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Polyembryony, brood chamber development and gender specialisation in cyclostome bryozoans

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Summary of the Dissertation

Investigations were conducted into aspects of the reproductive biology of the Cyclostomata, a relatively understudied ancient order of bryozoans.

Evidence for polyembryony (cloning of sexually produced embryos) in all three major cyclostome clades was obtained using a molecular genetic approach (Chapter 2) confirming historical inferences based on microscopy and supporting the widely held view that this apparently paradoxical reproductive mode characterises the order.

The paradoxical nature of polyembryony in the case of cyclostomes was investigated. Genotyping provided evidence for the prolonged production of genetically identical larvae from a single brood in *Filicrisia geniculata* and the presence of genetic diversity between broods within the same colony (Chapter 5). By cloning multiple progeny genotypes at a given time and testing each against varying environmental conditions over a substantial period, polyembryony may be less paradoxical in this group than first assumed.

Variation in gender roles among colonies of *Filicrisia geniculata* was investigated using cultured material (Chapter 4). Two distinct categories of colony were discovered. Sperm were produced exclusively by 'male' colonies, composed solely of autozooids. 'Female' colonies comprised regular autozooids and gonozooids. These observations are consistent with at least very pronounced gender specialisation, apparently amounting to outright gonochorism, at the colony level. This is the first properly documented example of separate sexes in bryozoans.

Mating trials investigating the effect of exposure to conspecific allosperm on brood chamber development revealed variation in reproductive traits between the two cyclostome species studied (Chapter 3). *Tubulipora plumosa* demonstrated greater production of gonozooids and larvae in the presence of conspecific allosperm, but with evidence of some selfing when in reproductive isolation. Similar investigations with *Filicrisia geniculata* revealed that, in female colonies, completion of gonozooid development and brooding occurred exclusively in the presence of allosperm.

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Chapter 1: **Introduction to the mating systems of cyclostome bryozoans.**

1.1 Spermcast mating in sessile colonial invertebrates.

Many marine hard substrate communities are dominated by sessile, colonial, suspension-feeding invertebrates (Jackson, 1977). Examples include hydroids, ascidians and bryozoans. Colonial growth is achieved through asexual budding, creating a colony of connected modular units. The mating systems of these organisms often involves simultaneous hermaphroditism, at either the level of the individual module or the colony as a whole. There are three recognised fertilisation mechanisms in marine invertebrates: external fertilisation, more generally known as broadcast or free spawning; direct mating via copulation or pseudocopulation; and spermcast mating (Bishop & Pemberton, 2006). While the first two processes are widely recognised, the third has only recently received any great focus (Pemberton *et al.*, 2003; Bishop & Pemberton, 2006). Spermcast mating involves the fertilisation of a retained egg by water-borne sperm (Pemberton *et al.*, 2003; Bishop & Pemberton, 2006). In contrast, external fertilisation involves the spawning of both sperm and eggs into the water column, where fertilisation then takes place.

Spermcast mating occurs in a phylogenetically diverse group of sessile marine invertebrates including some sponges, some corals and hydroids, all bryozoans, the majority of colonial, and some unitary, ascidians, and as recognised only recently, some stalked barnacles (Pemberton *et al.*, 2003; Barazandeh *et al.*, 2013). Many of these organisms brood embryos post-fertilisation. In contrast to broadcast spawners, spermcasters typically release short-lived lecithotrophic larvae that disperse over shorter distances (Jackson, 1986).

Spermcast mating in sessile colonial marine invertebrates is analogous to wind pollination in plants (Bishop *et al.*, 2000; Pemberton *et al.*, 2004; Hughes, 2005). Shared characteristics include: a sessile adult habit, remote mating involving the dispersal of only male genetic material, internal fertilisation, and embryonic brooding often with ongoing maternal investment/nutrition (e.g. Ostrovsky (2013) for cheilostome bryozoans) (Bishop *et al.*, 2000; Pemberton *et al.*, 2004). The retention of eggs in spermcasters is proposed to lead to different evolutionary pressures on reproductive traits, creating fundamental differences in life history strategies compared to those of broadcasters (Pemberton *et al.*, 2003). Broadcast spawning is associated with the threat of reduced fertilisation success due to sperm

limitation, caused by dilution of short-lived sperm by turbulence in the water column (Levitan & Petersen, 1995). This has promoted the evolution of strategies to enhance fertilisation success, for example, synchronous spawning and aggregative behaviour (Levitan, 1995; Levitan & Petersen, 1995). In contrast, spermcast mating systems appear less susceptible to sperm limitation due to the accumulation and, in some cases, storage of long-lived spermatozoa from dilute suspension (Hughes *et al.*, 2002; Pemberton *et al.*, 2003; Johnson & Yund, 2004). Other characteristics common to spermcast mating systems include the control of female investment by receipt of compatible allosperm, and the presence of mechanisms to prevent or reduce self-fertilisation. These mechanisms involve interactions between sperm and the maternal tissue of the recipient, whereas in broadcast systems, avoidance of self-fertilisation generally involves gametic incompatibility (Bishop & Pemberton, 2006).

1.2 The Bryozoa.

Bryozoans are modular, suspension-feeding, aquatic invertebrates found in a variety of both marine and freshwater environments, often forming a major component of sessile epifaunal communities (Hayward & Ryland, 1985). Their colonies are composed of numerous repeated units, or zooids, whose patterns of budding produce a range of growth forms. Bryozoan colonies can significantly contribute to biodiversity by providing habitat complexity, which supports a diverse range of invertebrates (e.g. Wood *et al.*, 2012).

Bryozoan colonies are hermaphroditic, exhibiting either zooidal hermaphroditism (bisexual zooids) or zooidal gonochorism (single-sex zooids within a single colony) (Ryland, 1970; Reed, 1991; Ostrovsky *et al.*, 2008). Zooidal or colonial protandry, protogyny and simultaneous hermaphroditism may occur in different species, reflecting variation in the timing of the appearance and functioning of gonads (Reed, 1991; Ostrovsky *et al.*, 2008). Cross-fertilisation is internal, either intraovarian or intracoelomic, and occurs via spermcast mating. Most bryozoans produce short-lived lecithotrophic larvae that are brooded, though some gymnolaemates release pelagic, planktotrophic larvae called ‘cyphonautes’ (Ström, 1977; Reed, 1991; Ostrovsky *et al.*, 2008). Bryozoans exhibit a variety of reproductive patterns, most comprehensively reviewed and updated by Reed (1991) and Ostrovsky *et al.* (2008).

1.3 An introduction to cyclostome bryozoans.

Colonies of the exclusively marine bryozoan Order Cyclostomata are formed from autozooids of simple cylindrical morphology (Borg, 1926; Hayward & Ryland, 1985). A wide variety of colony forms is achieved through different patterns of budding and growth, creating distinctive morphologies at both generic and specific level (Hayward & Ryland, 1985). These heavily calcified colonies are often small and exhibit limited zooidal polymorphism compared to non-cyclostomatous bryozoans (Reed, 1991). However, some degree of polymorphism is present with the enlargement of female zooids to form voluminous chambers, termed gonozooids, for the incubation of multiple embryos (Borg, 1926). Cyclostomes are viviparous as embryonic development (and incubation) takes place intra-coelomically inside the gonozooid (within the maternal body cavity, enclosed by the membranous sac), where it is facilitated by the extra-embryonic nutrition of embryos (Ostrovsky *et al.*, 2009). Gonozooids attain a greater size than regular autozooids and are often of a characteristic shape, making them of prime taxonomic importance. They can be distinguished further by the presence of the ooeciostome, an opening through which larvae escape. Variation in gonozooid morphology is observed across the order, from the simple, discrete gonozooids of the Crisiidae (Harmer, 1893), to the more irregular, lobed growth form of the Tubuliporidae, where the gonozooid develops between rows of autozooids (Harmer, 1898). The Lichenoporidae possess more complex brood 'spaces' formed through the fusion of alveolar spaces surrounding the maternal zooid, creating a central brood cavity (Harmer, 1896, 1928; Borg, 1926, 1933). These lichenoporid colonial larval chambers are not homologous to the gonozooids of other cyclostome families as the membranous sac, which encloses the developing embryos and nutritive tissue in all cyclostomes, extends beyond the original gonozooid and throughout the colony's entire internal cavity (Borg, 1926).

After a period of incubation and nourishment within the gonozooid, larvae awaiting release accumulate beneath the ooeciostome (Reed, 1991). These lecithotrophic larvae are then liberated through this opening and swim for a short time before settling onto the substratum by everting an adhesive sac (Nielsen, 1970; 2012). Larvae metamorphose soon after settlement. At first a primary disc is formed followed by the development of the first autozooid, thereby forming the ancestrula (Nielsen, 1970). Colony development (astogeny) commences with the budding of autozooids directly from the ancestrula and continues with the replication of zooidal units.

The Cyclostomata are now the only living representatives of the Class Stenolaemata; the other four orders are thought to have become extinct in the Late Permian or Triassic (251-199 Ma) (Taylor, 2000; Waeschenbach *et al.*, 2009). The Stenolaemata first appeared in the Early Ordovician (488-479 Ma) and also provide the first fossil evidence for the phylum Bryozoa (Hu & Spjeldnaes, 1991; Feng-Sheng *et al.*, 2007). Cyclostomes were once the major order of bryozoans, dominating for more than 100 million years throughout the Jurassic and Early Cretaceous (e.g. Lidgard *et al.*, 1993). They are now very subordinate to cheilostomes, in terms of both diversity and abundance, with cyclostomes thought to make up <10% of the extant bryozoan fauna (Taylor, 2000; Waeschenbach *et al.*, 2012).

Five suborders of extant cyclostomes are now recognised: Tubuliporina, Articulata, Cancellata, Cerioporina and Rectangulata, along with the family Cinctiporidae, whose phylogenetic position is uncertain (Boardman *et al.*, 1992; Waeschenbach *et al.*, 2009). The relative scarcity of phylogenetically informative morphological characters among cyclostomes (compared to cheilostomes) is acknowledged, and previous attempts to reconstruct phylogeny using skeletal characters in this ancient order revealed high levels of homoplasy (Taylor & Weedon, 2000). The first molecular phylogeny of the Cyclostomata revealed three well-supported major clades, although their interrelationships are uncertain (Waeschenbach *et al.*, 2009). Further molecular phylogenetic analysis with limited additional taxon sampling (four extra taxa) yielded conflicting topologies, and interrelationships between cyclostome clades remain unresolved (Waeschenbach *et al.*, 2012).

1.4 Reproduction in cyclostome bryozoans.

1.4.1 Gonad and gonozooid development.

Primary sex cells in cyclostomes originate exclusively at the growing edge of the colony (Robertson, 1903; Borg, 1926). These germ cells arise prior to the development of polypide buds in this growing zone, and, at this stage, oogonia and spermatogonia are indistinguishable (Borg, 1926; Harmer, 1928). Some germ cells become incorporated with an invagination of the colony margin, which forms the first stage of the delineation of a new zooid, and one or two germ cells will become connected to the proximal end of the young polypide bud (Borg, 1926). Germ cells that do not become associated with developing polypide buds degenerate.

Primary spermatogonia divide repeatedly to form a mass of tissue that constitutes the testis (Borg, 1926). The testis remains at the proximal end of the developing polypide bud, where the caecum of the polypide eventually develops. The cyclostome testis is unique among the Bryozoa in being a well-defined structure with a distinct location within the zooid (Borg, 1926).

Cyclostome oogenesis has been best studied in the Crisiidae (Articulata). Further investigations have identified a high level of similarity in the basic process of oogenesis at the ordinal level (Harmer, 1893, 1896, 1898; Borg, 1926). Primary oogonia become associated with a young polypide bud at an early stage of polypide development or they degenerate (Harmer, 1893; Borg, 1926). Most commonly, only a single ovum becomes connected to a polypide bud and, as with the developing testis, is positioned at the proximal end of the young bud. At this stage, the incipient gonozooid is indistinguishable from a regular zooid (Harmer, 1928). Not all polypide buds with ova will develop further into gonozooids; indeed the majority of ova will degenerate. Similar development characterises the species of Tubuliporidae and Lichenoporidae studied to date, with only a (small) fraction of the polypides associated initially with ova becoming gonozooids (Harmer, 1893, 1896, 1898; Borg, 1926).

The mechanism determining which developing polypides become gonozooids is not currently understood (Reed, 1991; Ryland, 2000), but potential female zooids undergo one of two developmental pathways (Borg, 1926). In the majority, oocytes/ova degenerate, a normal functional polypide develops, and the zooid becomes a regular feeding autozooid (Borg, 1926; Harmer, 1928). The remaining female zooids will become gonozooids and, in the Crisiidae at least, a specialised, abbreviated development of the polypide occurs, where a transient lophophore develops with only a rudimentary gut (Borg, 1926). The associated oocyte begins to enlarge and, upon fertilisation (presumably) and the onset of embryogenesis, degeneration of the young fertile polypide commences (Borg, 1926). Subsequent to the formation of this primary embryo, nutritive tissues develop and the zooid itself undergoes its transformation into the enlarged gonozooid so characteristic of the Cyclostomata. Contrary to the Crisiidae, the incipient gonozooid of the Tubuliporidae and Lichenoporidae develops a functional i.e. feeding polypide which then degenerates following fertilisation (Harmer, 1896, 1898; Borg 1926). In the Tubuliporidae, this functional polypide is indistinguishable in terms of size and zooecial morphology from regular autozooids, except

for the presence of the oocyte itself at the proximal end of the polypide (Harmer, 1898; Borg, 1926).

1.4.2 Gender allocation and fertilisation.

Whilst the Cyclostomata are understood to exhibit colonial hermaphroditism, individual zooids within colonies are gonochoristic (Nielsen, 2012). Spermatogonia are found only in zooids without oogonia (Harmer, 1928). Monoecious colonies (i.e. with gonochoric zooids) of *Tubulipora* were described by Harmer (1898), with colonies of *T. lilacea* and *T. phalangea* possessing zooids with testes at various stages of development (including some with ripe sperm) together with other zooids containing eggs (Harmer, 1898; Borg, 1926). However, some evidence of zooidal hermaphroditism has been found among the Lichenoporidae (Harmer, 1896; Borg, 1926). Thus, within a single colony, separate male and female zooids occur alongside zooids containing both sexual elements (Harmer, 1896).

Fertilisation itself has not been observed in any cyclostome but is assumed to be internal, with a retained egg fertilised by waterborne sperm (i.e. spermcast mating). The release of sperm has been observed in two cyclostome species (Silén, 1972) and uptake by the recipient colony is assumed to be via entrainment in feeding currents as in gymnolaemate bryozoans (Temkin, 1994). The role of the transitory lophophore in the developing gonozooid as a potential entry route for sperm has been proposed (Silén, 1972).

1.4.3 Polyembryony.

The Cyclostomata are thought to be characterised by the highly specialised reproductive phenomenon of polyembryony, which is believed to occur within the gonozooids of all living species (Harmer, 1893, 1896, 1898; Calvet, 1900; Robertson 1903; Borg, 1926). Polyembryony, or embryonic fission, is the splitting of a zygote into multiple genetically identical clones (Craig *et al.*, 1995) and was first described in *Crisia* by Harmer (1893) and subsequently confirmed by Robertson (1903) and Borg (1926) in a range of families across the group. These observations from microscopy revealed the iterative budding of a primary embryo, formed at the proximal end of the developing gonozooid, into multiple secondary embryos, with only small differences identified between families. Secondary embryos are then brooded within gonozooids and receive extra-embryonic nutrition from the nutritive syncytium that surrounds them (Borg, 1926).

The occurrence of gonozooids in most extant cyclostome species suggests an association with embryonic cloning (Borg, 1926). Evidence of gonozooids in cyclostomes since the late Triassic (McKinney & Taylor, 1997) suggests that polyembryony may have existed as a reproductive strategy since this time and may be a plesiomorphic character of most post-Palaeozoic cyclostome bryozoans (Taylor, 2000). One exception is the family Cinctiporidae, for which no brood chambers have been recorded (Boardman *et al.*, 1992). However, species representing this group are characterised by autozooids whose large size may enable them to brood multiple larvae generated by polyembryony (Taylor, 2000).

Polyembryony has been described as an arguably paradoxical reproductive mode, as it clones an unproven genotype at the expense of genetic diversity within the brood (Craig *et al.*, 1995; 1997). The paradox is that genetically variable offspring are not produced to face environmental variation, instead the mother 'bets' only on an unproven genotype (Craig *et al.*, 1995; 1997). This is based on Williams' inference that genetically diverse, therefore sexually produced, offspring are necessary for survival in changing environmental conditions (Williams, 1975). Whereas sex provides genetic diversity in changing environments, asexual reproduction may be beneficial in more stable environments and enables the mother's relatively successful genotype to be replicated (Craig *et al.*, 1997). Polyembryony, by combining both contrasting reproductive modes, appears to compromise their respective benefits (Hughes *et al.*, 2005). Despite this, polyembryony has been reported in over 18 taxa from six animal phyla, including parasitoid wasps and parasitic flatworms in addition to cyclostome bryozoans (Craig *et al.*, 1997).

The evolution and persistence of polyembryony has puzzled many scientists and has prompted theoretical discussions regarding its apparently paradoxical nature. Craig *et al.* (1995; 1997) outlined certain conditions predicted to favour polyembryony. Thus, it is predicted to occur in circumstances where offspring have more information regarding environmental quality, and therefore optimal clutch size, compared to the mother e.g. in some parasites or parasitoids (Craig *et al.*, 1997). In the case of cyclostomes, Craig *et al.* (1997) suggest polyembryony may enhance reproductive success, especially if sperm are limited, as many embryos are produced from a single fertilised egg. Furthermore, polyembryony allows a rapid increase in offspring when food is plentiful, enabling flexibility in brood size. This is particularly important for suspension feeders, such as cyclostomes, as food supply can be irregular (Craig *et al.*, 1997).

Ryland (1996) proposed a hypothesis for the occurrence of polyembryony in cyclostome bryozoans based on the very low-dispersal life history expected for sessile colonial marine invertebrates (Jackson, 1986). For such organisms, local populations are expected to be genetically homogeneous (low diversity) due to limited dispersal of larvae. Consequently there will be little genetic difference between potential mates, resulting in broods of 'conventional' sexual progeny of low diversity (Ryland, 1996). In this scenario, the predicted reduction in offspring diversity caused by polyembryony will be less drastic and there may be little difference between separately-fertilised and cloned (polyembryonous) broods, since both broods would be of low genetic diversity.

