL ₁ * cal ₄	No	-
CAL ₄ * cal ₁	Yes	>100
CAL ₂ * cal ₃	Yes	>100
CAL ₃ * cal ₂	Yes	>100
AML ₁ * aml ₂	Yes	>100
AML ₂ * aml ₁	Yes	>100
AML ₃ * aml ₄	Yes	>100
AML ₄ * aml ₃	Yes	>100

Table A.30. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Californian and Amlwch Harbour populations.

Genotype	Were embryos produced?	No. of larvae released
CAL ₁	No	-
CAL ₂	No	-
CAL ₃	No	-
CAL ₄	No	-
AML ₁	No	-
AML ₂	No	-
AML ₃	No	-
AML ₄	Yes	0

Table A.31. Between-clades mating. Outcome of the mating between genotypes from the Californian and Woods Hole populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
CAL ₁ * whe ₃	No	-	-	-	-
CAL ₃ * whe ₂	No	-	-	-	-
WHE ₂ * cal ₃	Yes	>100	100	10	-
WHE ₃ * cal ₁	Yes	>100	100	10	-

Table A.32. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Californian and Woods Hole populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
CAL ₁ * cal ₃	Yes	22
CAL ₃ * cal ₁	Yes	76
WHE ₂ * whe ₃	Yes	>100
WHE ₃ * whe ₂	Yes	>100

Table A.33. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Californian and Woods Hole populations.

Genotype	Were embryos produced?	No. of larvae released
CAL ₁	No	-
CAL ₃	No	-
WHE ₂	Yes	>100
WHE ₃	Yes	>100

Table A.34. Between-clades mating. Outcome of the mating between genotypes from the Alaskan and Oban populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
ALA ₂ * obn ₁	No	-	-	-	-
ALA ₃ * obn ₄	No	-	-	-	-
OBN ₁ * ala ₂	No	-	-	-	-
OBN ₄ * ala ₃	No	-	-	-	-

Table A.35. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Alaskan and Oban populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
ALA ₂ * ala ₃	Yes	>100
ALA ₃ * ala ₂	Yes	>100
OBN ₁ * obn ₄	Yes	54
OBN ₄ * obn ₁	Yes	>100

Table A.36. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Alaskan and Oban populations.

Genotype	Were embryos produced?	No. of larvae released
ALA ₂	No	-
ALA ₃	No	-
OBN ₁	No	-
OBN ₄	No	-

Table A.37. Between-clades mating. Outcome of the mating between genotypes from the Alaskan and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
ALA ₁ * ice ₁	No	-	-	-	-
ALA ₂ * ice ₂	No	-	-	-	-
ALA ₃ * ice ₃	No	-	-	-	-
ALA ₄ * ice ₄	No	-	-	-	-
ICE ₁ * ala ₁	No	-	-	-	-
ICE ₂ * ala ₂	No	-	-	-	-
ICE ₃ * ala ₃	No	-	-	-	-
ICE ₄ * ala ₄	No	-	-	-	-

Table A.38. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Alaskan and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
ALA ₁ * ala ₄	Yes	>100
ALA ₄ * ala ₁	Yes	>100
ALA ₂ * ala ₃	Yes	>100
ALA ₃ * ala ₂	Yes	>100
ICE ₁ * ice ₄	Yes	>100
ICE ₄ * ice ₁	Yes	>100
ICE ₂ * ice ₃	Yes	>100
ICE ₃ * ice ₂	Yes	>100

Table A.39. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Alaskan and Icelandic populations.

Genotype	Were embryos produced?	No. of larvae released
ALA ₁	No	-
ALA ₂	No	-
ALA ₃	No	-
ALA ₄	No	-
ICE ₁	No	-
ICE ₂	No	-
ICE ₃	No	-
ICE ₄	No	-

Table A.40. Between-clades mating. Outcome of the mating between genotypes from the Alaskan and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
ALA ₁ * aml ₁	No	-	-	-	-
ALA ₂ * aml ₂	No	-	-	-	-
ALA ₃ * aml ₃	No	-	-	-	-
ALA ₄ * aml ₄	No	-	-	-	-
AML ₁ * ala ₁	No	-	-	-	-
AML ₂ * ala ₂	No	-	-	-	-
AML ₃ * ala ₃	No	-	-	-	-
AML ₄ * ala ₄	Yes	0	-	-	-

Table A.41. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Alaskan and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
ALA ₁ * ala ₄	Yes	>100
ALA ₄ * ala ₁	Yes	>100
ALA ₂ * ala ₃	Yes	>100
ALA ₃ * ala ₂	Yes	>100
AML ₁ * aml ₂	Yes	>100
AML ₂ * aml ₁	Yes	>100
AML ₃ * aml ₄	Yes	>100
AML ₄ * aml ₃	Yes	>100

Table A.42. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Alaskan and Amlwch Harbour populations.