Recent empirical studies revealed genetically heterogeneous populations of *Crisia denticulata* over small spatial scales, suggesting receipt of sperm from genetically diverse mates despite predicted restricted sperm and larval dispersal (Pemberton *et al.*, 2007). This result counters Ryland's (1996) hypothesis, which is based on genetic similarity between parents and offspring. An alternative explanation for polyembryony in cyclostomes focuses on the level of the genet (Hughes *et al.*, 2005; Pemberton *et al.*, 2007). Here, the genet is the single brood, represented by multiple cloned larvae of an identical genotype (ramets) (Pearse *et al.*, 1989). Selection acts at the level of the genet as a whole; therefore polyembryony allows dispersal of one genet among many independent units (larvae in this case), spreading risk and enhancing the genet's fecundity (Pearse *et al.*, 1989). Furthermore, evidence from studies of *Crisia denticulata* suggests that genetic diversity of offspring can be maintained by producing multiple broods of differing genotype within a single colony (Hughes *et al.*, 2005; Pemberton *et al.*, 2007).

1.5 Areas of investigation.

Many questions arise from our current (limited) knowledge of the mating systems of cyclostomes, as outlined above. The aim of this thesis is to address some of these outstanding questions. Below I briefly outline the questions addressed in each chapter.

Documenting polyembryony: Historical observations of polyembryony based on microscopy have been confirmed genetically in a single species (*Crisia denticulata* (Hughes *et al.*, 2005)). Additional molecular evidence supporting microscopy observations across the cyclostome phylogeny is required in order to confirm the widely cited inference that this specialised reproductive mode characterises this ancient order. Chapter 2 addresses this

issue by documenting embryonic cloning in *Hornera robusta, Plagioecia patina* and *Tubulipora plumosa*.

The paradox of polyembryony: The sequential release of cloned larvae from multiple broods of different genotype from the same colony may make polyembryony less paradoxical in cyclostomes (Pemberton *et al*., 2007). In this case, a colony would be testing multiple genotypes at a given time but also each genotype would be tested against varying environmental conditions over time. Therefore, in terms of spreading the risk, polyembryony in this group may effectively converge on normal sexual reproduction, which tests multiple genotypes at once. Chapter 5 describes investigations into the longevity of primary embryos and the ability to release larvae over time.

Trigger for gonozooid development and female investment: In the cheilostome bryozoan *Celleporella hyalina*, the receipt of conspecific allosperm triggers brood chamber development and thereby influences female investment (Hughes *et al*., 2002). Chapter 3 investigates the possibility of a similar trigger in another major group of bryozoans with an alternative reproductive mode, the Cyclostomata.

Gender roles: It is generally observed among cyclostomes that colonies are often encountered with few or no gonozooids, suggesting exclusive investment in male reproductive function (e.g. Harmer, 1896). Wide variation in female investment, in terms of differences in gonozooid number between colonies, has also been reported in some cyclostomes (Pemberton *et al*., 2011). Chapter 4 examines variation in gender roles among colonies of *Filicrisia geniculata* by investigating cultured material.

Enhancing our understanding of the mating systems of cyclostomes: Employment of both laboratory culturing techniques and molecular genetic methods has the potential to reveal new insights into polyembryony and other aspects of the reproductive biology of this relatively understudied order of bryozoans. Such investigations may also provide information on outcrossing and self-fertilisation among cyclostomes thus significantly contributing to our understanding of hermaphroditism in this phylum of sessile colonial marine invertebrates. The final general chapter of this thesis (Chapter 6) explores these issues.

Chapter 2: **Molecular confirmation of polyembryony in cyclostome bryozoans.**

2.1 Introduction.

Polyembryony is the production of multiple genetically identical embryos from a single sexually produced zygote. This combination of asexual and sexual reproductive mode appears paradoxical, as the mother 'bets' only on a single unproven genotype at the expense of both (sexual) brood genetic diversity and her own relatively successful genotype (Craig *et al.*, 1995, 1997). By combining contrasting reproductive modes, their respective benefits seem compromised (Hughes *et al.*, 2005). Despite this, polyembryony has persisted, having evolved numerous times in a diverse range of taxa including some rust fungi, algae, plants and animals. In the Metazoa alone, it has been reported from six phyla, including cnidarians, platyhelminths, and bryozoans, where it is thought to characterise an entire order, the Cyclostomata (see Craig *et al.*, 1997; Hughes *et al.*, 2005).

Cyclostome colonies are characterised by the presence of voluminous brood chambers (gonozooids), each containing a brood of multiple larvae. Individual broods are produced by the iterative budding of a primary embryo and are nourished within the brood chamber until emerging as independent larvae (Borg, 1926). The occurrence of enlarged brood chambers in all Recent families of cyclostomes, with the exception of the Cinctiporidae (Boardman *et al.*, 1992), suggests an association with embryonic cloning (Borg, 1926; Ström, 1977).

Early histological observations provided the first evidence of polyembryony in cyclostomes. Harmer identified 'embryonic fission' first in the genus *Crisia* (Crisiidae) (1890, 1893) and then in *Lichenopora verrucaria* (Lichenoporidae) (1896) and the genus *Tubulipora* (Tubuliporidae) (1898). Work by Calvet (1900), Robertson (1903) and Borg (1926, 1933) supported these inferences, the last providing additional evidence from *Berenicia* (now *Plagioecia*) *patina* (Plagioeciidae), *Hornera lichenoides* (Horneridae) and the Heteroporidae. Overall, this early work established the occurrence of embryonic fission in five cyclostome suborders and consequently, in all three major clades as indicated by our most recent understanding of cyclostome phylogeny (Waeschenbach *et al.*, 2009). However, in order to rule out the possibilities of parthenogenesis and multiple fertilisations within each brood chamber, evidence of the genetic composition of both individual broods and maternal colonies is required.

The first genetic evidence was provided recently by utilising microsatellite markers and confirmed polyembryony in a single cyclostome species, *Crisia denticulata*. Genotyping of brooded embryos indicated a single genotype present within each brood chamber. Individual broods were genetically distinct from the brood-parent and from each other, therefore indicating outcrossing via water-borne sperm (Craig *et al.*, 2001; Hughes *et al.*, 2005). However, to confirm the historical inferences of polyembryony throughout the group, further genetic evidence is needed. This study set out to provide such evidence and utilised ISSRs (Inter-simple sequence repeats) rather than microsatellite markers.

ISSRs are a class of molecular genetic markers being used increasingly outside of botanical studies where they were first adopted (see Wolfe & Liston 1998). Their popularity stems from their proven effectiveness and ease of application in a wide variety of taxonomic and population genetic investigations. ISSRs are found genome-wide and are regions of DNA sequence located between closely spaced and inversely oriented microsatellite (SSR) loci (Zietkiewicz *et al.*, 1994). The amplification of these regions relies on a single targeted primer comprised of a short microsatellite sequence and a short oligonucleotide 'anchor' (1-3 nucleotides) at either the 3' or 5' end. Amplifications yield highly reproducible, highly polymorphic, multilocus banding patterns, which are easily visualised by gel electrophoresis (Zietkiewicz *et al.*, 1994; Bornet & Branchard, 2001). The use of this semi-arbitrary technique has several advantages over similar alternative markers (RAPDs, AFLPs and microsatellites). Unlike microsatellites, ISSRs can be used without prior DNA sequence information, allowing cost-effective and rapid development. The use of ISSRs is also desirable over AFLPs due to their ease of development and screening and their ability to work successfully when the amount of template DNA is limited (Zietkiewicz *et al.*, 1994; Bornet & Branchard, 2001). Furthermore, the highly reliable and reproducible nature of ISSRs is advantageous compared to RAPDs. Applications beyond plant science have involved a wide variety of metazoan taxa, with an increasing number of studies relating to marine invertebrates, such as corals, barnacles, polychaetes, and bivalve and gastropod molluscs (Casu *et al.*, 2005, 2006, 2008; Cossu *et al.*, 2012; Maltagliati *et al.*, 2005; Hou *et al.*, 2006; Trucco & Lasta, 2007; Varela *et al.*, 2007; de Aranzamendi *et al.*, 2008, 2009; Pannacciulli *et al.*, 2009).

Given the intention of this study to test the occurrence of polyembryony in a range of taxa using a molecular genetic approach, the application of ISSR markers here seems appropriate, particularly as prior genomic information is not required and extensive and costly

development is avoided. Larvae within broods, covering all major cyclostome clades, were analysed in order to provide evidence for polyembryony across the entire Order.

2.2 Materials and Methods.

2.2.1 Sampling and species collection.

Taxa used in this study, along with their classification and sampling localities, are detailed in Table 1. Collections were made from UK, Sweden and New Zealand. Despite intensive sampling effort, collections of *Disporella hispida* (Suborder Rectangulata, Family Lichenoporidae) colonies yielded few brood chambers and no larvae. Polyembryony has been confirmed previously in *Crisia denticulata* using microsatellite loci (Hughes *et al.*, 2005). Therefore, *C. denticulata* was included here to verify the validity of ISSRs as reliable alternative markers.

Larvae were collected from both live and RNAlater-preserved specimens. Individual brood chambers from each colony were isolated and transferred to a watch glass, filled with either autoclaved, filtered, UV-sterilised seawater (live specimens) or RNAlater (preserved specimens). Where it was not possible to easily isolate multiple individual brood chambers (e.g. some *Tubulipora plumosa* and *Plagioecia patina* colonies), only a single brood chamber per colony was opened. With live specimens, brood chambers were opened and individual swimming larvae, released from the membranous sac, were captured in pulled glass capillary tubes. With RNAlater-preserved specimens, brood chambers were opened and individual larvae were dissected from the membranous sac. In both cases due to the use of non-specific primers, care was taken to avoid contamination by maternal tissue and only well-differentiated and clearly distinct individual larvae were used, to ensure no transfer of attached 'brood-mate' tissue. In both cases, individual larvae were rinsed in a drop of RNAlater on a clean petri dish and then finally transferred into 5μl RNAlater in a 0.2ml Eppendorf tube. Care was also taken to transfer each larva into the Eppendorf tube in as little liquid as possible. Larvae were then stored at -20°C until DNA extraction.

Collections of whole brood tissue were also made for use during methodology optimisation. Individual brood chambers were isolated from colonies as above. Calcified brood chamber walls were carefully removed and the membranous sac, containing the primary embryo and developing larvae, was then extracted from the chamber, rinsed in drop of RNAlater and then transferred to 10μl RNAlater in an 0.5ml Eppendorf tube. This tissue was stored at -20°C until DNA extraction.

2.2.2 DNA extraction, PCR and product visualisation.

DNA Extraction.

Total genomic DNA (gDNA) was extracted from individual larvae following the modified DNeasy animal tissue extraction protocol as detailed in Webster (2009). In this case, DNA extraction is performed directly on the RNAlater-preserved larvae, using one-quarter volumes of reagents for digestion and DNA precipitation steps 1-4 of the manufacturer's instructions, added directly to individual sample tubes. This protocol is particularly advantageous when working with very small samples, such as larvae, as it does not require samples to be recaptured and removed from preservative before DNA extraction, which is time-consuming and may result in sample damage or loss. The presence of a larva in a tube was checked, using a microscope, prior to DNA extraction, preventing PCR failures due to capture problems, saving time and resources (Webster, 2009). Genomic DNA was extracted from the whole brood samples using the (unmodified) DNeasy Blood & Tissue extraction kit following manufacturer's instructions (QIAGEN).

ISSR amplification.

ISSR primers utilised in analysis (Table 2; see section 2.2.3) were selected from an original set of 20 primers found at http://www.biosci.ohio-state.edu/~awolfe/ISSR/protocols.ISSR.html. PCRs were conducted in 25μl reaction volumes using Thermoprime (or DreamTaq for *Plagioecia patina* only) kits, 1μl of 10μM of each primer and up to 12ng genomic DNA. A single primer was utilised per PCR. PCR cycling conditions were as follows: initial denaturation for 3 min at 94°C, followed by 50 cycles of 30 s at 94°C, 30 s at Ta°C (see Table 2), 2 min at 72°C, and completed by 10 min at 72°C; the comparatively large number of PCR cycles was conducted to compensate for often low gDNA concentrations and limited amount of gDNA elute. For each primer, all PCRs of whole brood and individual larval extracts were conducted in triplicate to verify repeatability of results. Positive and negative controls were included.

Visualisation of amplified PCR products.

PCR products were first visualised by electrophoresis using 0.8% agarose gels in 1x TBE buffer (50 V for 2 h) with HyperLadder I molecular weight marker (Bioline) and loading buffer

containing Gel Red (Biotium) gel stain and viewed with a UVP Gel Doc system. Successful PCR products were purified using Millipore filter plates (Merck Millipore; processed by NHM Sequencing Facility) and concentration of DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

After purification, high-resolution visualisation of PCR products was achieved by polyacrylamide gel electrophoresis (PAGE). Gels were cast using Hoefer SE600 cooled vertical electrophoresis apparatus. Glass plates (18 x 16 cm) were cleaned with 70% ethanol and dried prior to assembly (according to manufacturer's instructions) using 1.5mm thick spacers and a 20-well gel comb. 19% polyacrylamide gels were poured in a fume hood, with the initial solution being briefly degassed (3 min) prior to the addition of the polymerisation agents/catalysts (TEMED (32.5μl) and 10% ammonium persulphate solution (250μl)). Purified PCR products were run with loading buffer and ladder in 1x TBE buffer (150v for 8-9h). After electrophoresis, gels were silver-stained in a plastic tray on a rocking platform as follows: 2 x 3 min in Solution A (360ml distilled water, 40ml ethanol, 2 ml acetic acid), 1 x 10 min in Solution B (200ml distilled water, 0.2g silver nitrate), 2 x 3 min in distilled water, 1 x 10 min in Solution C (300ml distilled water, 4.5g sodium hydroxide, 0.03g sodium borohydride, 1.2ml formaldehyde), 1 x 3 min in Solution A, and stored in Solution A. After staining, gels were sealed in plastic wrapping film for storage and scanned to obtain an electronic record.

Automated electrophoresis analysis.

Following silver-stained PAGE, selected PCR products were further analysed using an Experion automated electrophoresis station with the Experion DNA 1K kit (Biorad). This methodology obtains a high-resolution virtual gel image from which band sizes are easily read. This facilitates comparison and accurate scoring of banding patterns, particularly where closely spaced bands are present.

2.2.3 ISSR marker selection.

Preliminary trials of ISSR markers utilised whole brood extracts from various cyclostome species; these extracts were used at this stage due to higher DNA concentrations and greater elution volumes compared to individual larval extracts, enabling more markers to be tested with a single extract. Initial testing of primers using larval extracts of *Filicrisia geniculata* and *Crisia denticulata* revealed amplification difficulties. Thus, this preliminary

work was continued with *Tubulipora plumosa* larval extracts, alongside whole brood extracts, which identified a subset of five markers, from the initial set of 20 (see section 2.2.2), that were polymorphic between species and indicated some degree of polymorphism between broods within the same species. Following this, for each species, single larvae from four broods were screened with all five markers to test their potential to identify between-brood polymorphism. The most informative two-three ISSRs were then used in subsequent screening of broods. Good repeatability of ISSRs was confirmed by performing PCRs in triplicate. The final protocol, as detailed above and in the following section, was developed to overcome issues encountered during marker selection. These included working with low and limited quantities of DNA (increased no. of PCR cycles) and the visualisation of clear, well-separated and easily scorable PCR products (PAGE and automated electrophoresis).

2.2.4 Brood screening protocol.

Analysis was conducted for comparisons at three levels: (1) within broods, (2) between broods from different colonies, and (3) between broods from the same colony. Evidence of embryonic cloning is sought by (1) and (2): an identical profile within a brood confirms cloning of an embryo and comparisons of broods from different colonies demonstrate marker variability (different patterns between broods indicate that the identical pattern within a brood is real). Comparison (3) provides evidence for polyembryony. Genotyping numerous larvae from multiple broods from different colonies does not in itself confirm polyembryony, only embryonic cloning. Maternal colony tissue also needs to be genotyped in order to confirm the occurrence of sex, thus ruling out apomictic parthenogenesis. Due to the non-specific nature of these primers, maternal colony tissue was not analysed due to possible contamination by food particles, attached detritus, epibionts or associated bacteria or fungi. Consequently, it was not possible to genotype broods and maternal colonies, as done in the *Crisia denticulata* microsatellite analysis (Hughes *et al.*, 2005), and to confirm polyembryony in this way. However, genotyping different broods from the same colony can obtain evidence of polyembryony, as differences between brood genotypes will indicate genetic reorganisation (sex) and exclude apomictic parthenogenesis. Therefore, this evidence combined with that from comparisons (1) and (2) will indicate the incidence of polyembryony. The following procedure was applied to all species and Table 3 details samples analysed and ISSRs used.

Multiple larvae from different broods were screened using the two-three polymorphic ISSRs selected for that species. For all larvae, PCRs were conducted in triplicate. Where replicates

for a single larva gave different banding patterns or PCRs failed (probably an indication of contamination or problem with PCR itself), larvae were discounted from analysis. For within-brood comparisons of each ISSR, purified larval PCR products for each brood were visualised by silver staining PAGE to verify that banding patterns were identical between them. Between-brood comparisons (including those from within the same colony) were conducted per marker on an automated electrophoresis station with selected larvae from each brood analysed together (see section 2.2.5).

Comparisons between broods from the same colony were made for *Hornera robusta* (Broods E and F), *Plagioecia patina* (Brood H and WB01 ('whole brood')) and *Tubulipora plumosa* (Brood M and WB02 ('whole brood')) (Table 3). 'Whole brood' tissue PCRs were performed in triplicate (as for larvae). The *T. plumosa* brood WB02 was dissected and divided into four separate tissue samples that were each analysed separately.

2.2.5 Data analysis.

For each species, all possible pair-wise comparisons of banding patterns were made between broods for each ISSR marker, using data obtained from automated electrophoresis.

ISSRs are dominant diallelic Mendelian markers and scored on the basis of band presence/absence (Casu *et al*., 2005). Presence of a dominant allele determines the presence of a band. A primer annealing site is likely repeated throughout the genome in which case multiple loci are amplified simultaneously; different-sized alleles are produced by variation in the distance between priming sites on the opposite DNA strands at different loci, and thus in amplicon length. Absence of a band of a particular size indicates either a mismatch at one or both priming sites, resulting in non-amplification, or variation in length of the intervening sequence amplified due to indel(s) (Zietkiewicz *et al*., 1994; Wolfe & Liston, 1998).

Scoring criteria for virtual gels:

A maximum of 11 samples can be analysed per automated electrophoresis run. In most cases for each marker, three larvae per brood were analysed, allowing direct comparison of banding patterns between a maximum of three broods (i.e. utilising nine wells, the remaining two wells being empty).

Only bands scored by Experion software were used in the analysis and any bands that did not meet the criteria outlined below were discounted. The Experion analysis kit used measures bands up to 1.5Kb. Therefore, any bands visible on virtual gels above this size were not scored.

Initially, banding patterns from larvae within the same brood were compared and band sizes verified, so that bands regarded visually as the same were within the +/- 10% sizing accuracy limits specified by the manufacturer. The average band size was then derived from these sizes and used in comparisons between broods.

In pair-wise comparisons between broods, bands were scored on a presence/absence basis on the virtual gels. Shared bands were identified as bands that were visually identical and whose sizes were within the +/- 10% sizing accuracy limits as specified by the manufacturer. Two levels of scrutiny were applied to comparisons:

Level One (Conservative scoring): bands difficult to differentiate between broods were grouped together so that the broods possessing them were scored equally as having that band but could still be scored against the third brood devoid of that band.

Level Two (Ultra-conservative scoring, more rigorous than the previous category): any 'difficult' bands were ignored and disregarded from comparisons altogether.

Numbers of differences between broods were identified by these pair-wise comparisons.

2.3 Results.

For comparisons within broods (1) and between broods from different colonies (2), multiple larvae from three different broods were screened with multiple ISSR primers selected for each species (*Hornera robusta* = two ISSRs; other species = three ISSRs). There were some exceptions to this: with *Crisia denticulata*, one brood (Brood C) was analysed with only two ISSRs (UBC827 & UBC850) as the initial run with UBC850 had to be repeated due to first-run PCR failure, resulting in insufficient DNA available to perform analysis with UBC884; similarly with *Tubulipora plumosa*, one brood (Brood K) was analysed with only two primers (UBC817 and UBC855) as insufficient DNA was available to repeat analysis with UBC850 due to PCR failure of the initial run (Table 3).

(1) Within-brood comparison:

All PAGE runs revealed identical profiles for all larvae and replicates within each brood with each primer (Figure 1; see Appendix I for all other PAGE gels). In the analysis of *Crisia denticulata* broods, two larvae from Brood A were discounted from further analysis with primer UBC850. PCR replicates for these larvae gave different banding profiles i.e. different bands for replicates of the same larva, suggesting PCR failure as identical profiles were found for all larva replicates with the other two markers. In analysis of *Hornera robusta* and *Plagioecia patina* broods, two larvae each from Broods F and G respectively, were excluded from further analysis due to PCR failures and differences in banding profiles between PCR replicates of the same larva with all primers used, indicating probable sample contamination.

Virtual gels from automated electrophoresis runs with each primer confirmed identical banding patterns within broods (Figure 2 & 3 and Appendix IV).

(2) Between-brood comparison:

Virtual gels revealed differences between broods for all pair-wise comparisons of all species (Table 4; see Appendix II for an example of larval banding profile scoring; see Appendix III for Level 1 scoring). Figure 2 provides an example set of virtual gels for *Plagioecia patina* (see Appendix IV for all other virtual gels). In comparisons of *Tubulipora plumosa* broods, primers UBC817 and UBC855 revealed differences between all three broods (Table 4). However, with primer UBC850, Brood J PCR products had degraded over time and were too

weak to be scored by automated electrophoresis alongside Brood L. Repeat analysis of these PCR products by PAGE with those from Brood L indicated some differences between these broods (Appendix I: Figure 10ii) but the PAGE gel was not scored.

(3) Between broods from same colony comparison:

Polyembryony can be confirmed in *Hornera robusta* due to differences in banding profiles between Brood E and F (which are from the same colony) (Table 4). Furthermore, analysis of *Tubulipora plumosa* Brood M and the four tissue extracts from WB02, (whole brood from the same colony; all four extracts shared identical banding profiles (Appendix I: Figure 12)), revealed differences between these broods, thus providing evidence for polyembryony (Table 4; Figure 3). For *Plagioecia patina*, attempts were made to confirm polyembryony by analysing a sample of whole brood tissue (WB01) from the same colony as Brood H. Unfortunately, these PCR products were weak, making it difficult to visualise differences on PAGE gels (Appendix I: Figure 8) and analysis by automated electrophoresis failed to score the samples. Therefore, comparisons cannot be made and polyembryony cannot be confirmed here.

2.4 Discussion.

2.4.1 Molecular evidence for polyembryony across distinct cyclostome lineages.

This study provides the first molecular evidence for the occurrence of polyembryony in cyclostome bryozoans other than *Crisia denticulata*. Identical banding patterns, and thus larval genotypes, within broods were discovered in four species, *C. denticulata*, *Hornera robusta*, *Plagioecia patina* and *Tubulipora plumosa*, whereas genotypes differed consistently between broods (Table 4, Figure 2). Due to the non-specific nature of ISSR primers, the decision was made to not analyse maternal colony tissue due to possible contamination by food particles and attached foreign organisms. Consequently, it was not possible to genotype broods and maternal colonies, as done in the *Crisia denticulata* microsatellite analysis (Hughes *et al.*, 2005) to confirm polyembryony. Therefore, the approach was taken to genotype multiple broods from the same colony where possible. Results here from analysis of *H. robusta* and *T. plumosa* indicate that in such instances genotypes differed between broods from the same colony (Table 4, Figure 3). These results provide evidence for the cloning of progeny following genetic reorganisation within a brood, thus the genotype of progeny must differ to that of the brood-parent. This excludes apomictic parthenogenesis and indicates the likely occurrence of conventional sexual reproduction (although selfing cannot be ruled out). This evidence for polyembryony in a range of cyclostome bryozoans representing all three major clades, in addition to that from the previous molecular study (Hughes *et al.*, 2005), confirms historical inferences and suggests this phenomenon likely extends across the whole group, with the possible exception of the Cinctiporidae (Boardman *et al.*, 1992). However, the occurrence of polyembryony in the Lichenoporidae still requires confirmation and should be subject to future investigation.

2.4.2 ISSR methodology critique.

Analysis of *Crisia denticulata* broods here confirmed former findings gathered using microsatellites (Hughes *et al.*, 2005) and therefore supports the ISSR method. However, the investigation conducted here is not without limitations. Due to the limited volume of DNA eluted during extraction, it was not always possible to repeat PCRs or to run analysis with a full set of markers if repeats were required. Despite eluting DNA in 2 x 25μl elution buffer during extraction, this column-based protocol yields only approximately 40-45μl of DNA extract. Larval PCRs were performed in triplicate per primer with 3-4μl DNA per PCR.

Therefore, where 4μl DNA per PCR was used (for *Crisia denticulata* and *Tubulipora plumosa*), only nine PCRs per larva could be conducted (three PCRs per primer x three primers = 36μl DNA required), as there was insufficient DNA to repeat analysis (in triplicate) with a particular primer (which requires a further 12μl DNA).

Furthermore, analysis was limited due to the number of samples that could be analysed per run on the automated electrophoresis apparatus. The fact that only 11 samples could be analysed in a single run was a real limitation to genotype scoring. Analysis kits are expensive and can be temperamental (although perhaps this latter point applies specifically to their use in the machine used here – superior machines are apparently available). Furthermore, complementing the existing dataset with data generated using the Experion DNA 12K kit may provide further alleles for genotyping broods. An alternative method using fluorescently labelled ISSRs (Prince, 2009), where PCRs are analysed on a sequencer, is available but would likely increase costs and require lengthy optimisation.

In comparison with alternative methods, ISSRs use non-specific primers and consequently are not favoured by all. Microsatellites are viewed as a superior alternative PCR-based method to ISSRs, with their major advantage being their species-specificity. However, they are expensive to employ and require lengthy development, which is disadvantageous when attempting to screen multiple taxa (as in the case here). AFLPs require a minimum amount of starting material as their development starts with a restriction digest. This essentially precludes development based on larval material. Furthermore, although AFLPs do not require prior knowledge of genomic sequences, development can be lengthy, as with microsatellites. RAPDs are another alternative and have been used successfully in genomic fingerprinting (among other applications). They are arbitrary primers and, like ISSRs, do not require prior genomic sequence knowledge so have broad taxonomic application. However, ISSR markers are viewed as an improvement over RAPDs, in terms of reliability and reproducibility, due to their longer primers (which reduces mis-priming) and as they are anchored and targeted to microsatellite sequences (Zietkiewicz *et al.*, 1994; Casu *et al.*, 2005). Overall, the application of ISSRs here seems appropriate, as this investigation involved small quantities of template DNA and required analysis of several taxa for which markers had not been developed.

This present study marks the first application of ISSR primers to the study of polyembryony in metazoans. To date, only three studies investigating polyembryonic plants have utilised

ISSRs: to study mango tree genetic diversity (Rocha *et al.*, 2012) and for citrus seedling diagnosis (Shareefa *et al.*, 2009; Golein *et al.*, 2011). Most studies of marine invertebrates that utilise ISSRs focus on studies of genetic diversity, genetic differentiation, population structure or taxonomic distinction (inter- and intra-specific genetic relationships) (see Pannacciulli *et al.*, 2009 and references therein).

In conclusion, the confirmation of polyembryony in species representative of all major clades of cyclostome bryozoans has implications for our understanding of the evolution and retention of this apparently paradoxical reproductive mode in an ancient order. This will be addressed further in the General Discussion (Chapter 6) in the context of all the research presented in this thesis.

Table 1: Classification and sample localities of species used in genotyping analysis. WB = whole brood tissue sample.

	Species	Collection locality	Samples
Class Stenolaemata			
Order Cyclostomata			
Suborder Articulata			
Family Crisiidae	*Crisia denticulata*	Wembury, nr Plymouth, Devon, UK, intertidal	Broods A, B, C
Suborder Cancellata			
Family Horneridae	*Hornera robusta*	Shag Point, Otago, New Zealand, 85 m	Broods D, E, F
Suborder Tubuliporina			
Family Plagioeciidae	*Plagioecia patina*	Gullmar fjord, Kristineberg, Sweden, 16-20 m	Broods G, H, I, WB01
Family Tubuliporidae	*Tubulipora plumosa*	Hannafore Point, Looe, Cornwall, UK, intertidal	Brood J
		Hoe Foreshore, Plymouth, Devon, UK, intertidal	Brood K, L, M, WB02

Table 2: ISSR primers used in brood genotyping analysis. Tm = primer melting temperature, Ta = primer annealing temperature.

Primer	Sequence (5' - 3')	Tm (°C)	Ta (°C)
UBC 817	$(CA)_8A$	52.7	45
UBC 827	$(AC)_8G$	54.9	50
UBC 850	$(GT)_8YC$	53.0	50
UBC 855	$(AC)_8YT$	51.9	45
UBC 884	$HBH(AG)_7$	41.9	35

Table 3: Brood screening information. Details of the number of larvae screened per brood and the ISSR primers used for each species. Colony information illustrates broods that originated from the same colony. WB = whole brood tissue sample.

Species	Colony	Brood ID	No. of larvae	ISSR markers
Crisia denticulata	1	A	7	UBC 827, UBC 850, UBC 884
	2	B	5	
	3	C	6	UBC 827, UBC 850
Hornera robusta	1	D	10	UBC 817, UBC 855
	2	E	10	
	2	F	10	
Plagioecia patina	1	G	10	UBC 827, UBC 850, UBC 855
	2	H	9	
	2	WB 01	whole brood	
	3	I	10	
Tubulipora plumosa	1	J	6	UBC 817, UBC 850, UBC 855
	2	K	12	UBC 817, UBC 855
	3	L	10	UBC 817, UBC 850, UBC 855
	4	M	8	
	4	WB 02	whole brood	

Table 4: Overview of brood scoring and pair-wise comparison for each species. a) Brood scoring table. (i) *Crisia denticulata*, (ii) *Hornera robusta*, (iii) *Plagioecia patina*, and (iv) *Tubulipora plumosa*. Green cell = present.

a)

(i) *Crisia denticulata*				
	Band size (bp)	Brood C	Brood A	Brood B
UBC 827				
1	720			
2	751			
3	820			
4	843/862			
5	970			
6	1202			
7	1252			
8	1357			
UBC 850				
1	312			
2	451			
3	468/470			
4	490/492.5			
5	575			
6	583/585			
7	652			
8	670			
9	691.5/699			
10	814			
11	825			
12	857			
13	946/951			
14	990			
15	1308			
16	1351			
UBC 884				
1	402/408	n/a		
2	677/686	n/a		
3	732.5	n/a		
4	968	n/a		
5	1002	n/a		
6	1283	n/a		
7	1327	n/a		

a) cont'd

(ii) *Hornera robusta*

	Band size (bp)	Brood D	Brood E	Brood F
UBC 817				
1	557			■
2	559/571	■	■	
3	841		■	
4	848/861	■		■
5	1038/1043		■	■
6	1087	■		
7	1172		■	
8	1212/1264	■		■
UBC 855				
1	644/645		■	■
2	654	■		
3	726/728		■	■
4	749.5	■		
5	858/862		■	■
6	882	■		
7	981		■	
8	1100/1105		■	■
9	1129	■		
10	1267/1271		■	■
11	1372			■

a) cont'd

(iii) *Plagioecia patina*				
	Band size (bp)	Brood G	Brood H	Brood I
UBC 827				
1	448	■		
2	677			■
3	701	■		
4	824			■
5	871		■	
6	993	■		
7	1049.5		■	
8	1097/1113	■		■
9	1164		■	
UBC 850				
1	421/430	■		■
2	466	■		
3	471/474		■	■
4	699/727.5	■		■
5	819		■	
6	798/854	■		■
7	1086/1099		■	■
8	1192/1212	■		■
UBC 855				
1	563			■
2	634/638		■	■
3	660/668		■	■
4	679	■		
5	733/740		■	■
6	765	■		
7	855			■
8	885/902	■	■	
9	1218/1256/1282	■	■	■

a) cont'd

(iv) *Tubulipora plumosa*				
	Band size (bp)	Brood J	Brood K	Brood L
UBC 817				
1	426		■	
2	441			■
3	613		■	
4	641			■
5	688		■	
6	872		■	
7	1013			■
8	1058.2	■		
9	1240	■		
10	1296			■
11	1341.5/1342	■	■	
UBC 855				
1	354			■
2	874			■
3	983		■	
4	1002			■
5	1165			■
6	1205.5	■		
7	1302/1325	■		■
	Band size (bp)	Brood M	WB02	-
UBC 817				
1	461/463	■	■	-
2	477		■	-
3	678	■		-
4	781		■	-
5	879	■		-
6	906		■	-
7	1121	■		-
8	1205		■	-
UBC 850				
1	541	■		-
2	616		■	-
3	622	■		-
4	644.5		■	-
5	656	■		-
6	691		■	-
7	755/762	■	■	-
8	836	■		-
9	985.5/992	■	■	-
10	1115	■		-
11	1200		■	-
12	1294.5	■		-
UBC 855				
1	481	■		-
2	518	■		-
3	581	■		-
4	625/627.5	■	■	-
5	778.5	■		-
6	931/932	■	■	-
7	1056.5/1076	■	■	-

Table 4 continued: b) Pair-wise comparison matrices of the number of differences between broods based on the brood scoring table. Capital letters refer to brood identities; WB = whole brood. () = Level Two scoring (ultra-conservative); where no Level Two scoring is shown, there is no difference to basic scoring (for Level One scoring, see Appendix III).

b)

(i) *Crisia denticulata*

UBC 827				UBC 850				UBC 884			
	C	A	B		C	A	B		A	B	
C	-			C	-						
A	(1) 2	-		A	(7) 10	-		A	-		
B	(5) 7	(4) 7	-	B	(9) 12	(8) 10	-	B	5	-	

(ii) *Hornera robusta*

UBC 817				UBC 855							
	D	E*	F*		D	E*	F*				
D	-			D	-						
E*	(2) 6	-		E*	(6) 10	-					
F*	(2) 4	(4) 6	-	F*	(6) 10	2	-				

(iii) *Plagioecia patina*

UBC 827				UBC 850				UBC 855			
	G	H	I		G	H	I		G	H	I
G	-			G	-			G	-		
H	7	-		H	8	-		H	(1) 5	-	
I	5	6	-	I	3	5	-	I	(4) 8	(3) 3	-

(iv) *Tubulipora plumosa*

UBC 817				UBC 855							
	J	K	L		J	K	L				
J	-			J	-						
K	(5) 6	-		K	3	-					
L	(5) 7	(8) 9	-	L	5	6	-				

UBC 817				UBC 850				UBC 855			
	M*	WB02*			M*	WB02*			M*	WB02*	
M*	-			M*	-			M*	-		
WB02*	7	-		WB02*	10	-		WB02*	4	-	

* = Pair-wise comparisons between broods from the same colony.