Genotype	Were embryos produced?	No. of larvae released
ALA ₁	No	-
ALA ₂	No	-
ALA ₃	No	-
ALA ₄	No	-
AML ₁	No	-
AML ₂	No	-
AML ₃	No	-
AML ₄	Yes	0

Table A.43. Between-clades mating. Outcome of the mating between genotypes from the Oban and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
OBN ₁ * ice ₁	No	-	-	-	-
OBN ₄ * ice ₄	No	-	-	-	-
ICE ₁ * obn ₁	No	-	-	-	-
ICE ₄ * obn ₄	No	-	-	-	-

Table A.44. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Oban and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
OBN ₁ * obn ₄	Yes	>100
OBN ₄ * obn ₁	Yes	>100
ICE ₁ * ice ₄	Yes	>100
ICE ₄ * ice ₁	Yes	>100

Table A.45. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Oban and Icelandic populations.

Genotype	Were embryos produced?	No. of larvae released
OBN ₁	No	-
OBN ₄	No	-
ICE ₁	No	-
ICE ₄	No	-

Table A.46. Between-clades mating. Outcome of the mating between genotypes from the Oban and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
OBN ₁ * aml ₁	No	-	-	-	-
OBN ₄ * aml ₂	No	-	-	-	-
AML ₁ * obn ₁	No	-	-	-	-
AML ₂ * obn ₄	No	-	-	-	-

Table A.47. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Oban and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
OBN ₁ * obn ₄	Yes	>100
OBN ₄ * obn ₁	Yes	>100
AML ₁ * aml ₂	Yes	>100
AML ₂ * aml ₁	Yes	>100

Table A.48. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Oban and Amlwch Harbour populations.

Genotype	Were embryos produced?	No. of larvae released
OBN ₁	No	-
OBN ₄	No	-
AML ₁	No	-
AML ₂	No	-

Table A.49. Between-clades mating. Outcome of the mating between genotypes from the Woods Hole and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
WHE ₂ * ice ₃	Yes	>100	100	10	-
WHE ₃ * ice ₄	Yes	>100	100	10	-
ICE ₃ * whe ₂	No	-	-	-	-
ICE ₄ * whe ₃	No	-	-	-	-

Table A.50. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Woods Hole and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
WHE ₂ * whe ₃	Yes	>100
WHE ₃ * whe ₂	Yes	>100
ICE ₃ * ice ₄	Yes	50
ICE ₄ * ice ₃	Yes	50

Table A.51. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Woods Hole and Icelandic populations.

Genotype	Were embryos produced?	No. of larvae released
WHE ₂	Yes	>100
WHE ₃	Yes	>100
ICE ₃	No	-
ICE ₄	No	-

Table A.52. Between-clades mating. Outcome of the mating between genotypes from the Woods Hole and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
WHE ₂ * aml ₂	Yes	>100	100	10	-
WHE ₃ * aml ₃	Yes	>100	100	10	-
AML ₂ * whe ₂	No	-	-	-	-
AML ₃ * whe ₃	No	-	-	-	-

Table A.53. Between-clades mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Woods Hole and Amlwch Harbour populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
WHE ₂ * whe ₃	Yes	>100
WHE ₃ * whe ₂	Yes	>100
AML ₂ * aml ₃	Yes	>100
AML ₃ * aml ₂	Yes	>100

Table A.54. Between-clades mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Woods Hole and Amlwch Harbour populations.

Genotype	Were embryos produced?	No. of larvae released
WHE ₂	Yes	>100
WHE ₃	Yes	>100
AML ₂	No	-
AML ₃	No	-

Table A.55. Within-clade mating. Outcome of the mating between genotypes from the Amlwch Harbour and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
AML ₁ * ice ₁	No	-	-	-	-
AML ₂ * ice ₂	No	-	-	-	-
AML ₃ * ice ₃	No	-	-	-	-
AML ₄ * ice ₄	Yes	0	-	-	-
ICE ₁ * aml ₁	No	-	-	-	-
ICE ₂ * aml ₂	No	-	-	-	-
ICE ₃ * aml ₃	No	-	-	-	-
ICE ₄ * aml ₄	No	-	-	-	-

Table A.56. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Amlwch Harbour and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
AML ₁ * aml ₂	Yes	>100
AML ₂ * aml ₁	Yes	>100
AML ₃ * aml ₄	Yes	>100
AML ₄ * aml ₃	Yes	>100
ICE ₁ * ice ₄	No	-
ICE ₄ * ice ₁	Yes	28
ICE ₂ * ice ₃	No	-
ICE ₃ * ice ₂	Yes	56

Table A.57. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Amlwch Harbour and Icelandic populations.

Genotype	Were embryos produced?	No. of larvae released
AML ₁	No	-
AML ₂	No	-
AML ₃	No	-
AML ₄	Yes	0
ICE ₁	No	-
ICE ₂	No	-
ICE ₃	No	-
ICE ₄	No	-

Table A.58. Within-clade mating. Outcome of the mating between genotypes from the Lough Hyne and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
IRL ₁ * ice ₁	No	-	-	-	-
IRL ₂ * ice ₂	Yes	0	-	-	-
IRL ₃ * ice ₃	No	-	-	-	-
IRL ₄ * ice ₄	No	-	-	-	-
ICE ₁ * irl ₁	No	-	-	-	-
ICE ₂ * irl ₂	No	-	-	-	-
ICE ₃ * irl ₃	No	-	-	-	-
ICE ₄ * irl ₄	No	-	-	-	-

Table A.59. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Lough Hyne and Icelandic populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
IRL ₁ * irl ₂	Yes	>100
IRL ₂ * irl ₁	Yes	>100
IRL ₃ * irl ₄	Yes	>100
IRL ₄ * irl ₃	Yes	>100
ICE ₁ * ice ₄	No	-
ICE ₄ * ice ₁	Yes	28
ICE ₂ * ice ₃	No	-
ICE ₃ * ice ₂	Yes	>100

Table A.60. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Lough Hyne and Icelandic populations.