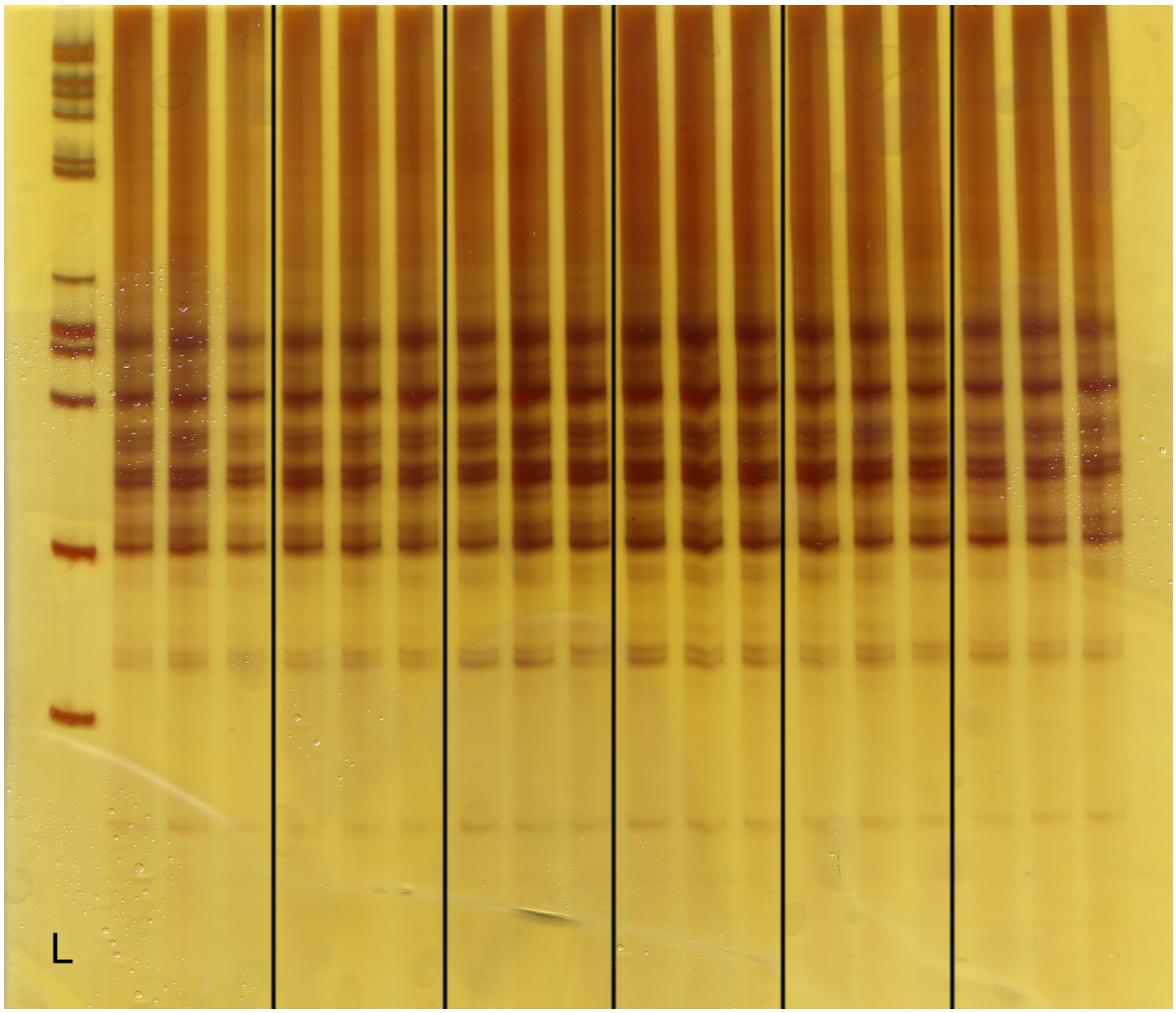


Figure 1. Example of a PAGE gel from genotyping analysis of *Tubulipora plumosa*: within-brood comparison. Gel image shows banding profiles of six larvae (in triplicate) from Brood L screened with the ISSR primer UBC 850. L = ladder.

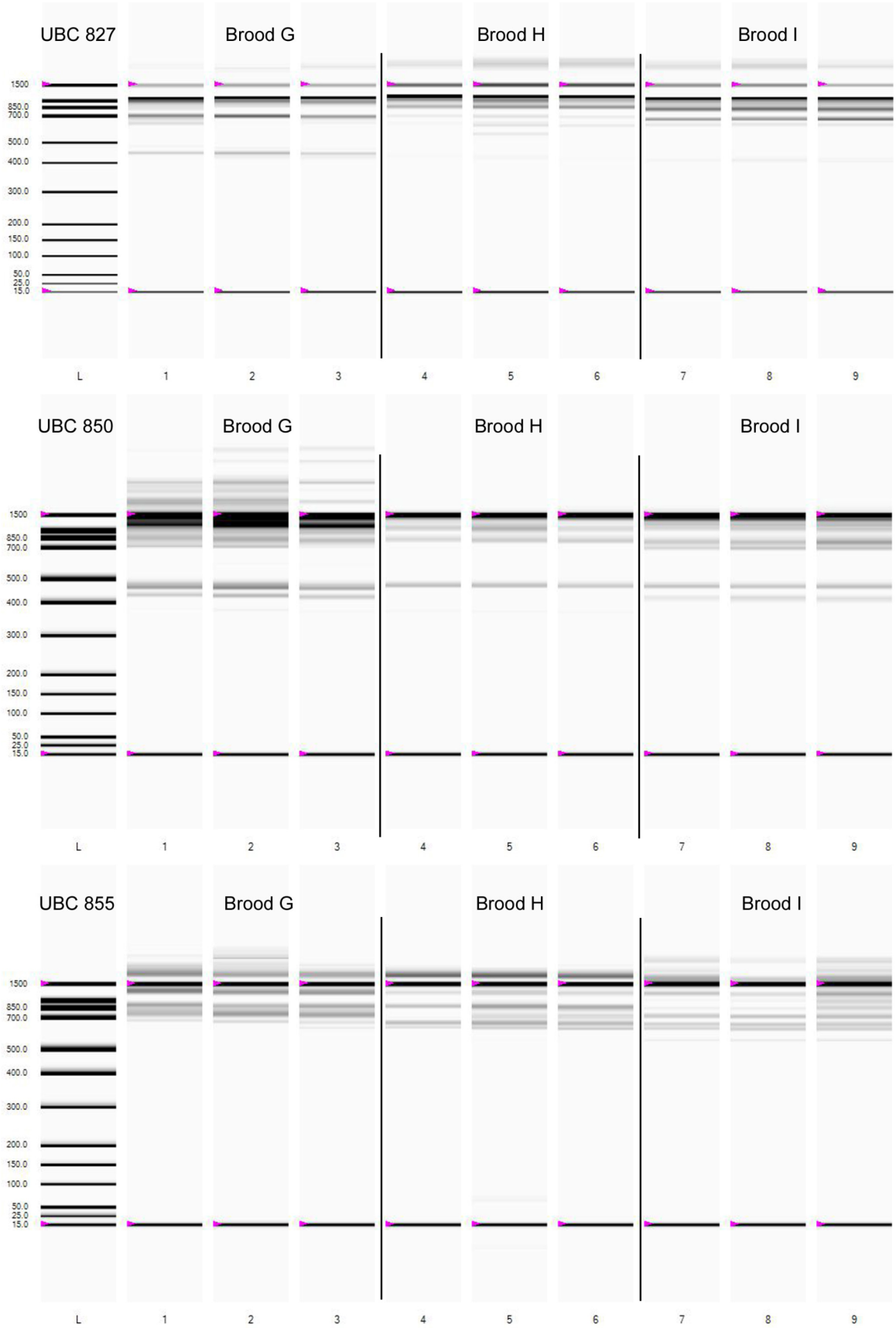


Figure 2: Set of virtual gels from genotyping analysis of *Plagioecia patina*: comparisons between broods from different colonies. Each gel image shows banding profiles, from one ISSR primer, of three individuals from three different broods. Note: larger bands (> 1.5 Kb) outside of the sizing range were not scored. L = ladder. Upper internal size marker = 1500 bp, lower internal size marker = 15 bp.

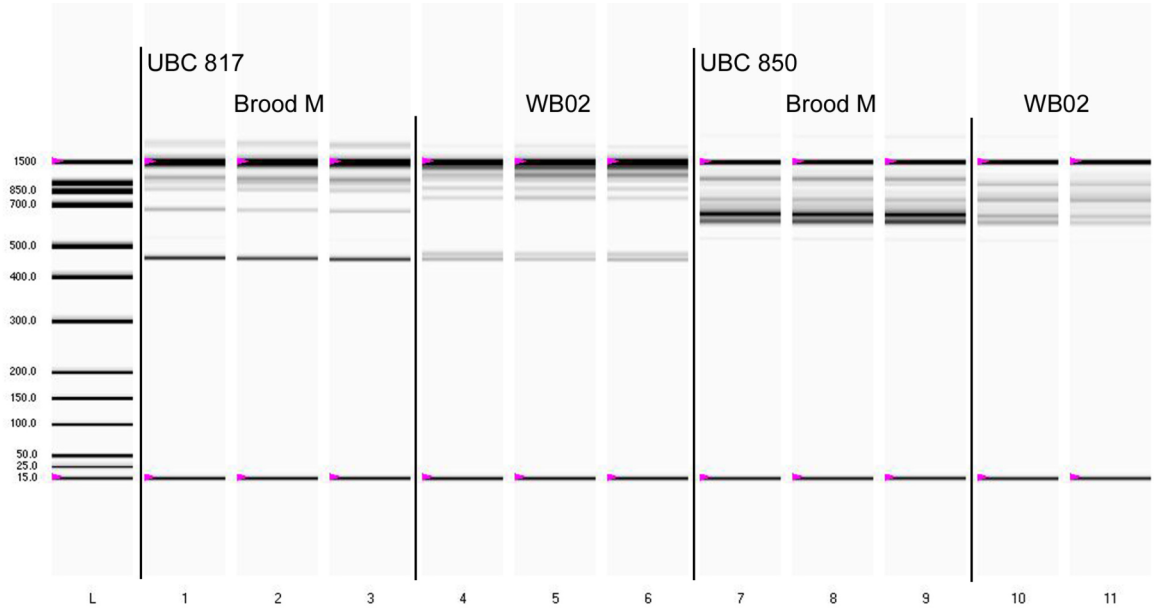


Figure 3: Virtual gel from genotyping analysis of *Tubulipora plumosa*: comparisons between broods from the same colony. Gel image shows banding profiles, from two ISSR primers, for three individuals from Brood M and two-three different tissue extracts from the same brood WB02 ('whole brood' sample). Note: larger bands (>1.5Kb) outside of the sizing range were not scored. L = ladder.
Upper internal size marker = 1500 bp, lower internal size marker = 15 bp.

Chapter 3: **Brood chamber development and the control of female investment in cyclostome bryozoans.**

3.1 Introduction.

Many members of the phylum Bryozoa exemplify polymorphism between modules, a feature often exhibited by other colonial invertebrates. Zooidal polymorphism facilitates division of labour in these modular organisms, with specialised modules for feeding, support, defence and reproduction. Spatial constraints and other external cues may influence the development of these zooidal polymorphs (e.g. Harvell, 1994; Hughes *et al.*, 2003). For instance, the receipt of conspecific allosperm is known to trigger the development of female zooids in the cheilostome bryozoan *Celleporella hyalina* (Bishop *et al.*, 2000; Hughes *et al.*, 2002). A similar triggering of female investment by allosperm is also observed in the colonial ascidian *Diplosoma listerianum*, although this occurs in the absence of zooidal polymorphism (Bishop *et al.*, 2000).

A delay in female investment until male genetic input is assured in colonial invertebrates such as bryozoans and ascidians is a trait shared with flowering plants. A modular architecture facilitates this ability to direct resources between vegetative growth and reproduction and is just one of several life history analogies identified between some modular animals and angiosperms (see Richards, 1997; Bishop *et al.*, 2000).

The present study extends investigations of female investment in bryozoans to a different major clade with a contrasting reproductive mode — polyembryony. The possibility that allosperm triggers maternal investment is examined in two bryozoan species from the order Cyclostomata, *Tubulipora plumosa* (Tubuliporina) and *Filicrisia geniculata* (Articulata). Cyclostomes lack the degree of zooidal polymorphism found in other bryozoans such as the previously studied *Celleporella hyalina,* but in addition to feeding zooids, possess female zooids (brood chambers or gonozooids) for brooding multiple larvae. Cyclostome larvae are nourished within gonozooids by specialised nutritive tissue, the cost of which is likely to create competition for resources within the colony.

Here, investigations sought to test the hypothesis that the receipt of conspecific allosperm triggers brood chamber development in cyclostome bryozoans. However, observations of

gonozooid or incipient gonozooid development (without progeny in either case) in *Tubulipora plumosa* and *Filicrisia geniculata* respectively, when in reproductive isolation prior to experimentation, suggested that allosperm may not be required to initiate gonozooid development in these species. Consequently, the original hypothesis was modified to test whether *complete* gonozooid development *and* subsequent progeny production was triggered by the presence of allosperm. Ramets of *T. plumosa* and *F. geniculata* were exposed to a source of conspecific allosperm in laboratory culture and its effect on brood chamber development, and ultimately female investment, is reported.

3.2 Materials and Methods.

3.2.1 Biology of *Tubulipora plumosa* (Tubuliporina) and *Filicrisia geniculata* (Articulata).

Tubulipora plumosa Thompson in Harmer, 1898 and *Filicrisia geniculata* (Milne Edwards, 1838) are cyclostome bryozoans of contrasting colony form and gonozooid morphology (Figure 1: a-c, *T. plumosa*; d-f, *F. geniculata*). Because they represent different cyclostome suborders, they lend a phylogenetic perspective to the investigation. *T. plumosa* is an encrusting bryozoan, forming colonies of a single, broad lobe or multiple, narrower lobes (Figure 1a). Autozooids are arranged in radiating, linear, comb-like rows within the lobes. Gonozooids are often extensive, extending between rows of autozooids, entirely or partially occupying lobes (Figure 1b). Colonies of *T. plumosa* are common in shallow water rocky habitats, where they are found encrusting a variety of substrata, including various algal species (Hayward & Ryland, 1985). *F. geniculata* is an erect bryozoan with a rather straggly or weedy colony form (Figure 1d). Branches are formed from a single series of long and slender zooids, successive zooids being separated by a non-calcified joint. Gonozooids are inflated and club-shaped (clavate) (Figure 1f). Colonies of *F. geniculata* are often found entangled with other crisiids among the sessile sward communities of the low shore, located below large boulders and overhangs (Hayward & Ryland, 1985).

3.2.2 Material collection and founding of clones.

3.2.2.1 *Tubulipora plumosa*.

In August 2010, *Tubulipora plumosa* colonies were collected on fronds of the non-native brown alga *Sargassum muticum* from the Plymouth Hoe foreshore, Devon. Isolated wild colonies with single gonozooids, each from different *S. muticum* plants, were placed in separate crystallising dishes filled with aged, 0.2μm-filtered, UV-sterilised natural seawater (FSW) and lined with seawater-preconditioned acetate sheet. Larvae released overnight subsequently settled and metamorphosed onto the acetate. Multiple ancestrulae were founded from each wild (parental) colony. Individual metamorphs (at the ancestrula stage) were isolated on trimmed acetate, mounted onto a larger piece of acetate fixed to a microscope slide and clipped into separate stirred tanks. Colonies were grown on, and cloned by artificially dividing and re-culturing the sections to form a set of independent

'subcolonies' (ramets). Only a single colony (clone) per parental colony, represented by a set of equal-sized ramets, was selected for experimentation. Henceforth, a 'clone' refers to a genetically distinct genet represented in this case by a set of ramets.

3.2.2.2 *Filicrisia geniculata*.

In August and September 2010, *Filicrisia geniculata* colonies were collected from Wembury, Devon and Hannafore Point, Looe, Cornwall. Individual small colony fragments with a single gonozooid were mounted onto a piece of acetate sheet on a microscope slide, held in place by a loop of very fine fishing line, and clipped into separate stirred tanks (one fragment per tank). Tanks were filled with aged, 0.2μm-filtered, UV-sterilised natural seawater (FSW) and lined with seawater-preconditioned acetate sheet. Colony fragments were maintained in culture and the acetate sheet was monitored daily for ancestrulae. After ~10 days, individual metamorphs were isolated into separate stirred tanks as described for *Tubulipora plumosa*. Colonies were maintained in culture conditions until attaining a suitable size for experimentation. Multiple colonies were founded from a single gonozooid, but only a single colony (clone) from each was selected for further experimentation. Each experimental clone was divided into a set of equal-sized ramets, as in *T. plumosa*.

3.2.2.3 Culturing conditions.

Tubulipora plumosa and *Filicrisia geniculata* ramets were maintained in stirred tanks (for *T. plumosa*: two ramets per tank) filled with ~850ml FSW at 16^{o}C±1^{o}C with 15:9 hour light:dark regime, and fed twice daily with a mixture of *Rhinomonas reticulata* and *Isochrysis galbana*. Water was replaced weekly and ramets were observed and regularly cleaned with a soft artist's brush (~one week intervals).

Precautions were taken against any unwanted transfer of sperm between tanks. Thus, stirrers were used to circulate water within each tank rather than aeration to prevent aerosol particles, potentially containing sperm, being formed and transferred between tanks. During water changing, hands were washed and dried with a heated air dryer between tanks. Plastic pipettes, one per tank, were used to deliver food and these were dried between feeds, with separate sets for morning and afternoon feeding.

3.2.3 Experimental procedure.

3.2.3.1 General experimental details.

I conducted 'single-clone' and 'mixed-clone' treatments. In the 'single-clone' treatment, two ramets from the same clone were placed within a tank. The 'mixed-clone' treatment consisted of a single ramet from each of two different clones being placed within a tank (Figure 2). Consequently, each tank contained two ramets therefore the degree of crowding was equal in both treatments. Furthermore, for each species, the number of tanks in each treatment was equal, as were the number of ramets per clone in each treatment (these varied between the species due to number of available clones). Experiments were conducted under culturing conditions identical to those used previously to maintain ramets. Tank order on shelves was randomised to reduce any potential effects of shelf position. All tanks were subject to an equal number of water changes so opportunities for the potential loss of sperm and larvae were equal.

3.2.3.2 Species-specific information.

Tubulipora plumosa:
Five clones provided material for this experiment. Ramets of each clone were divided in half four times at four week intervals to obtain 16 ramets per clone. Some gonozooids (but not larvae) were produced during this 'growth' phase by some clones but these were avoided when producing the final ramets for the experiment (at the fourth 'cut'). Two equal-sized ramets were placed in each tank. The single-clone treatment comprised four tanks per clone (total no. ramets per clone = eight) and the mixed-clone treatment comprised 20 tanks, two tanks per cross (total no. ramets per clone = eight) (Table 1).

Filicrisia geniculata:
Only four clones achieved a size large enough to provide the required number of ramets at the required size. Two colony types had been identified whilst rearing colonies in isolation prior to experimentation: 'Type 1' colonies, composed of only regular autozooids, and 'Type 2' colonies, which also developed incomplete gonozooids (Figure 3). The following experiment involved two clones of each colony type: Clones A & D were Type 1 colonies and Type 2 colonies were represented by Clones B & C. Each clone was divided into 12 ramets, each with ~8-12 branch tips with feeding autozooids. Two ramets were placed in each tank.

For the single-clone treatment, three tanks per clone were set up (total no. ramets per clone = six). The mixed-clone treatment comprised 12 tanks, two tanks per cross (total no. ramets per clone = six) (Table 1).

3.2.3.3 Complementary study – exposure of *Filicrisia geniculata* to a single dose of allosperm.

Six fragments of Type 2 Clone C, all with developing gonozooids (in addition to autozooids), were mounted onto separate slides as described in Section 3.2.2.2. Two experimental treatments were conducted: 'exposure' and 'control'. In the 'exposure' treatment, three fragments were placed into a tank containing allosperm in suspension (but not Type 1 colonies). The presence of allosperm in suspension was confirmed using techniques described by Bishop (1998) and explained in detail in Chapter 4. In the 'control' treatment, the remaining three fragments were placed in a tank containing clean FSW only. Tanks were maintained under standard culture conditions as described in Section 3.2.2.3 and the fragments were monitored for completion of gonozooids.