Genotype	Were embryos produced?	No. of larvae released
IRL ₁	No	-
IRL ₂	Yes	0
IRL ₃	No	-
IRL ₄	No	-
ICE ₁	No	-
ICE ₂	No	-
ICE ₃	No	-
ICE ₄	No	-

Table A.61. Within-clade mating. Outcome of the mating between genotypes from the Icelandic and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
ICE ₁ * spn ₁	No	-	-	-	-
ICE ₂ * spn ₂	No	-	-	-	-
ICE ₃ * spn ₃	No	-	-	-	-
ICE ₄ * spn ₄	No	-	-	-	-
SPN ₁ * ice ₁	Yes	0	-	-	-
SPN ₂ * ice ₂	Yes	0	-	-	-
SPN ₃ * ice ₃	No	-	-	-	-
SPN ₄ * ice ₄	No	-	-	-	-

Table A.62. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Icelandic and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
ICE ₁ * ice ₄	Yes	>100
ICE ₄ * ice ₁	Yes	>100
ICE ₂ * ice ₃	Yes	57
ICE ₃ * ice ₂	Yes	>100
SPN ₁ * spn ₂	Yes	>100
SPN ₂ * spn ₁	Yes	>100
SPN ₃ * spn ₄	Yes	>100
SPN ₄ * spn ₃	Yes	>100

Table A.63. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Icelandic and Ría de Ferrol (var. normal) populations.

Genotype	Were embryos produced?	No. of larvae released
ICE ₁	No	-
ICE ₂	No	-
ICE ₃	No	-
ICE ₄	No	-
SPN ₁	Yes	0
SPN ₂	Yes	0
SPN ₃	Yes	0
SPN ₄	No	-

Table A.64. Within-clade mating. Outcome of the mating between genotypes from the Icelandic and Ría de Ferrol (var. *reticulata*) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
ICE ₁ * ret ₁	No	-	-	-	-
ICE ₂ * ret ₂	No	-	-	-	-
ICE ₄ * ret ₄	No	-	-	-	-
RET ₁ * ice ₁	No	-	-	-	-
RET ₂ * ice ₂	Yes	0	-	-	-
RET ₄ * ice ₄	Yes	0	-	-	-

Table A.65. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Icelandic and Ría de Ferrol (var. *reticulata*) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
ICE ₁ * ice ₄	Yes	>100
ICE ₄ * ice ₁	Yes	>100
ICE ₂ * ice ₃	Yes	57
ICE ₃ * ice ₂	Yes	>100
RET ₁ * ret ₄	Yes	>100
RET ₄ * ret ₁	Yes	>100
RET ₂ * ret ₄	Yes	>100
RET ₄ * ret ₂	Yes	>100

Table A.66. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Icelandic and Ría de Ferrol (var. *reticulata*) populations.

Genotype	Were embryos produced?	No. of larvae released
ICE ₁	No	-
ICE ₂	No	-
ICE ₃	No	-
ICE ₄	No	-
RET ₁	No	-
RET ₂	Yes	0
RET ₄	Yes	0

Table A.67. Within-clade mating. Outcome of the mating between genotypes from the Amlwch Harbour and Lough Hyne populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
AML ₁ * irl ₁	Yes	>100	100	10	10
AML ₂ * irl ₂	Yes	>100	100	10	9
AML ₃ * irl ₃	Yes	51	100	10	10
AML ₄ * irl ₄	Yes	20	85	10	?
IRL ₁ * aml ₁	Yes	>100	100	10	8
IRL ₂ * aml ₂	Yes	>100	100	10	9
IRL ₃ * aml ₃	Yes	>100	100	10	9
IRL ₄ * aml ₄	Yes	>100	100	10	6

Table A.68. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Amlwch Harbour and Lough Hyne populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
AML ₁ * aml ₂	Yes	>100
AML ₂ * aml ₁	Yes	>100
AML ₃ * aml ₄	Yes	>100
AML ₄ * aml ₃	Yes	>100
IRL ₁ * irl ₂	Yes	>100
IRL ₂ * irl ₁	Yes	>100
IRL ₃ * irl ₄	Yes	>100
IRL ₄ * irl ₃	Yes	>100

Table A.69. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Amlwch Harbour and Lough Hyne populations.

Genotype	Were embryos produced?	No. of larvae released
AML ₁	No	-
AML ₂	No	-
AML ₃	No	-
AML ₄	Yes	0
IRL ₁	No	-
IRL ₂	Yes	0
IRL ₃	No	-
IRL ₄	No	-

Table A.70. Within-clade mating. Outcome of the backcross matings to the maternal population using the F1^s produced in the matings between the Amlwch Harbour and Lough Hyne populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – AML₁IRL₁ would have been brooded by the genotype AML₁ and fathered by the genotype IRL₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
AML ₁ IRL ₁ * aml ₄	Yes	>100	100
AML ₂ IRL ₂ * aml ₃	Yes	>100	100
AML ₃ IRL ₃ * aml ₂	Yes	>100	100
AML ₄ IRL ₄ * aml ₁	No	-	-
AML ₄ * aml ₁ irl ₁	Yes	80	85
AML ₃ * aml ₂ irl ₂	Yes	>100	100
AML ₂ * aml ₃ irl ₃	No	-	-
AML ₁ * aml ₄ irl ₄	Yes	>100	100
IRL ₁ AML ₁ * irl ₄	Yes	>100	100
IRL ₂ AML ₂ * irl ₃	Yes	>100	100
IRL ₃ AML ₃ * irl ₂	Yes	>100	100
IRL ₄ AML ₄ * irl ₁	Yes	>100	100
IRL ₄ * irl ₁ aml ₁	Yes	50	100
IRL ₃ * irl ₂ aml ₂	Yes	>100	100
IRL ₂ * irl ₃ aml ₃	Yes	>100	100
IRL ₁ * irl ₄ aml ₄	Yes	>100	100

All but 1 of the F1 genotypes shown in the above table (and in table A.71) are known to be the product of outcrossing between Amlwch Harbour and Lough Hyne genotypes (the paternity analysis on AML₄IRL₄ was inconclusive) (A. Gomez pers. comms.).

Table A.71. Within-clade mating. Outcome of the crosses between the F1^s produced in the matings between the Amlwch Harbour and Lough Hyne populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – AML₁IRL₁ would have been brooded by the genotype AML₁ and fathered by the genotype IRL₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
AML ₁ IRL ₁ * irl ₄ aml ₄	Yes	>100	100
AML ₂ IRL ₂ * irl ₃ aml ₃	Yes	>100	100
AML ₃ IRL ₃ * irl ₂ aml ₂	Yes	>100	100
AML ₄ IRL ₄ * irl ₁ aml ₁	No	-	-
IRL ₄ AML ₄ * aml ₁ irl ₁	Yes	>100	100
IRL ₃ AML ₃ * aml ₂ irl ₂	Yes	>100	100
IRL ₂ AML ₂ * aml ₃ irl ₃	Yes	>100	100
IRL ₁ AML ₁ * aml ₄ irl ₄	Yes	>100	100

Table A.72. Within-clade mating. Outcome of the mating between genotypes from the Amlwch Harbour and Plymouth populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
AML ₁ * ply ₁	Yes	>100	100	10	4
AML ₂ * ply ₂	Yes	60	100	10	9
AML ₃ * ply ₃	Yes	65	100	10	10
AML ₄ * ply ₄	Yes	16	100	10	2
PLY ₁ * aml ₁	Yes	50	100	10	4
PLY ₂ * aml ₂	Yes	>100	100	10	6
PLY ₃ * aml ₃	Yes	>100	98	10	10
PLY ₄ * aml ₄	Yes	>100	100	10	5

Table A.73. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Amlwch Harbour and Plymouth populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
AML ₁ * aml ₂	Yes	>100
AML ₂ * aml ₁	Yes	>100
AML ₃ * aml ₄	Yes	>100
AML ₄ * aml ₃	Yes	>100
PLY ₁ * ply ₂	Yes	>100
PLY ₂ * ply ₁	Yes	>100
PLY ₃ * ply ₄	Yes	>100
PLY ₄ * ply ₃	Yes	>100

Table A.74. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Amlwch Harbour and Plymouth populations.

Genotype	Were embryos produced?	No. of larvae released
AML ₁	No	-
AML ₂	No	-
AML ₃	No	-
AML ₄	Yes	1
PLY ₁	No	-
PLY ₂	No	-
PLY ₃	No	-
PLY ₄	No	-

Table A.75. Within-clade mating. Outcome of the backcross matings to the maternal population using the F1^s produced in the matings between the Amlwch Harbour and Plymouth populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – AML₁PLY₁ would have been brooded by the genotype AML₁ and fathered by the genotype PLY₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
AML ₁ PLY ₁ * aml ₄	Yes	>100	100
AML ₂ PLY ₂ * aml ₃	Yes	>100	100
AML ₃ PLY ₃ * aml ₂	Yes	>100	100
AML ₄ PLY ₄ * aml ₁	Yes	>100	100
AML ₄ * aml ₁ ply ₁	Yes	>100	100
AML ₃ * aml ₂ ply ₂	Yes	>100	95
AML ₂ * aml ₃ ply ₃	Yes	>100	100
AML ₁ * aml ₄ ply ₄	Yes	>100	100
PLY ₁ AML ₁ * ply ₄	Yes	>100	100
PLY ₂ AML ₂ * ply ₃	Yes	>100	100
PLY ₃ AML ₃ * ply ₂	Yes	>100	100
PLY ₄ AML ₄ * ply ₁	Yes	>100	100
PLY ₄ * ply ₁ aml ₁	Yes	>100	100
PLY ₃ * ply ₂ aml ₂	Yes	>100	97
PLY ₂ * ply ₃ aml ₃	Yes	>100	100
PLY ₁ * ply ₄ aml ₄	Yes	>100	100

All but 2 of the F1 genotypes shown in the above table (and in table A.76) are known to be the product of outcrossing between Amlwch Harbour and Lough Hyne genotypes (the paternity analysis on AML₄PLY₄ and PLY₁AML₁ were inconclusive) (A. Gomez pers. comms.).