3.2.4 Data collection and analysis.

All colonies were monitored for the appearance of gonozooids and progeny. Counts were made of the number of completed gonozooids per ramet and the number of progeny produced per tank. Only settled progeny could be recorded as swimming larvae may be lost during water changes. Any bias in the effect of larval loss was minimised by undertaking an equal number of water changes for all tanks.

Statistical analysis of count data was conducted where possible (*T. plumosa* only) to assess the effect of conspecific allosperm on gonozooid development and progeny production. A replicated G-test was conducted to assess the overall effect of the treatments on gonozooid production and on the overall response of clones (McDonald, 2009). Progeny production between treatments was assessed using a Mann-Whitney U test in Minitab.

3.3 Results.

3.3.1 *Tubulipora plumosa*.

Gonozooids were first observed developing by Week 4 in some single- and some mixed-clone ramets. Only a few gonozooids were complete at this stage, occurring as densely punctate patches (calcified outer walls) developing at the colony's growing edge. No progeny were observed at this time. At Week 8, counts of gonozooids and settled progeny were made. Most progeny at this time were newly settled, either at primary disc or ancestrula stage. Only a few had reached three-four autozooids in size. Adult ramets were transferred onto new slides in new tanks at this time to reduce the risk of cross-fertilisation with developing progeny and to enable counting of further progeny. At Week 12, final counts of gonozooids per ramet and progeny per tank were made.

Gonozooids were produced by all clones in the mixed-clone treatment (present in 30 ramets out of 40) and by four clones in the single-clone treatment (present in 16 ramets out of 40). The presence of gonozooids in the single-clone treatment provides evidence of gonozooid development in the absence of allosperm. One clone (Clone 4) developed only a single gonozooid in the mixed-clone treatment and none in the single-clone treatment; however, in the mixed-clone treatment, gonozooids and progeny were produced by the companion clone.

Overall, results indicated that gonozooid production depended on treatment (replicated G-test, pooled $G = 211.8$, d.f. = 1, $p<0.0001$). Thus, the number of gonozooids produced differed between treatments, with more in the mixed-clone treatment (mean = 6.625, SD = 6.02, n = 40) than in the single-clone treatment (mean = 0.775, SD = 1.230, n = 40) (Table 2; see Appendix V for all raw data).

Overall, the clones did not differ in their response to the two treatments (replicated G-test, heterogeneity $G = 7.693$, d.f. = 4, $p = 0.1035$) — gonozooid production increased in ramets in the mixed-clone treatment in all clones. Figure 4 shows this relatively homogenous response across clones and that clones rank the same in both treatments.

Progeny counts per tank give an estimate of progeny production per gonozooid in each treatment. Overall, progeny production was greater in mixed-clone tank gonozooids (mean= 69.2, SD = 56.1, n = 20) compared to those from single-clone tanks (mean = 15.56, SD =

20.62, n = 9) (Mann-Whitney U, W = 3620, p = 0.0037) (Table 3). An absolute figure of progeny production per gonozooid was not possible as: (a) progeny cannot be assigned to a particular ramet or gonozooid within a tank (due to multiple gonozooids present within each tank); (b) progeny cannot be counted directly *in situ* within a gonozooid (as prevented by the opaque, calcified outer skeleton); and (c) some larvae may be lost through water changing (an effect minimised by the equal number of water changes undergone by all tanks). Counts of metamorphosed i.e. settled larvae per tank were used to estimate larval production per gonozooid by assuming that all gonozooids contributed equally. There was wide variation in progeny per gonozooid between tanks in both treatments (Table 3). Progeny production in single-clone tanks provides evidence for self-fertilisation, which is only undertaken in Clones 1, 2, and 5 (although gonozooids were produced, no progeny were recorded from single-clone tanks of Clone 3). The overall frequency of tanks with progeny depended on treatment (Chi-squared: X^2 = 15, d.f = 1, p<0.001), with 18 out of 20 mixed-clone tanks and 6 out of 20 single-clone tanks having progeny.

3.3.2 *Filicrisia geniculata*.

After 20 weeks, no completed gonozooids developed in any ramet in the single-clone treatment in any clone. In the mixed-clone treatment, only Clones B and C produced completed gonozooids but only when crossed with ramets of Clones A and D; in single-clone tanks, these clones (B & C) produced incomplete gonozooids. Clones A and D developed only autozooids in all ramets in both treatments.

Gonozooid production depended on clone with completed gonozooids only produced in Clones B and C (Chi-squared: X^2 = 161.738, d.f. = 3, p<0.0001) (Table 4). A very clear-cut pattern of two distinct colony types was thus observed. Type 1 colonies (Clones A & D) were composed solely of autozooids; Type 2 colonies (Clones B & C) formed incomplete gonozooids, in addition to autozooids, in reproductive isolation and when reared with another Type 2 colony (Figure 3a). Completed gonozooids were only produced in Type 2 colonies in the presence of Type 1 colonies (Figure 3d).

Progeny were recorded from a total of three tanks over the duration of the experiment, all from the mixed-clone treatments (Table 5). However, despite efforts to thoroughly examine colonies, metamorphs could potentially settle onto branches of ramets and be difficult to count or be obscured altogether (even in tanks where gonozooids were present but no

progeny were scored). Therefore, alongside the reasons outlined in the above *T. plumosa* section, progeny counts should be considered as estimates.

Branches with individual brood chambers (representing all successful crosses) were isolated and retained in culture to continue to monitor progeny production over time (see Chapter 5 for details).

3.3.2.1 Observations of gonozooid development in Type 2 colonies of *F. geniculata*.

The development of gonozooids was observed only in Type 2 colonies. This was first recognised under culture conditions when colonies were reared in reproductive isolation during the preparatory stages of the experiment. Monitoring the progress of gonozooid growth among ramets of Type 2 colonies indicated a succession of key developmental stages. Gonozooids first became evident at the growing tips of branches as the newly formed female zooid budded from the preceeding autozooid (Figure 3a). Here, the early gonozooid appeared as a slightly widened structure with a densely punctate outer wall. As growth progressed, the zooid became more expanded to form a cup-shaped structure. At this stage, a polypide with a transitory lophophore was present, with short lophophore tentacles visible emerging from the 'cup' (Figure 3b). Following this, development appeared to progress in one of two possible directions depending on culturing conditions. When reared in reproductive isolation or in the presence of another Type 2 colony, any further gonozooid growth was aborted and the zooids became non-functional. The incomplete gonozooid remained either as a cup-shaped structure, with the opening sealed over, or with a short wide autozooid-like opening extending from it (Figure 3c). Feeding was not observed in either form, nor was the lophophore, suggesting degeneration of the polypide. However, gonozooid development continued when this colony type was cultured in the presence of Type 1 colonies, resulting in the production of fully formed functional gonozooids (Figure 3d). Broods of mature larvae were released from these zooids and successfully metamorphosed into functional colonies.

3.3.3 Complementary study – exposure of *Filicrisia geniculata* to a single dose of allosperm.

Exposing fragments of Type 2 Clone C to a single dose of allosperm, confirms that it is allosperm that triggers the completion of developing gonozooids. Following exposure to

allosperm, completed gonozooids were found only in those fragments reared in the ‘exposure’ tank (a single completed gonozooid was present in two of the three fragments), with incomplete gonozooids continuing to be formed beyond these. No completed gonozooids were produced in any of the three control fragments.

3.4 Discussion.

These investigations revealed variation in reproductive traits between the two species. The encrusting *Tubulipora plumosa* (suborder Tubuliporina) demonstrated a greater degree of female investment in the presence of conspecific allosperm, along with some evidence of selfing when in reproductive isolation. There was no evidence of selfing in the erect species *Filicrisia geniculata* (suborder Articulata). Complete gonozooids developed only in one of the two colony types identified and only when in the presence of the alternative colony form.

3.4.1 *Tubulipora plumosa*.

The response of *Tubulipora plumosa* clones to the two treatments was homogeneous. Exposure to conspecific allosperm, as demonstrated in the mixed-clone treatment, appears to greatly increase gonozooid production (8.5-fold increase overall). With a single exception (Clone 4), all clones acted as both sperm donors and recipients as expected in functioning simultaneous hermaphrodites. Variation in overall female activity between clones was observed. The reproductive activity of one clone, Clone 4, suggested sole investment in sperm production or that female investment was rare. This clone did not (routinely) produce female zooids (only a single gonozooid developed out of all 16 ramets) and showed no evidence of selfing. However, it did appear to act as a sperm donor as suggested by the increased gonozooid production observed in companion (recipient) clones in the mixed-clone treatment.

Evidence of selfing in *Tubulipora plumosa* was identified here. Selfing is an advantage afforded by simultaneous hermaphroditism, providing reproductive assurance in isolation. However, this advantage may be compromised by reduced offspring production or survival (e.g. Johnson, 2010). Selfing activity here may have entailed some cost due to inbreeding regulation as significantly fewer gonozooids and metamorphosed progeny were produced by single-clone ramets. However, progeny production within treatments varied widely in both single- and mixed-clone tanks.

Evidence from the literature suggests that high levels of selfing are generally avoided in hermaphroditic colonial marine invertebrates (Ryland & Bishop, 1993; Knowlton & Jackson, 1993; Bishop *et al.*, 1996; Hoare & Hughes, 2001; Hoare *et al.*, 1999). Selfing has been reported in some reproductively isolated colonies of the gymnolaemate bryozoan *Celleporella*

hyalina, with populations from different geographic locations apparently varying in selfing ability (Hughes *et al.*, 2002). Despite clear evidence of inbreeding depression in some populations (Hoare & Hughes, 2001), all *C. hyalina* colonies in a minority of populations were reproductively self-compatible and selfed progeny showed no sign of inbreeding depression (Hughes *et al.*, 2002). In another bryozoan, *Bugula stolonifera*, selfing entails reduced larval production and survival and those selfed progeny that did survive did not successfully reproduce (Johnson, 2010). The reproductive success of progeny was not investigated in the present study and provides an opportunity for further work, particularly in terms of inbreeding depression.

The findings presented here provide evidence for greater female investment in the presence of conspecific allosperm. However, maternal investment is not completely deferred until cross-fertilisation is assured, since selfing occurred when colonies are reproductively isolated. This suggests that colonies are able to regulate maternal investment in relation to availability of allosperm. Colonies are therefore apparently able to avoid directing a substantial proportion of resources to female function until receipt of conspecific allosperm. This is in accordance with evidence from the cheilostome bryozoan *Celleporella hyalina* (Bishop *et al.*, 2000; Hughes *et al.*, 2002).

3.4.2 *Filicrisia geniculata*.

Results presented here are entirely consistent with the existence of colonies of separate sexes in *Filicrisia geniculata*. Colonies composed solely of autozooids (Type 1), and that show no tendency to form gonozooids at all, should be considered putative males. Colonies of this type failed to develop gonozooids under either experimental treatment. The completion of gonozooids in the alternative colony form when reared in the presence of Type 1 colonies suggested that these (Type 1) colonies act as sperm donors. On the basis of this evidence, colonies that have a tendency to form gonozooids even in isolation (Type 2) should be considered putative females. The occurrence of incomplete gonozooids when in isolation or when reared only with other putative females, suggests that (1) sperm are not produced, (2) autosperm do not have same affect as allosperm, or (3) the production of incompatible allosperm which trigger an inbreeding regulatory response in the recipient colony if, by chance, the Type 2 colonies are more closely related to each other than to the Type 1 colonies. These alternative scenarios for sperm production in Type 2 colonies, in addition to

the evidence of potential gonochorism in *F. geniculata* overall, requires further investigation and will be addressed in the following chapter.

Observations of gonozooid development in *Filicrisia geniculata* here confirmed the presence of a transitory lophophore in young female zooids as described by Borg (1926). Silén (1972) proposed that sperm acceptance was via the lophophore tentacles that can be seen extending from the developing gonozooid. Evidence from exposing putative female colony fragments to a single dose of allosperm suggests that incipient gonozooids may need to be at a particular developmental stage before being receptive to sperm uptake. Despite experimental fragments possessing many developing gonozooids, only one completed gonozooid was produced after exposure, suggesting the capture of allosperm by individual zooid(s) during a critical interval. However, before any firm inferences can be drawn, the fertilisation mechanisms of *F. geniculata* require further investigation, including confirmation of the transitory lophophore as the actual site of sperm uptake.

The observations detailed here indicate that some degree of female investment, in the form of incomplete gonozooids, is made prior to the receipt of allosperm in putative female colonies of *Filicrisia geniculata*. In this case, it is perhaps the greater investment in brooding offspring that is delayed until fertilisation is assured and this is what is controlled by availability of allosperm. Furthermore, the continued production of incomplete gonozooids after the formation of completed one(s) in the absence of allosperm (as shown in the single exposure to allosperm trial), suggests a zooid-by-zooid basis to gonozooid development. This also implies that sperm are not stored. Thus, from a developmental point of view, gonozooid development begins without fertilisation in *F. geniculata*. This is contrary to the view of Ryland (2000) that gonozooid formation occurs only after fertilisation in crisiids. This issue will be discussed further in the General Discussion (Chapter 6).

In conclusion, evidence from the investigations conducted here with two species of cyclostome bryozoan indicate that female investment per se is not completely delayed until fertilisation is assured. In addition, there is a slight but key difference in the control of female investment between the two species under investigation. In *Tubulipora plumosa*, the general degree of investment depends on allosperm availability; whereas in *Filicrisia geniculata*, the completion of gonozooids followed by brooding appears to be controlled by allosperm availability.

Table 1: All possible crosses performed between all clones in the mixed-clone treatment for each species (two tanks per cross).

Species	Clone	Clone
Filicrisia geniculata	A	B
	A	C
	A	D
	B	C
	B	D
	C	D
Tubulipora plumosa	1	2
	1	3
	1	4
	1	5
	2	3
	2	4
	2	5
	3	4
	3	5
	4	5

Table 2: The total number of gonozooids produced by *Tubulipora plumosa* clones in each treatment (from total of eight ramets per clone per treatment).

Clone	single-clone	mixed-clone
1	8	75
2	18	93
3	3	57
4	0	1
5	2	39

Table 3: Estimated progeny production in *Tubulipora plumosa* in both treatments. All replicates of crosses are shown in 'Tank' column ('b' = slide at back of tank; 'f' = slide at front of tank). Note: GZ = gonozooid.

Treatment	Tank	Total no. of GZ per tank	Total no. of progeny per tank	No. of progeny per GZ
MIXED	1b x 2f	27	2210	81.9
MIXED	1f x 2b	31	2464	79.5
MIXED	1b x 3f	14	1195	85.4
MIXED	1f x 3b	22	1926	87.5
MIXED	1f x 4b	20	594	29.7
MIXED	1b x 4f	10	1872	187.2
MIXED	1b x 5f	9	36	4.0
MIXED	1f x 5b	2	446	223.0
MIXED	2b x 3f	22	1807	82.1
MIXED	2f x 3b	21	1555	74.0
MIXED	2b x 4f	3	98	32.7
MIXED	2f x 4b	10	471	47.1
MIXED	2b x 5f	17	1729	101.7
MIXED	2f x 5b	29	1783	61.5
MIXED	3b x 4f	4	88	22.0
MIXED	3f x 4b	13	1003	77.2
MIXED	3b x 5f	2	0	0.0
MIXED	3f x 5b	2	0	0.0
MIXED	4b x 5f	4	226	56.5
MIXED	4f x 5b	3	154	51.3
SINGLE	1A x 1A	0	0	-
SINGLE	1B x 1B	0	0	-
SINGLE	1C x 1C	3	0	0.0
SINGLE	1D x 1D	5	9	1.8
SINGLE	2A x 2A	7	39	5.6
SINGLE	2B x 2B	2	124	62.0
SINGLE	2C x 2C	2	42	21.0
SINGLE	2D x 2D	7	200	28.6
SINGLE	3A x 3A	1	0	0.0
SINGLE	3B x 3B	2	0	0.0
SINGLE	3C x 3C	0	0	-
SINGLE	3D x 3D	0	0	-
SINGLE	4A x 4A	0	0	-
SINGLE	4B x 4B	0	0	-
SINGLE	4C x 4C	0	0	-
SINGLE	4D x 4D	0	0	-
SINGLE	5A x 5A	0	0	-
SINGLE	5B x 5B	0	0	-
SINGLE	5C x 5C	0	0	-
SINGLE	5D x 5D	2	42	21.0

Table 4: The total number of complete gonozooids produced by all *Filicrisia geniculata* clones in each treatment (from total of six ramets per clone per treatment).

Clone	Single-clone	Mixed-clone
A	0	0
B	0	24
C	0	79
D	0	0

Table 5: Total number of completed gonozooids and progeny produced by *Filicrisia geniculata* Type 2 clones (bold) in the mixed-clone treatment (crosses involving both colony types). Number of progeny recorded over the duration of experiment. Note: 'Rep' = replicate, 'GZ' = gonozooid, Type 1 colonies = Clones A & D, Type 2 colonies = Clones B & C.