Table A.76. Within-clade mating. Outcome of the crosses between the F1^s produced in the matings between the Amlwch Harbour and Plymouth populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – AML₁PLY₁ would have been brooded by the genotype AML₁ and fathered by the genotype PLY₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
AML ₁ PLY ₁ * ply ₄ aml ₄	Yes	>100	100
AML ₂ PLY ₂ * ply ₃ aml ₃	Yes	>100	100
AML ₃ PLY ₃ * ply ₂ aml ₂	Yes	>100	100
AML ₄ PLY ₄ * ply ₁ aml ₁	Yes	>100	100
PLY ₄ AML ₄ * aml ₁ ply ₁	Yes	>100	100
PLY ₃ AML ₃ * aml ₂ ply ₂	Yes	>100	100
PLY ₂ AML ₂ * aml ₃ ply ₃	Yes	>100	100
PLY ₁ AML ₁ * aml ₄ ply ₄	Yes	>100	100

Table A.77. Within-clade mating. Outcome of the mating between genotypes from the Amlwch Harbour and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
AML ₁ * spn ₁	Yes	>100	100	10	9
AML ₂ * spn ₂	Yes	>100	100	10	9
AML ₃ * spn ₃	Yes	>100	100	10	8
AML ₄ * spn ₄	Yes	4	0	-	-
SPN ₁ * aml ₁	Yes	34	100	10	9
SPN ₂ * aml ₂	Yes	>100	100	10	9
SPN ₃ * aml ₃	Yes	>100	100	10	8
SPN ₄ * aml ₄	Yes	>100	100	10	9

Table A.78. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Amlwch Harbour and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
AML ₁ * aml ₂	Yes	>100
AML ₂ * aml ₁	Yes	>100
AML ₃ * aml ₄	Yes	>100
AML ₄ * aml ₃	Yes	>100
SPN ₁ * spn ₂	Yes	>100
SPN ₂ * spn ₁	Yes	>100
SPN ₃ * spn ₄	Yes	>100
SPN ₄ * spn ₃	Yes	>100

Table A.79. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Amlwch Harbour and Ría de Ferrol (var. normal) populations.

Genotype	Were embryos produced?	No. of larvae released
AML ₁	No	-
AML ₂	No	-
AML ₃	No	-
AML ₄	Yes	0
SPN ₁	No	-
SPN ₂	No	-
SPN ₃	No	-
SPN ₄	No	-

Table A.80. Within-clade mating. Outcome of the backcross matings to the maternal population using the F1^s produced in the matings between the Amlwch Harbour and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – AML₁SPN₁ would have been brooded by the genotype AML₁ and fathered by the genotype SPN₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
AML ₁ SPN ₁ * aml ₃	Yes	>100	100
AML ₂ SPN ₂ * aml ₁	Yes	>100	100
AML ₃ SPN ₃ * aml ₂	Yes	>100	100
AML ₃ * aml ₁ spn ₁	Yes	24	100
AML ₁ * aml ₂ spn ₂	Yes	>100	100
AML ₂ * aml ₃ spn ₃	Yes	20	100
SPN ₁ AML ₁ * spn ₄	Yes	>100	100
SPN ₂ AML ₂ * spn ₃	Yes	>100	100
SPN ₃ AML ₃ * spn ₂	Yes	>100	100
SPN ₄ AML ₄ * spn ₁	Yes	>100	100
SPN ₄ * spn ₁ aml ₁	Yes	>100	100
SPN ₃ * spn ₂ aml ₂	Yes	>100	100
SPN ₂ * spn ₃ aml ₃	Yes	>100	100
SPN ₁ * spn ₄ aml ₄	Yes	>100	100

All of the F1 genotypes shown in the above table (and table A.81) are known to be the product of outcrossing between Amlwch Harbour and Ría de Ferrol (Var. normal) genotypes (A. Gomez pers. comms.).

Table A.81. Within-clade mating. Outcome of the crosses between the F1^s produced in the matings between the Amlwch Harbour and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – AML₁SPN₁ would have been brooded by the genotype AML₁ and fathered by the genotype SPN₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
AML ₁ SPN ₁ * spn ₄ aml ₄	Yes	>100	100
AML ₂ SPN ₂ * spn ₃ aml ₃	Yes	>100	100
AML ₃ SPN ₃ * spn ₂ aml ₂	Yes	>100	100
AML ₂ SPN ₂ * spn ₁ aml ₁	Yes	>100	100
SPN ₄ AML ₄ * aml ₁ spn ₁	Yes	>100	100
SPN ₃ AML ₃ * aml ₂ spn ₂	Yes	>100	97
SPN ₂ AML ₂ * aml ₃ spn ₃	Yes	>100	100
SPN ₁ AML ₁ * aml ₂ spn ₂	Yes	>100	100

Table A.82. Within-clade mating. Outcome of the mating between genotypes from the Amlwch Harbour and Ría de Ferrol (var. *reticulata*) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
AML ₁ * ret ₁	No	-	-	-	-
AML ₂ * ret ₂	No	-	-	-	-
AML ₃ * ret ₄	Yes	44	100	10	8
RET ₁ * aml ₁	Yes	44	100	10	8
RET ₂ * aml ₂	Yes	>100	100	10	8
RET ₄ * aml ₃	Yes	>100	100	10	8

Table A.83. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Amlwch Harbour and Ría de Ferrol (var. *reticulata*) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
AML ₁ * aml ₂	Yes	>100
AML ₂ * aml ₁	Yes	>100
AML ₃ * aml ₄	Yes	>100
AML ₄ * aml ₃	Yes	>100
RET ₁ * ret ₄	Yes	>100
RET ₄ * ret ₁	Yes	>100
RET ₂ * ret ₄	Yes	>100
RET ₄ * ret ₂	Yes	>100

Table A.84. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Amlwch Harbour and Ría de Ferrol (var. *reticulata*) populations.

Genotype	Were embryos produced?	No. of larvae released
AML ₁	No	-
AML ₂	No	-
AML ₃	No	-
RET ₁	No	-
RET ₂	No	-
RET ₄	No	-

Table A.85. Within-clade mating. Outcome of the mating between genotypes from the Lough Hyne and Plymouth populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
IRL ₁ * ply ₁	Yes	>100	100	10	10
IRL ₂ * ply ₂	Yes	>100	100	10	10
IRL ₃ * ply ₃	Yes	>100	100	10	5
IRL ₄ * ply ₄	Yes	>100	100	10	10
PLY ₁ * irl ₁	Yes	26	100	10	10
PLY ₂ * irl ₂	Yes	>100	100	10	9
PLY ₃ * irl ₃	Yes	>100	96	10	2
PLY ₄ * irl ₄	Yes	>100	100	10	10

Table A.86. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Lough Hyne and Plymouth populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
IRL ₁ * irl ₂	Yes	>100
IRL ₂ * irl ₁	Yes	>100
IRL ₃ * irl ₄	Yes	>100
IRL ₄ * irl ₃	Yes	>100
PLY ₁ * ply ₂	Yes	>100
PLY ₂ * ply ₁	Yes	>100
PLY ₃ * ply ₄	Yes	>100
PLY ₄ * ply ₃	Yes	>100

Table A.87. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Lough Hyne and Plymouth populations.

Genotype	Were embryos produced?	No. of larvae released
IRL ₁	No	-
IRL ₂	Yes	3
IRL ₃	No	-
IRL ₄	No	-
PLY ₁	No	-
PLY ₂	No	-
PLY ₃	No	-
PLY ₄	No	-

Table A.88. Within-clade mating. Outcome of the backcross matings to the maternal population using the F1^s produced in the matings between the Lough Hyne and Plymouth populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – IRL₁PLY₁ would have been brooded by the genotype IRL₁ and fathered by the genotype PLY₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
IRL ₁ PLY ₁ * irl ₄	Yes	>100	100
IRL ₂ PLY ₂ * irl ₃	Yes	>100	95
IRL ₃ PLY ₃ * irl ₂	Yes	>100	100
IRL ₄ PLY ₄ * irl ₁	Yes	>100	100
IRL ₄ * irl ₁ ply ₁	Yes	>100	100
IRL ₃ * irl ₂ ply ₂	Yes	>100	100
IRL ₂ * irl ₃ ply ₃	Yes	>100	100
IRL ₁ * irl ₄ ply ₄	Yes	>100	100
PLY ₁ IRL ₁ * ply ₄	Yes	91	100
PLY ₂ IRL ₂ * ply ₃	Yes	75	100
PLY ₃ IRL ₃ * ply ₂	Yes	>100	98
PLY ₄ IRL ₄ * ply ₁	Yes	>100	100
PLY ₄ * ply ₁ irl ₁	Yes	>100	100
PLY ₃ * ply ₂ irl ₂	Yes	>100	100
PLY ₂ * ply ₃ irl ₃	Yes	>100	100
PLY ₁ * ply ₄ irl ₄	Yes	>100	100

All but 2 of the F1 genotypes shown in the above table (and table A.89) are known to be the product of outcrossing between Lough Hyne and Plymouth genotypes (the paternity analysis on IRL₃PLY₃ and PLY₃IRL₃ were inconclusive) (A. Gomez pers. comms.).

Table A.89. Within-clade mating. Outcome of the crosses between the F1^s produced in the matings between the Lough Hyne and Plymouth populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – IRL₁PLY₁ would have been brooded by the genotype IRL₁ and fathered by the genotype PLY₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
IRL ₁ PLY ₁ * ply ₄ irl ₄	Yes	>100	100
IRL ₂ PLY ₂ * ply ₃ irl ₃	Yes	>100	100
IRL ₃ PLY ₃ * ply ₂ irl ₂	Yes	>100	100
IRL ₄ PLY ₄ * ply ₁ irl ₁	Yes	>100	100
PLY ₄ IRL ₄ * irl ₁ ply ₁	Yes	>100	100
PLY ₃ IRL ₃ * irl ₂ ply ₂	Yes	>100	100
PLY ₂ IRL ₂ * irl ₃ ply ₃	Yes	>100	100
PLY ₁ IRL ₁ * irl ₄ ply ₄	Yes	51	100