Cross	Tank	No. of completed GZ	No. of progeny
A x **B**	Rep 1	3	0
	Rep 2	10	1
B x D	Rep 1	2	0
	Rep 2	9	0
A x **C**	Rep 1	33	0
	Rep 2	20	116
C x D	Rep 1	16	9
	Rep 2	10	0

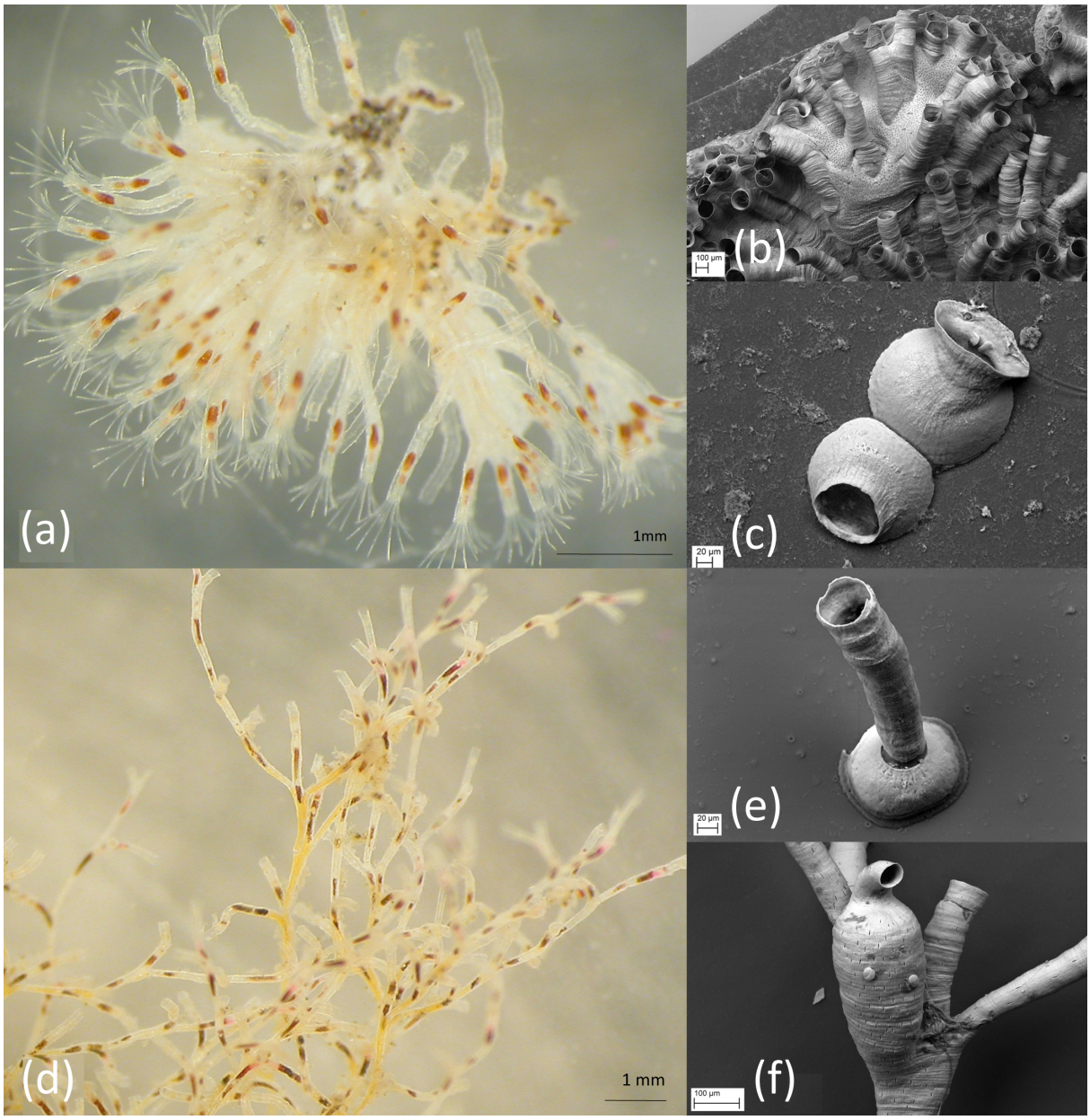


Figure 1: Images of *Tubulipora plumosa* and *Filicrisia geniculata* from light microscopy and scanning electron microscopy. *T. plumosa* (a) colony in culture, (b) part of colony with gonozooid, (c) ancestrulae. *F. geniculata* (d) colony in culture, (e) ancestrula, (f) complete gonozooid.

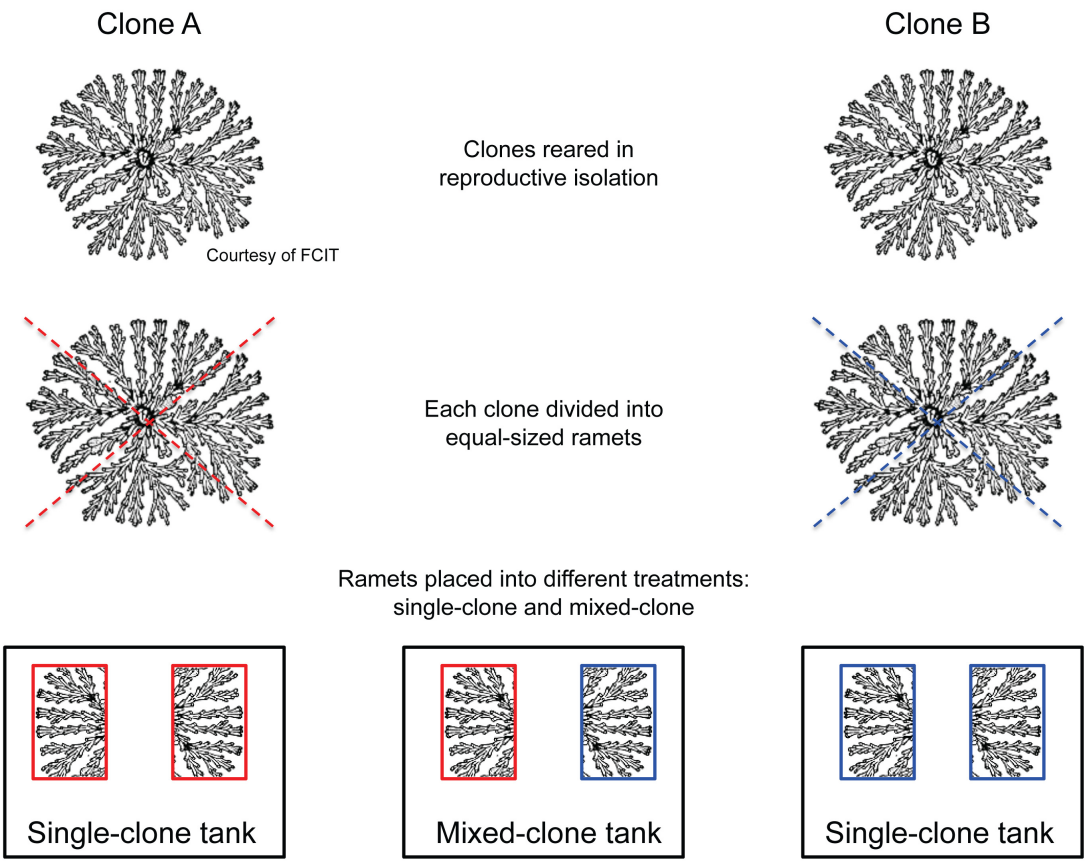


Figure 2: Diagram outlining experimental set-up.

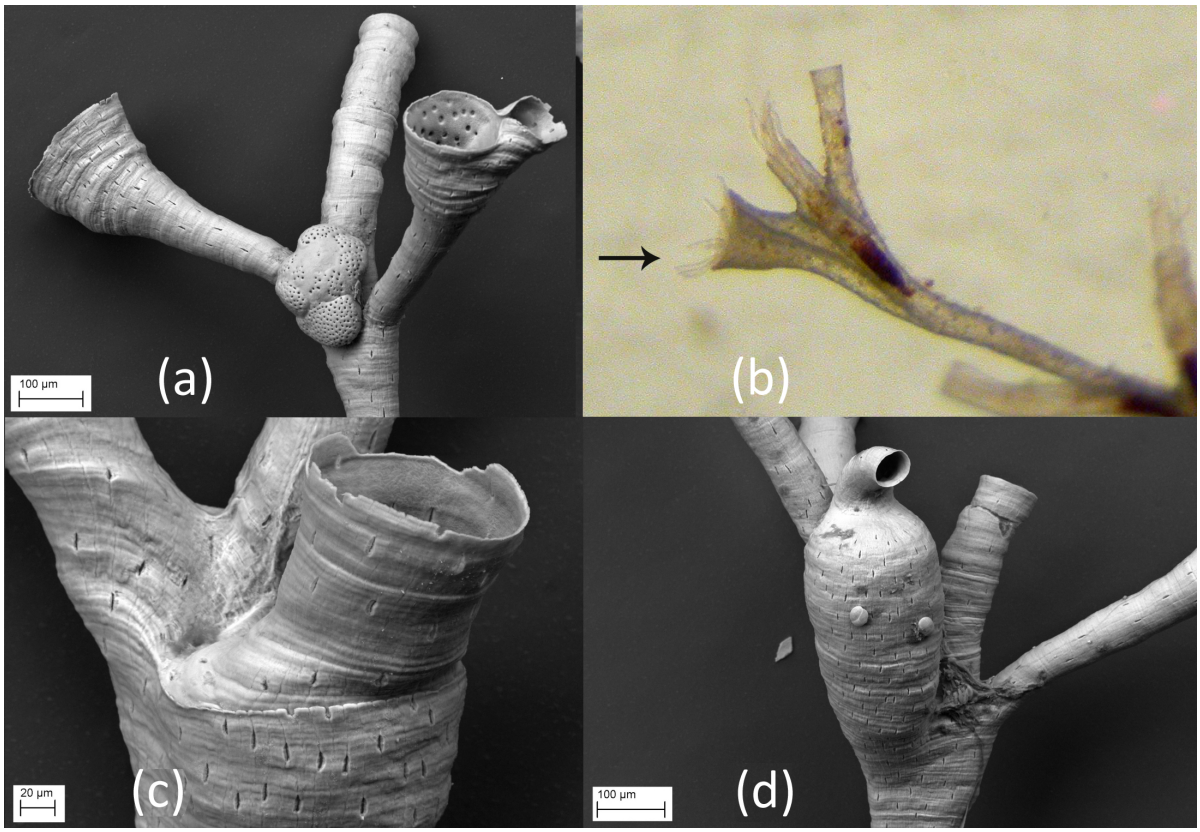


Figure 3: Gonozooid developmental stages in *Filicrisia geniculata* from light microscopy and scanning electron microscopy: (a) developing gonozooids, (b) transitory lophophore in cup-shaped gonozooid, (c) incomplete gonozooid, (d) completed gonozooid with ooeciostome (colony also bears foraminiferan).

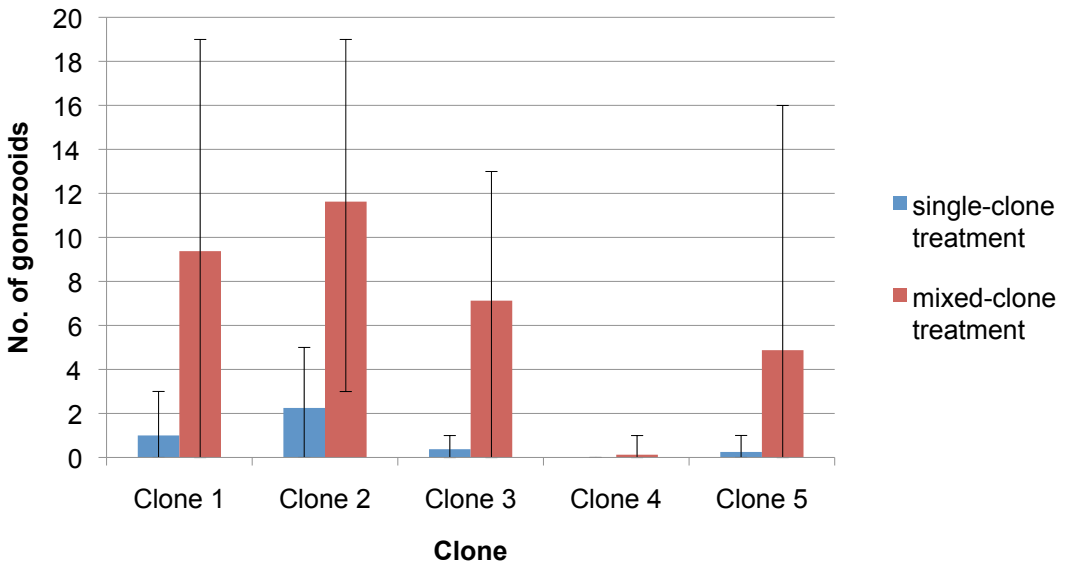


Figure 4: A graph of the mean number of gonozooids produced by *Tubulipora plumosa* clones in the two experimental treatments (from a total of eight ramets per clone per treatment). The range of gonozooid production among clonal ramets in each treatment is also shown, as indicated by error bars.

Chapter 4: **Gender specialisation in *Filicrisia geniculata*.**

4.1.Introduction.

Hermaphroditic organisms produce both male and female gametes, in contrast to gonochorists, where separate sexes exist. Many modular or colonial invertebrates are hermaphroditic and display variation in the condition at the zooid level. Thus, individual colony units (zooids) and consequently the colony itself can be hermaphroditic, or units can be single-sex, yielding a hermaphroditic colony (Ryland & Bishop, 1993). The latter form is equivalent to monoecy in plants and is widespread among colonial invertebrates — thus with plants and colonial invertebrates sharing a sessile adult habit there appears to have been parallel adaptation to this lifestyle in the two groups (Hughes, 2005). Hermaphroditism in both groups may be simultaneous, with gametes of both types produced at the same time. In colonial invertebrates hermaphroditism may also be sequential, with zooids first being functionally male (protandry) or female (protogyny); sequential hermaphroditism is very rare in plants (Avise, 2011).

Bryozoan colonies are hermaphroditic, with individual autozooids either bisexual (zooidal hermaphrodites) or single-sex within the same colony (zooidal gonochorists) (Ryland, 1970; Ostrovsky *et al*., 2008). Zooidal gonochorism may be a characteristic of cyclostome bryozoan colonies as eggs only develop in gonozooids or brood chambers (i.e. female zooids) (Hayward & Ryland, 1985). Dioecy or gonochorism (separate sexes) at the colony level is not known in bryozoans (Ryland & Bishop, 1993). However, in Chapter 3, evidence gathered from an investigation into gonozooid development, and therefore the control of female investment, pointed to the existence of separate sexes in *Filicrisia geniculata*. Two distinct colony types were identified, with complete gonozooids and progeny produced in one colony type (putative females: 'Type 2') but only in the presence of the other colony type (putative males: 'Type 1'). In the latter, colonies were composed solely of autozooids whereas the former had a tendency to form incomplete gonozooids, in addition to autozooids, when in reproductive isolation.

The basis for the occurrence of separate sexes as detailed in the previous chapter relates only to evidence of female reproductive function. In order to gain further support for this assertion, information relating to male reproductive output in *F. geniculata* is required. The

aim of this study was to assess sperm production in both colony types and to ascertain the extent of gender specialisation in this species.

4.2.Materials and Methods.

4.2.1 Experimental specimen details.

Specimens utilised in the experiment consisted in part of newly settled colonies obtained as described in section 4.2.2 below (identified as 2011 in Table 1). This material was supplemented by adult ramets (identified as 2010 in Table 1) and from progeny (identified as Progeny in Table 1) derived from experiments detailed in Chapter 3. Both of these sources of supplementary material were reared in reproductive isolation. Samples included both of the colony types described in Chapter 3, based on the presence or absence of incomplete gonozooids: 'Type 1' colonies consist only of autozooids; 'Type 2' colonies develop both autozooids and incomplete gonozooids when reared in reproductive isolation.

4.2.2 Founding of additional colonies.

In July and August 2011, *Filicrisia geniculata* colonies were collected from Wembury, Devon. Small colony fragments with brood chambers were placed in separate crystallising dishes filled with aged, 0.2*μ*m-filtered, UV-sterilised natural seawater (FSW) and lined with seawater-preconditioned acetate sheet. Larvae were released overnight, and subsequently settled and metamorphosed onto the acetate. Acetate was trimmed around metamorphs (at the ancestrula stage), mounted onto a larger piece of acetate fixed to a microscope slide and clipped into separate stirred tanks. Colonies were maintained under culture conditions until attaining a suitable size for experimentation, as described in Chapter 3 (Materials and Methods, section 3.2.2.3). Multiple colonies were founded from a single gonozooid but only a single colony from each was selected for further experimentation. Colonies of both types were founded (Table 1).

4.2.3 Filtering sperm from seawater and sperm counting procedure.

The technique employed here is similar to that described by Bishop (1998). At each sampling event (see below), 30ml of culture tank water was collected in a disposable syringe and passed through a gridded 25mm diameter, 0.45um pore-size black nitrocelluose membrane filter (Swinex filter system). The filter was then positioned onto a glass microscope slide and 2 drops of 0.1mg ml^{-1} Hoechst 33342 stain (bis-benzimide trihydrochloride; Sigma) in distilled water was applied to the filter, before covering with a cover slip and sealing edges with nail

varnish. The preparation was illuminated with 330-380nm wavelength UV light emitted through a 420nm long pass filter using a Nikon Eclipse E1000 microscope in a darkened room.

For each filter, two transects, one vertical and one horizontal, were made (magnification = x200) and all sperm encountered between two graduations on an eye-piece graticule were counted. To ensure coverage of the maximum diameter of the filter, each transect passed through the centre of the filter.

4.2.4 Preliminary experiment – duration of sperm in suspension.

Colonies were placed in stirred tanks containing clean aged FSW for 24h. After this period, a sample of water was collected, filtered and stained as detailed above (section 4.2.3). Colonies were removed from tanks at this point but the water was retained in the tanks and stirred overnight. The following day (after 48h), a further water sample was taken and processed as before. These preliminary investigations demonstrated that sperm remained visible on filters 24h and 48h after the colony was removed from a tank. Water samples therefore could be collected at any time, as any sperm released within the previous 24h period remained visible. This information is necessary in case 'females' release sperm at different time to 'males'.

4.2.5 Estimation of sperm production.

Individual colonies were placed in separate stirred tanks filled with clean aged FSW for 24h. After 24h, a 30ml sample of tank water was taken from each culture tank and processed as detailed above (section 4.2.3) and sperm present on filters was counted. Water in tanks containing colonies was replaced following each sampling event and the procedure was repeated twice over the following two days, resulting in a total of three water samples per colony being taken over a three-day period.

Type 1 colonies were then rinsed in distilled water, dried at room temperature and weighed on an analytical balance, in order to calculate sperm production per unit dry weight.

In order to investigate the incidence of incomplete gonozooids in reproductive isolation, Type 2 colonies were preserved in 100% ethanol and counts were made of the number of

autozooids and incomplete gonozooids present in 10 randomly sampled branches per colony. The proportion of incomplete gonozooids in each colony was determined as a proportion of the total number of zooids (autozooids + gonozooids) pooled across the 10 branches sampled per colony. Colonies were then dried and weighed.

4.2.6 Statistical analysis.

For each Type 1 colony, sperm counts were averaged over the two sweeps and the estimated number of sperm per unit dry weight was calculated for each repeated measurement (i.e. three values for sperm mg^{-1} dry weight per colony). Data were tested for equality of variances using Levene's test and Bartlett's test and for normality using Kolmogorov-Smirnov test. Bartletts's and Kolmogorov-Smirnov test statistics were significant and remained so after log transformation (Levene's test statistics were non-significant). The non-parametric Friedman test for repeated measures was therefore performed on the untransformed data in Minitab (factor = colony, repeated measure = day (or sampling event) and vice-versa to make the test a 'two-way' analysis).