Table A.90. Within-clade mating. Outcome of the mating between genotypes from the Lough Hyne and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
IRL ₁ * spn ₁	Yes	>100	100	10	10
IRL ₂ * spn ₂	Yes	>100	100	10	10
IRL ₃ * spn ₃	Yes	>100	100	10	10
IRL ₄ * spn ₄	Yes	>100	100	10	10
SPN ₁ * irl ₁	Yes	7	0	-	-
SPN ₂ * irl ₂	Yes	0	-	-	-
SPN ₃ * irl ₃	No	0	-	-	-
SPN ₄ * irl ₄	Yes	>100	100	10	7

Table A.91. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Lough Hyne and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
IRL ₁ * irl ₂	Yes	>100
IRL ₂ * irl ₁	Yes	>100
IRL ₃ * irl ₄	Yes	>100
IRL ₄ * irl ₃	Yes	>100
SPN ₁ * spn ₂	Yes	>100
SPN ₂ * spn ₁	Yes	>100
SPN ₃ * spn ₄	Yes	>100
SPN ₄ * spn ₃	Yes	>100

Table A.92. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Lough Hyne and Ría de Ferrol (var. normal) populations.

Genotype	Were embryos produced?	No. of larvae released
IRL ₁	No	0
IRL ₂	Yes	0
IRL ₃	No	-
IRL ₄	No	-
SPN ₁	No	-
SPN ₂	No	-
SPN ₃	No	-
SPN ₄	No	-

Table A.93. Within-clade mating. Outcome of the backcross matings to the maternal population using the F1^s produced in the matings between the Lough Hyne and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – IRL₁SPN₁ would have been brooded by the genotype IRL₁ and fathered by the genotype SPN₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
IRL ₁ SPN ₁ * irl ₄	Yes	>100	100
IRL ₂ SPN ₂ * irl ₃	Yes	4	0
IRL ₃ SPN ₃ * irl ₂	Yes	>100	100
IRL ₄ SPN ₄ * irl ₁	Yes	>100	100
IRL ₄ * irl ₁ spn ₁	Yes	76	100
IRL ₃ * irl ₂ spn ₂	Yes	>100	97
IRL ₂ * irl ₃ spn ₃	Yes	52	100
IRL ₁ * irl ₄ spn ₄	Yes	>100	98
SPN ₄ IRL ₄ * spn ₁	Yes	>100	100
SPN ₁ * spn ₄ irl ₄	Yes	>100	100

All but 1 of the genotypes shown in the above table (and table A.94) are known to be the product of outcrossing between Lough Hyne and Ría de Ferrol (var. normal) genotypes (the paternity analysis on IRL₂SPN₂ was inconclusive) (A. Gomez pers. comms.).

Table A.94. Within-clade mating. Outcome of the crosses between the F1^s produced in the matings between the Lough Hyne and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – IRL₁SPN₁ would have been brooded by the genotype IRL₁ and fathered by the genotype SPN₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
IRL ₁ SPN ₁ * spn ₄ irl ₄	Yes	>100	100
IRL ₂ SPN ₂ * spn ₄ irl ₄	Yes	>100	100
IRL ₃ SPN ₃ * spn ₄ irl ₄	Yes	>100	100
IRL ₄ SPN ₄ * spn ₄ irl ₄	Yes	>100	100
SPN ₄ IRL ₄ * irl ₁ spn ₁	Yes	26	100
SPN ₄ IRL ₄ * irl ₂ spn ₂	Yes	>100	100
SPN ₄ IRL ₄ * irl ₃ spn ₃	Yes	>100	100
SPN ₄ IRL ₄ * irl ₄ spn ₄	Yes	63	100

Table A.95. Within-clade mating. Outcome of the mating between genotypes from the Plymouth and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
PLY ₁ * spn ₁	Yes	75	100	10	10
PLY ₂ * spn ₂	Yes	>100	100	10	9
PLY ₃ * spn ₃	Yes	>100	100	10	7
PLY ₄ * spn ₄	Yes	>100	100	10	10
SPN ₁ * ply ₁	Yes	>100	99	10	10
SPN ₂ * ply ₂	Yes	>100	100	10	8
SPN ₃ * ply ₃	Yes	>100	100	10	9
SPN ₄ * ply ₄	Yes	>100	99	10	7

Table A.96. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Plymouth and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
PLY ₁ * ply ₂	Yes	>100
PLY ₂ * ply ₁	Yes	>100
PLY ₃ * ply ₄	Yes	>100
PLY ₄ * ply ₃	Yes	>100
SPN ₁ * spn ₂	Yes	>100
SPN ₂ * spn ₁	Yes	>100
SPN ₃ * spn ₄	Yes	>100
SPN ₄ * spn ₃	Yes	>100

Table A.97. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Plymouth and Ría de Ferrol (var. normal) populations.