For each Type 2 colony, the proportion of zooids that were incomplete gonozooids was calculated for each branch (10 per colony). Data (untransformed and then arcsine square-root transformed) were tested for equality of variances using Bartlett's test and Levene's test but both datasets yielded significant test statistics. Therefore, the non-parametric Kruskal-Wallis test was performed on the untransformed data (individual branches were treated as replicates for each colony) in Minitab (factor = colony).

4.3 Results.

Measurements of colony dry weight indicated that colonies of both types were of a similar size range and included both large and small colonies (Type 1: mean = 35.6mg, SD = 35.2, n = 6, range = 11 –103.3mg; Type 2: mean = 51.8, SD = 36.9, n = 8, range = 9.6 – 121.6mg (Table 1)). Dry weight did not differ significantly between the two colony types (Two-sample t-test: t = 0.8307, d.f. = 11, p = 0.4235).

Sperm production was observed in all Type 1 colonies but not in any Type 2 colonies, indicating that sperm production is associated with the absence of gonozooids and therefore depends on colony type (as based on presence/absence of incomplete gonozooids) (Fisher's exact test, p = 0.0003) (see Appendix VI for raw sperm count data). Within Type 1 colonies, sperm production appeared consistent over time, but average sperm production varied 24-fold across all colonies (2.44 – 58.6 sperm mg^{-1} dry weight) (Table 2). Statistical analysis indicated a significant difference in sperm production per unit dry weight between colonies (Friedman test, S = 14.62, d.f. = 5, p = 0.012), which was not affected by different sampling events (Friedman test, S = 1.00, d.f. = 2, p = 0.607).

There was 5-fold variation in the proportion of gonozooids between colonies (proportion range: 0.03 – 0.15) (Table 3; see Appendix VII for raw count data) and incomplete gonozooid production depended on colony identity (Kruskal-Wallis, H = 56.61, d.f. = 7, p <0.001).

One notable general observation was the sequential production of multiple incomplete gonozooids, forming 'stacks', in some branches in some Type 2 colonies (Figure 1). Such branches appeared to lack autozooids, and subsequently died off perhaps due to insufficient support as a result of lack of autozooids, thereby potentially sacrificing overall colony health.

4.4 Discussion.

4.4.1 Colony types and the development of male and female function in crisiids.

The analysis of sperm production presented here confirms that Type 1 and Type 2 colonies should be considered 'male' and 'female' respectively and provides evidence to support the conclusion made in Chapter 3, that separate sexes exist in *Filicrisia geniculata*. Furthermore, the founding of new colonies of both types suggests that the findings described in Chapter 3 were not anomalies. Finally, these present findings rule out the alternative scenarios outlined in Chapter 3 to explain the lack of completed gonozooids and progeny produced in reproductive isolation or in crosses involving two Type 2 colonies. The alternative scenarios ruled out are (1) that autosperm do not have the same affect as allosperm, and that (2) when paired, inbreeding regulation prevents successful fertilisation if, by chance, Type 2 colonies were more closely related to each other than to Type 1 colonies.

Borg (1926) described zooidal gonochorism in crisiids, with developing polypide buds associating with either male or female germinal cells at the growing zone of the colony, and only at this early stage of polypide development. However, the apparent lack of sperm production in female colonies of *Filicrisia geniculata* reported here, suggests that primary spermatogonia are not associated with developing polypide buds in colonies of this type. Thus, autozooids in the two colony types appear to be functionally distinct: autozooids in males produce sperm; those in females do not. This implies that in female colonies of *F. geniculata* at least, developing polypide buds associate only with oocytes, at least initially. Borg (1926) and Ryland (2000) both described two developmental pathways for female zooids within the Crisiidae. In the majority of cases, all the oocytes connected to a developing polypide bud will degenerate (all ova are resorbed) and the zooid then becomes a feeding autozooid with a functional polypide. A few polypide buds remain associated with oocytes and will undergo further development to eventually become gonozooids. This would suggest that the development of all autozooids in female colonies of *F. geniculata* involves such a temporary allocation to female function. Evidence from histological investigations should be sought before any further inferences as to the nature of autozooid development in *F. geniculata* colonies can be made.

4.4.2 Variation in reproductive investment within colony types.

In the present investigation, it is difficult to attribute a cause (genetic or environmental) to the observed variation in sperm production found amongst male colonies and the variation in incomplete gonozooids observed amongst female colonies of *Filicrisia geniculata*. Multiple ramets of the same colony were not investigated here and therefore differences cannot be ascribed to genotype.

Wide variation in female investment, in terms of the number of gonozooids per colony, even in colonies of a similar size, has been previously reported in *Crisia denticulata* (Pemberton *et al*., 2011). Female investment in *C. denticulata* appears to be more variable than that observed here in *Filicrisia geniculata*. The cause of this variation requires further investigation. Furthermore, the study of *C. denticulata* identified a pattern of continuous variation in female investment (in absence of sperm production data), which differs from the pattern of definite colony states (male OR female) in *F. geniculata*.

4.4.3 Gender specialisation at the colony level.

Gender specialisation in hermaphroditic organisms is defined as the specialisation in one gender at the expense of the other, resulting in the asymmetrical division of resources between male and female reproductive structures (Robbins & Travis, 1986). This term, used almost exclusively in the botanical literature, might be safer to use here at this time for describing *Filicrisia geniculata* than gonochorism, given the lack of anatomical evidence for the presence or absence of testes in autozooids of Type 2 (female) colonies. Gender specialisation appears to differ from models of sex allocation proposed for simultaneous hermaphrodites (Charnov, 1982). Flexibility in resource allocation to different sexual functions is considered a characteristic of simultaneous hermaphrodites, and an advantage over gonochorism (Charnov, 1982; Michiels, 1998). Allocation is not always equal and can be predicted by models of sex allocation, based on ‘gain curves’ (Charnov, 1982). These models involve a trade-off in male and female investment, as resources are shifted between alternative sexual functions. This does not appear to be the case in *F. geniculata* since the extreme bias in gender allocation appears to be fixed. Perhaps a gradual imbalance in gender allocation has led to gender specialisation over time in this species and may signify a transition from simultaneous colonial hermaphroditism. Whilst the situation in *F. geniculata* is

consistent with effective gonochorism, the term 'gender specialisation' offers a degree of flexibility of interpretation in the absence of further information.

It is difficult to attribute a cause for gender specialisation or, effectively, sex determination, observed in *Filicrisia geniculata* based on this present study. Investigations in Chapter 3 employed multiple ramets of the same clone (genotype) and revealed a consistent 'behaviour' of colonies as either male or female, which could indicate a genetic basis for sexuality of colonies. Environmental or external controls could also influence colony gender. Although all colonies were reared in 'common garden' conditions, the possibility of a very sensitive environmental switch or maternal carry-over determining sexual function cannot be ruled out.

As early as the late 19th and early 20th centuries, the existence of separate sex colonies in crisiids was suggested. Thus, Harmer (1893), as noted by Borg (1926), remarked that spermatozoa occur in *Crisia* generally "in colonies without ovicells". Robertson (1903) identified *Crisia eburnea* and *Crisia occidentalis* (though the evidence was less conclusive in the latter) as dioecious species, because "two kinds of genital products [were] never found in the same colony". More recently, Beauchamp (1984) used the observation of equal numbers of similar-sized colonies with and without ovicells in *Crisia franciscana* as evidence of gonochorism, and Pemberton *et al.* (2011) reported that a large proportion of *Crisia denticulata* colonies lack brood chambers even in the largest size categories.

Borg (1926) invoked 'colonial protandrism' in order to explain the difficulty in finding testes and gonozooids at the same time in *Crisiella producta* and *Crisia eburnea*. He disputed the claims of dioecious colonies reported by Harmer (1893) and Robertson (1903) and proposed that careful re-assessment would indicate 'colonial protandrism' i.e. sequential hermaphroditism. Evidence from long-term culturing rules out the occurrence of sequential hermaphroditism in *Filicrisia geniculata*. Gender specialisation of colonies remains fixed over time.

In conclusion, the evidence present here is consistent with existence of at least extreme gender specialisation, if not gonochorism at the colony level, in *Filicrisia geniculata*. However, the incidence of gonochorism can only be fully confirmed after further investigation. Furthermore, the nature of gender determination in this species is currently unclear and

requires further work. The implications of the possible transition towards gonochorism revealed here will be further considered in the General Discussion (Chapter 6).

Table 1: Details of *Filicrisia geniculata* specimens used, including colony type: Type 1 = 'male', Type 2 = 'female', 'Progeny' = colonies derived from Chapter 3 experiments.

Sample	Year	Colony Type	Dry weight(mg)
Clone C	2010	Type 2	121.6
Clone D	2010	Type 1	103.2
104 x 4 A	Progeny	Type 1	43.7
107 x 104 B	Progeny	Type 1	26.2
107 x 104 C	Progeny	Type 2	70.7
July 4D	2011	Type 2	9.6
July 6	2011	Type 1	18.2
August 2A	2011	Type 1	11.5
August 4A	2011	Type 2	28
August 4B	2011	Type 1	11
August 6A	2011	Type 2	44.1
August 7B	2011	Type 2	55.3
August 10A	2011	Type 2	13.1
August 12A	2011	Type 2	71.8

Table 2: Sperm production data for all Type 1 colonies of *Filicrisia geniculata*. Figures are sperm mg^{-1} dry weight.

Colony	COUNT 1	COUNT 2	COUNT 3	Average
July 6	0.38	4.18	2.77	2.4
107x104 B	17.77	34.92	38.55	30.41
Clone D	8.87	7.33	7.22	7.8
Aug 2A	68.52	49.13	58.17	58.6
Aug 4B	1.55	4.82	3.86	3.4
104x4 A	47.59	52.52	42.44	47.52

Table 3: Average counts for each Type 2 colony of *Filicrisia geniculata* of autozooids and incomplete gonozooids (as calculated from 10 randomly sampled branches) and the proportion of gonozooids.

Colony	Autozooids	Incomplete gonozooids	Proportion of gonozooids
Aug 4A	105.3	12.1	0.10
Aug 7B	81.7	15.3	0.16
Aug 6A	86.8	5.8	0.06
Clone C	129.1	24.1	0.16
107x104 C	130	3.6	0.03
Aug 12A	126.5	22.6	0.15
July 4D	97.9	5.5	0.05
Aug 10A	121	5.1	0.04

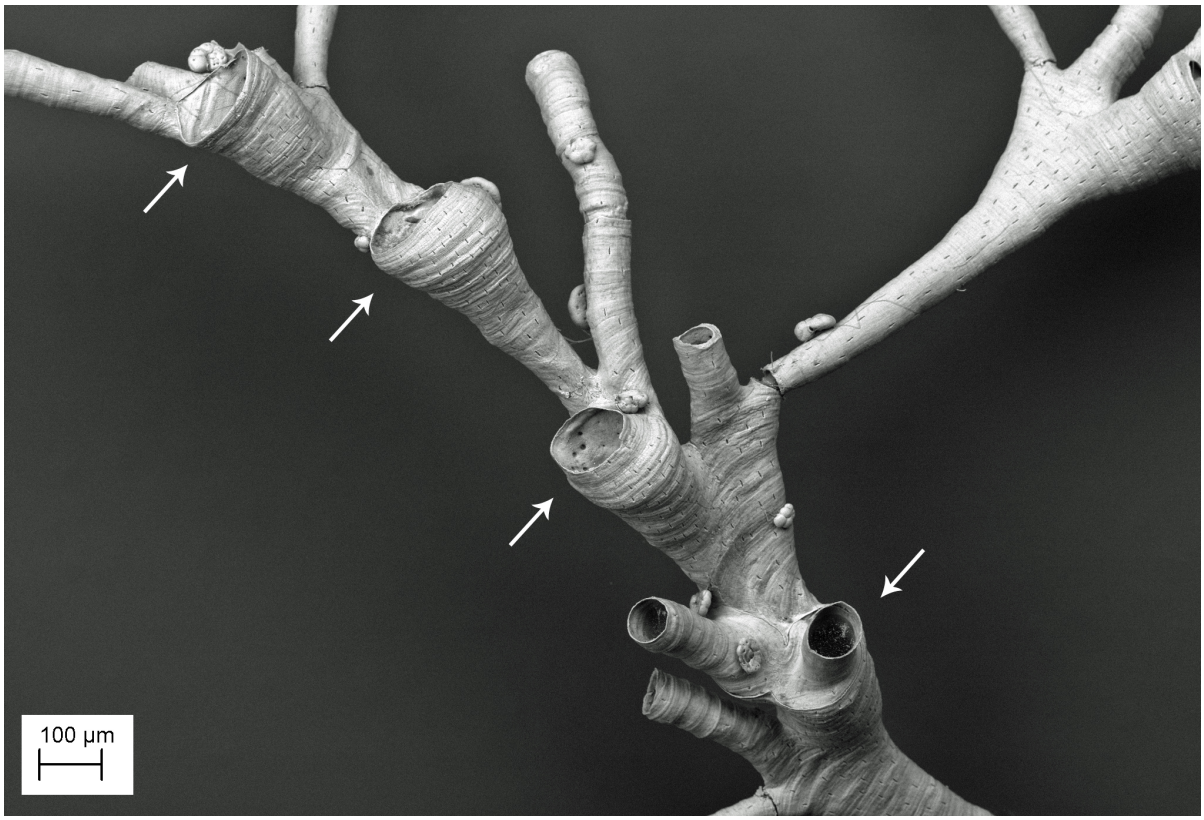


Figure 1: Scanning electron micrograph depicting a branch of *Filicrisia geniculata* with four incomplete aborted gonozooids forming a ‘stack’.

Chapter 5: **Investigating the paradox of polyembryony in the case of cyclostome bryozoans.**

5.1 Introduction.

Polyembryony is the production of broods of identical genotype through the cloning of multiple embryos from a single sexually produced zygote. It has been considered paradoxical due to its seemingly disadvantageous combination of sexual and asexual reproduction (Craig *et al.*, 1995, 1997; Hughes *et al.*, 2005). Craig *et al.* (1995) were first to outline this paradox, the underlying basis of which is that genetically diverse, therefore sexually produced, offspring are necessary for survival in changing environmental conditions (Williams, 1975). In the case of polyembryony, the mother 'bets' on a single unproven genotype at the expense of brood genetic diversity (Craig *et al.*, 1995, 1997). Whereas sex provides genetic diversity in changing environments, asexual reproduction (cloning) may be beneficial in more stable environments as it enables the mother's relatively successful genotype to be replicated. Polyembryony, by combining both contrasting reproductive modes, appears to compromise their respective benefits (Hughes *et al.*, 2005).

In the case of cyclostome bryozoans, polyembryony may be less paradoxical if genetically identical larvae are released from a single brood sequentially over time, rather than in a single event (Pemberton *et al.*, 2007). In such a case, a single genotype would be repeatedly tested against a changing environment over an extended period of brood release. Furthermore, if multiple broods of different genotype are present within a colony, multiple genotypes may be tested at a given time. Therefore, in terms of spreading risk, polyembryony in this case may be more similar to regular sexual reproduction, which tests multiple genotypes at once.

Microsatellites are powerful, highly polymorphic Mendelian molecular markers used widely in a range of ecological and evolutionary studies at both the individual and population level (Goldstein & Schlötterer, 1999; Sunnucks, 2000). More specifically, they are important genetic tools for deducing patterns of parentage through the comparison of parent and offspring genotypes at multiple polymorphic loci (Jones & Arden, 2003; Avise, 2004). Microsatellite DNA loci are composed of repeat sequences of 2-6 nucleotides. Multiple alleles (of differing length) may be present at a single locus due to variation in the number of repeats

between different individuals (Avise, 2004). However, one major drawback of microsatellites is that they require *de novo* development (Abdelkrim *et al.*, 2009).

With the advent of next-generation sequencing (NGS) technologies and their subsequent application to microsatellite discovery, particularly for non-model species, the *de novo* development of these markers is being streamlined (Abdelkrim *et al.*, 2009; Allentoft *et al.*, 2009; Malausa *et al.*, 2011). NGS is fast becoming the preferred method for microsatellite development (Gardner *et al.*, 2011). Pyrosequencing using the 454 GS-FLX Titanium platform has proved efficient and cost-effective not only because a large number of polymorphic loci are recovered in a single run but also because the large average fragment size obtained increases the chance of sequencing microsatellite motif flanking regions, facilitating primer design (Gardner *et al.*, 2011). Enriching DNA libraries for microsatellites prior to pyrosequencing can enhance primer design further by increasing the number of reads recovered per locus, and has proved particularly beneficial for the isolation of polymorphic loci in non-model species (Guichoux *et al.*, 2011; Malausa *et al.*, 2011).

The aim of this study was to test the hypothesis that the cyclostome bryozoan *Filicrisia geniculata* demonstrates extended release of genetically identical offspring from a single gonozooid over time – a study that derived directly from investigations of gonozooid formation in Chapter 3. To this end, novel molecular markers for *F. geniculata* were developed using NGS techniques in order to document the repeated (cloned) genotype of the progeny released from individual gonozooids over time.

5.2 Materials and Methods.

5.2.1 Sample collection.

Branches with single fully developed gonozooids, representing all successful controlled crosses detailed in Chapter 3, were isolated and retained in culture to monitor progeny production over time.

Branches were mounted onto slides (held in place with a loop of very fine fishing line) and placed in acetate-lined tanks, maintained under standard culture conditions (as described in Chapter 3). Larvae that settled and metamorphosed on the acetate grew into small colonies that were collected and genotyped. Colonies were isolated on a small piece of the surrounding acetate and preserved in 100% Ethanol. For DNA extraction, a single, long branching fragment was dissected from each offspring colony, directly from the ancestrula in most cases to ensure a single colony had been sampled. Any attached debris e.g. foraminifera or attached metamorphs (brood-mates) was removed.

Two successive batches of progeny were collected from each gonozooid (‘brood`). For the first batch, the isolated colony fragment with the completed gonozooid was removed from its tank and placed into a clean tank that was lined with fresh acetate after 30 days (Broods I & III) or 35 days (Broods II & IV). The existing progeny produced by the gonozooid before it was removed from its original tank were allowed to grow on into small colonies before preservation. The new tank was monitored for newly settled progeny, which formed the second batch of offspring. The second batch was preserved after a period of 108 days (Brood II), 147 days (Brood III), 176 days (Brood IV) and 182 days (Brood I) since the transfer to the new tank. For more details see Results section 5.3.1.