Genotype	Were embryos produced?	No. of larvae released
PLY ₁	No	-
PLY ₂	No	-
PLY ₃	No	-
PLY ₄	No	-
SPN ₁	No	-
SPN ₂	No	-
SPN ₃	No	-
SPN ₄	No	-

Table A.98. Within-clade mating. Outcome of the backcross matings to the maternal population using the F1^s produced in the matings between the Plymouth and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – PLY₁SPN₁ would have been brooded by the genotype PLY₁ and fathered by the genotype SPN₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
PLY ₁ SPN ₁ * ply ₄	No	-	-
PLY ₂ SPN ₂ * ply ₃	Yes	37	97
PLY ₃ SPN ₃ * ply ₂	Yes	>100	100
PLY ₄ SPN ₄ * ply ₁	Yes	>100	100
PLY ₄ * ply ₁ spn ₁	Yes	80	100
PLY ₃ * ply ₂ spn ₂	Yes	>100	100
PLY ₂ * ply ₃ spn ₃	Yes	>100	100
PLY ₁ * ply ₄ spn ₄	Yes	38	100
SPN ₁ PLY ₁ * spn ₄	No	-	-
SPN ₂ PLY ₂ * spn ₃	Yes	>100	100
SPN ₃ PLY ₃ * spn ₂	Yes	>100	98
SPN ₄ PLY ₄ * spn ₁	Yes	80	100
SPN ₄ * spn ₁ ply ₁	Yes	>100	100
SPN ₃ * spn ₂ ply ₂	Yes	>100	100
SPN ₂ * spn ₃ ply ₃	Yes	>100	100
SPN ₁ * spn ₄ ply ₄	Yes	>100	100

The four F1 genotypes released by colonies from the Ría de Ferrol (var. normal) population shown in the above table (and table A.99) are known to be the product of outcrossing between Plymouth and Ría de Ferrol (var. normal) genotypes. Paternity analysis was unable to be performed on the other 4 F1 genotypes as the samples were lost (A. Gomez pers. comms.).

Table A.99. Within-clade mating. Outcome of the crosses between the F1^s produced in the matings between the Plymouth and Ría de Ferrol (var. normal) populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – PLY₁SPN₁ would have been brooded by the genotype PLY₁ and fathered by the genotype SPN₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
PLY ₁ SPN ₁ * spn ₄ ply ₄	No	-	-
PLY ₂ SPN ₂ * spn ₃ ply ₃	Yes	73	100
PLY ₃ SPN ₃ * spn ₂ ply ₂	Yes	>100	100
PLY ₄ SPN ₄ * spn ₁ ply ₁	Yes	>100	100
SPN ₄ PLY ₄ * ply ₁ spn ₁	Yes	>100	99
SPN ₃ PLY ₃ * ply ₂ spn ₂	Yes	>100	100
SPN ₂ PLY ₂ * ply ₃ spn ₃	Yes	>100	100
SPN ₁ PLY ₁ * ply ₄ spn ₄	No	-	-

Table A.100. Within-clade mating. Outcome of the mating between genotypes from the Ría de Ferrol (var. normal) and Ría de Ferrol (var. reticulata) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity	No. of colonies sent for paternity analysis	No. of colonies confirmed as outcrossed
SPN ₁ * ret ₁	Yes	9	0	-	-
SPN ₃ * ret ₂	No	-	-	-	-
SPN ₄ * ret ₄	Yes	>100	70	10	?
RET ₁ * spn ₁	Yes	>100	100	10	5
RET ₂ * spn ₃	Yes	>100	100	10	10
RET ₄ * spn ₄	Yes	>100	100	10	?

Table A.101. Within-clade mating. Outcome of the control matings to test for reproductive activity in the genotypes from the Ría de Ferrol (var. normal) and Ría de Ferrol (var. reticulata) populations. Capital letters denote the maternal genotype.

Genotype	Were embryos produced?	No. of larvae released
SPN ₁ * spn ₂	Yes	>100
SPN ₂ * spn ₁	Yes	>100
SPN ₃ * spn ₄	Yes	>100
SPN ₄ * spn ₃	Yes	>100
RET ₁ * ret ₄	Yes	>100
RET ₄ * ret ₁	Yes	>100
RET ₂ * ret ₄	Yes	>100
RET ₄ * ret ₂	No	-

Table A.102. Within-clade mating. Outcome of the controls to test for self-fertilisation activity in the genotypes from the Ría de Ferrol (var. normal) and Ría de Ferrol (var. reticulata) populations.

Genotype	Were embryos produced?	No. of larvae released
SPN ₁	No	-
SPN ₃	No	-
SPN ₄	No	-
RET ₁	No	-
RET ₂	No	-
RET ₄	No	-

Table A.103. Within-clade mating. Outcome of the backcross matings to the maternal population using the F1^s produced in the matings between the Ría de Ferrol (var. normal) and Ría de Ferrol (var. reticulata) populations. Capital letters denote the maternal genotype. (In the six letter code for the F1 genotypes the first three letters are taken from the colonies maternal genotype and the second three letters are taken from the colonies paternal genotype e.g. – SPN₁RET₁ would have been brooded by the genotype SPN₁ and fathered by the genotype RET₁).

Genotype	Were embryos produced?	No. of larvae settled	% of settled larvae reaching sexual maturity
RET ₁ SPN ₁ * ret ₄	Yes	>100	100
RET ₄ SPN ₄ * ret ₁	No	-	-
RET ₄ * ret ₁ spn ₁	No	-	-
RET ₁ * ret ₄ spn ₄	Yes	>100	100

Paternity analysis on both F1 genotypes in the above table were inconclusive (A. Gomez pers. comms.).