5.2.2 Microsatellite discovery using next-generation sequencing technology.

Gonozooid tissue rather than autozooid tissue was used as the source of DNA for microsatellite development as it is free of food particles (i.e. potential contaminants). Pooled gonozooid samples were expected to increase the overall intra-specific genetic diversity for optimal polymorphic primer design since each gonozooid within a colony contains larvae of a potentially different genotype. Gonozooids were fully developed and contained numerous developing larvae. Multiple gonozooids per colony were sampled from a total of three

colonies. All gonozooids dissected from a single colony were combined and total genomic DNA was extracted using a DNeasy animal tissue kit (QIAGEN), according to the manufacturer's instructions. DNA eluted from each of the three colonies was combined and submitted to GenoScreen (Lille, France) for microsatellite discovery.

Polymorphic microsatellite markers were generated by GenoScreen via a high-throughput technique using 454 GS-FLX technology (Roche Applied Science) (Malausa *et al.*, 2011). In brief, DNA libraries, highly enriched in microsatellite loci, were amplified using 454 GS-FLX Titanium pyrosequencing. Subsequent bioinformatic analysis of raw sequence data, performed using QDD software (Meglécz *et al.*, 2010), identified sequences containing microsatellites and designed associated primer pairs. This analysis generated a total of 28,177 raw data sequences, 7,205 of which contained microsatellite motifs. Two hundred and four bioinformatically validated primer pairs were designed. From these I selected an initial set of 34 primer pairs, based on the number of motif repeats (i.e. > 9) to ensure polymorphism (Schlötterer, 2004). These motifs included dinucleotides, trinucleotides and tetranucleotides (see Appendix VIII for full list).

5.2.3 Microsatellite marker selection.

A panel of eight individuals, which included the four brood parents involved in the controlled crosses, were used to test the initial set of 34 primer pairs, as selected from the GenoScreen data set. DNA was extracted from clean branches of these colonies including the growing tips.

Total genomic DNA was extracted using the DNeasy animal tissue extraction kit (QIAGEN), according to the manufacturer's instructions, and eluted in 100μl elution buffer. PCRs were conducted in a total reaction volume of 20μl using GoTaq Flexi DNA Polymerase kit (Promega), 250nM unlabelled forward primer, 250nM unlabelled reverse primer, 1X buffer, 1.5mM $MgCl_2$ solution, 250μM dNTP mix, 0.1mg/ml BSA, 0.5units Go Taq DNA Polymerase and 2μl template DNA.

PCR cycling conditions were as follows: initial denaturation for 5 min at 95^{o}C, followed by 35 cycles of 30 s at 95^{o}C, 30 s at *Ta*^{o} C (see Table 1), 30 s at 72^{o}C, and a final extension step of 10 min at 72^{o}C. PCR products were visualised on 2% agarose gels (100v for 40 mins) with loading buffer and Hyperladder IV molecular weight marker (Bioline) in TAE x1 buffer

solution. Loci producing promising banding patterns (i.e. one or two bands for each individual tested) were analysed by acrylamide electrophoresis. PCR products were run on a 10% polyacrylamide gel with loading buffer and Hyperladder V molecular weight marker (Bioline) in TAE x1 buffer (200v for 3h). After electrophoresis, gels were stained with 1μl SYBR Gold in 10ml TAEx1 for 15mins. Primer pairs that failed to amplify or that produced multiple fragments were discarded. This preliminary screening formed the basis of marker selection and identified the most informative polymorphic markers for the four brood parents. This final subset of four markers was used to generate data in fragment analysis (Table 1; see Appendix VIII for full list).

5.2.4 Microsatellite validation and genotyping of broods.

Initial fragment analysis was conducted on all brood parents and on progeny from an additional 'mixed brood' resulting from a third cross (Cross C x D; Brood V) in order to test loci and establish parental genotypes. This 'mixed brood' was composed of offspring collected at random from a tank containing multiple gonozooids.

For brood genotyping, all progeny collected from a total of four broods representing two successful crosses were genotyped with the four polymorphic loci identified above (Table 2). Total genomic DNA extraction of offspring was performed as described in section 5.2.1 using the extraction techniques detailed in section 5.2.3. The four loci analysed were FG08, FG12, FG13 and FG17 and were labelled with the fluorescent dyes PET, VIC, NED and 6-FAM, respectively. PCRs were performed as detailed above (section 5.2.3) but with 150nM labelled forward primer and 100nM unlabelled forward primer. For genotyping of progeny, PCRs were performed in simplex for each locus and combined for each individual to perform fragment analysis in multiplex. Parents were also included in the analysis of each brood. Fragment analysis was performed on an ABI 3130 Genetic Analyser (Applied Biosystems) and scored using Genemapper v 4.1 software (Applied Biosystems).

5.2.5 Data analysis.

For each brood, data were compiled and multilocus microsatellite genotypes were identified using the Multilocus Matches option in GenAlEx, ver. 6.3 (Peakall & Smouse, 2006, 2012).

5.3 Results.

5.3.1 Brood information.

Progeny were obtained from fragments of *Filicrisia geniculata* with isolated gonozooids from both replicates of the Crosses A x B (Broods I & II) and A x C (Broods III & IV) (Table 2).

All broods released larvae in the first and second tanks. The minimum estimated period of brood release is 18-21 days for Broods I, II & III (see below for Brood IV). This is based on records of progeny observed in the first tank and corresponds to the day on which the second tank was set up. It was not possible to rule out the possibility that progeny were released on the day on which the second tank was set up. Due to insufficiently detailed records, it was not possible to identify exactly when the progeny of the second batch were released. Progeny were recorded after 39 days (Brood III), 56 days (Brood II), 57 days (Brood IV) and 60 days (Brood I) in these second tanks. Observations for any further progeny production in these tanks continued until the second batch of progeny was collected (see Methods section 5.2.1) but no further progeny were released in any tank after the days stated above.

The longest period of brood release that could be verified from this information relates to Brood IV. Six metamorphs at the ancestrular stage were recorded in the second tank after 57 days, with their release estimated at approximately five days before observation was made (i.e. approximately 52 days after the second tank was set up). Therefore, in this example, the total duration of progeny release is estimated at 69 days (estimated first progeny release = after 17 days in Tank 1 (based on first observation of ancestrular stage metamorphs after 22 days since Tank 1 set up and allowing approximately five days to reach this growth stage) and estimated last progeny release = after 52 days in Tank 2). This provides the best indication of duration of progeny production.

Overall, only male offspring (Type 1 colonies; see Chapters 3 & 4 for definition) were produced as a result of these crosses with the single exception of Brood IV. In Brood IV, the first batch comprised solely male offspring whereas only females (Type 2 colonies; see Chapters 3 & 4 for definition) were produced in the second tank. Large and small colonies were collected in each of the two batches. All colonies (including small ones) in the second batch had incomplete gonozooids, enabling gender determination.

5.3.2 Genotyping analysis of broods resulting from controlled crosses.

5.3.2.1 Characteristics of newly developed polymorphic loci.

From the preliminary screening of the initial set of 34 candidate loci on agarose gels, 12 loci were selected for analysis by acrylamide electrophoresis due to their apparent polymorphism among the panel of eight individuals tested. On this basis, four loci were selected as sufficiently polymorphic for the four parents under scrutiny. These four informative loci were subjected to fragment analysis on the sequencer (Table 1).

Subsequent fragment analysis revealed the potential presence of null alleles at locus FG12. Difficulties in scoring and problems with amplification rendered this marker unreliable and resulted in its exclusion from genotyping analysis. Therefore, multilocus genotyping analysis combined data from only three loci – FG08, FG13 and FG17.

Eleven alleles in total were amplified across the whole data set (parents and progeny) from three loci. Alleles ranged from 110-118bp at FG08, 186-238bp at FG13, and from 176-228 bp at FG17, the most polymorphic locus (five alleles) (Figure 1).

5.3.2.2 Multilocus genotype (MLG) analysis.

An overview of the MLG analysis is provided in Table 3; see Appendix IX for all scores.

Validation of microsatellite loci: Progeny of both colony types (male and female) were produced and collected at random from a tank containing multiple gonozooids (approximately 16), forming the 'mixed brood' Brood V. Parental genotypes differed from each other at two loci (FG08 and FG17) and from progeny at all three loci, thus confirming outcrossing. Among progeny, seven MLG were identified: three were unique and four were shared. This analysis provides a preliminary confirmation that these microsatellite loci behave as expected from our existing understanding of reproduction in *Filicrisia geniculata*.

Cross A x B: Within each brood resulting from the Cross A x B, all progeny shared an identical MLG at all three loci, although individual F10 (Brood II) lacked data at locus FG17. However, the MLG differed between broods at two loci (FG13 and FG17). Parental genotypes differed at all three loci and from that of both broods. Progeny from Brood I

differed from both mother and father at two loci (FG08 and FG17, and FG08 and FG13 respectively). The progeny from Brood II, however, differed from their mother at two loci (FG08 and FG13) but from their father at all three loci.

Cross A x C: All progeny from broods resulting from the Cross A x C, both within and between broods, shared an identical MLG at all three loci, although individual B17 (Brood III) lacked data at locus FG17 and individuals C03 and C32 (Brood IV) lacked data at loci FG13 and FG17. The MLG of parents differed from each other at two loci (FG13 and FG17) and from that of both replicate broods. The brood MLG differed from that of both parents, from the mother at two loci (FG13 and FG17), and from the father at one locus (FG17).

In summary, genotyping analysis indicated that, for each cross, the MLG for parents differed from each other and from that of their offspring. The MLG of offspring within a brood remained constant over the two collections. Outcomes of this analysis include the confirmation of outcrossing and polyembryony, in addition to one example of genotypic diversity between broods from the same (maternal) colony (A x B).

5.4 Discussion.

The evidence from genotyping analysis presented here confirms the sequential release of larvae of the same genotype from a single brood of *Filicrisia geniculata* over an extended period of time, with a minimum estimate for duration of larval release of 18-21 days. The longest verifiable period of release was estimated as 69 days (Brood IV). Such a period of over two months would correspond to substantial changes in the environmental conditions experienced by larvae from a single polyembryonic brood. All progeny released from all broods were genotyped and were found to be genetically identical within each brood. There is thus no evidence of 'turnover' of larval genotype within a gonozooid in the experimental conditions of reproductive isolation following fertilisation.

Differences in genotype between different broods within a single colony indicate that genetic diversity can be generated amongst progeny of a colony through the production of multiple broods (Hughes *et al.*, 2005; Pemberton *et al.*, 2007). Other associated outcomes include confirmation of cross-fertilisation and polyembryony. This demonstration of polyembryony is consistent with work on another crisiid, *Crisia denticulata* (Hughes *et al.*, 2005; Pemberton *et al.*, 2007), and thus strongly suggests that this is characteristic of crisiids. An additional outcome of interest was the production of both male (Type 1) and female (Type 2) progeny of identical genotype by a single gonozooid (Brood IV). The reason for this switch is at present unclear and will be discussed in the General Discussion (Chapter 6) in the light of observations gained throughout this thesis.

The research presented is not without its limitations. The multilocus genotyping analysis combined data from only three loci (one locus (FG12) was unreliable). Exclusion of locus FG12 from analysis highlights the potential problems caused by the presence of null alleles. Despite this restriction, these loci were sufficiently polymorphic to confirm the unique genotypes of parents and offspring. This was possible because progeny were the result of controlled crosses between known parents. The small number of markers is therefore not an issue for the hypothesis tested here although the markers may not be so suitable if applied to other material and to test other hypotheses.

In future, should more microsatellite markers for this species be required, for instance for a population genetic level study, the preliminary screening of loci commenced here provides a good starting point for continued optimisation and selection of polymorphic markers. The

numerous candidate loci and associated flanking sequence information generated through the next-generation sequencing approach to microsatellite discovery provides scope for the development of further informative markers.

In conclusion, the confirmation of the sequential release of genetically identical larvae from a single gonozooid over an extended period of time, and of the presence of genetic diversity among broods within the same colony, suggests that polyembryony in *Filicrisia geniculata* may be effectively similar to regular sexual reproduction. Polyembryony may therefore be less paradoxical in this particular case than first suspected.

Table 1: Characteristics of the four microsatellite loci employed in genotyping analysis of *Filicrisia geniculata*. T_a(°C), annealing temperature; N_{cycl}, no. of PCR cycles at T_a; A range, size range of alleles (bp); $N_{t\,all}$, total no. of distinct alleles observed among four parents and progeny by fragment analysis.

Locus	Repeat motif	Primer sequence	T_a (°C)	N_{cycl}	A range	$N_{t\,all}$
FG08	$(CAAA)_{11}$	F: TCACCTCCTCATACACGCCT R: TCTGTGCTGTATTGTGAGCG	52	35	110-118	3
FG12	$(GTAT)_{11}$	F: TGTATGCATGTATGTATGGAATGG R: TACGATAAGCTCGCAGGACA	52	35	90-122	2
FG13	$(AC)_9$	F: ACATTAGACCCGGGATTTCG R: AAGTTGTGAAGTTAAGTTGTTCCAA	51	35	186-238	3
FG17	$(TGTA)_{23}$	F: TTTAAAATCCACACTCTATCGCC R: CAGGTACACTTACATGCCAACTACA	52	35	176-228	4

Table 2: Brood screening information for *Filicrisia geniculata* broods. Broods I & II and III & IV are replicate experimental broods of Cross A x B and Cross A x C, respectively. Brood V is the 'mixed brood' used to test loci. All scoring errors were non-amplifications at one locus (Broods II & III) or two loci (Brood IV).

Parents	Brood	No. of progeny	No. of progeny with scoring errors
A x B	Brood I	25	0
A x B	Brood II	16	1
A x C	Brood III	27	1
A x C	Brood IV	37	2
C x D	Brood V	12	0

Table 3: Overview of multilocus genotypes for *Filicrisia geniculata* parents and broods at each locus. In each table, rows 1 and 2 correspond to mother and father, respectively. The scores are allele sizes at a particular locus. Broods I & II and III & IV are the replicate broods of Cross A x B and Cross A x C, respectively. Brood V is the 'mixed brood' used to verify microsatellite loci. See Appendix IX for details of all scores.

Cross C x D

Brood V

Sample	FG08		FG13		FG17	
C	114	114	186	194	192	218
D	110	114	186	194	176	228
G01	114	114	186	194	176	192
G02	114	114	194	194	176	218
G03	114	114	186	194	176	218
G04	114	114	186	186	218	228
G05	114	114	186	194	176	218
G07	114	114	186	194	176	192
G09	110	114	186	194	192	228

Cross A x B

Brood I

Sample	FG08		FG13		FG17	
B	110	118	186	238	222	228
A	114	114	194	238	228	228
progeny x 25	110	114	186	238	228	228

Brood II

Sample	FG08		FG13		FG17	
B	110	118	186	238	222	228
A	114	114	194	238	228	228
progeny x 15	110	114	186	194	222	228

Cross A x C

Brood III

Sample	FG08		FG13		FG17	
C	114	114	186	194	192	218
A	114	114	194	238	228	228
progeny x 26	114	114	194	238	192	228

Brood IV

Sample	FG08		FG13		FG17	
C	114	114	186	194	192	218
A	114	114	194	238	228	228
progeny x 35	114	114	194	238	192	228

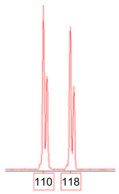
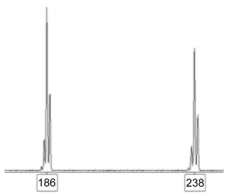
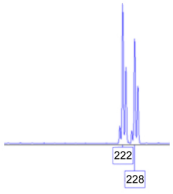
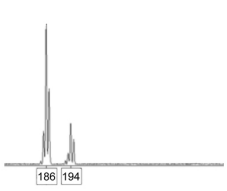
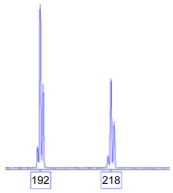
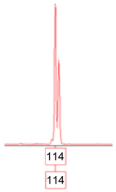
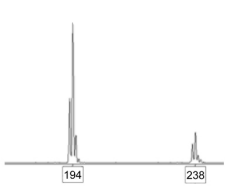
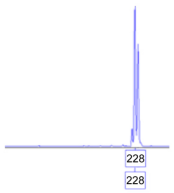
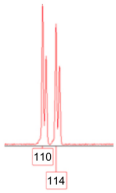
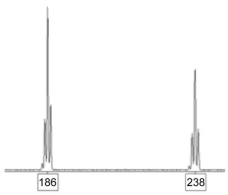
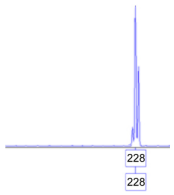
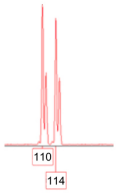
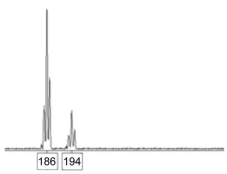
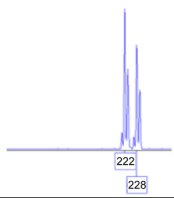
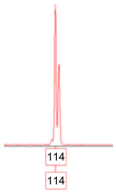
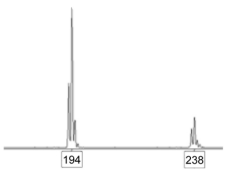
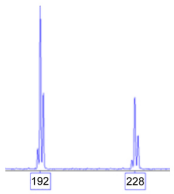
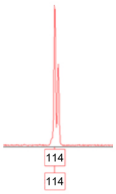
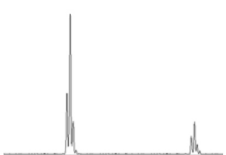
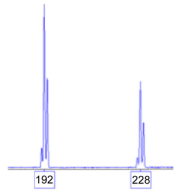
INDIVIDUAL	Locus FG08	Locus FG13	Locus FG17
Parent B (MOTHER)			
Parent C (MOTHER)			
Parent A (FATHER)			
A x B Brood I			
A x B Brood II			
A x C Brood III			
A x C Brood IV			

Figure 1: Example traces of parents and progeny at each locus used in genotyping analysis of broods resulting from controlled crosses.

Chapter 6: **General Discussion.**

6.1 Polyembryony in cyclostome bryozoans.

Evidence for the occurrence of polyembryony in all three major cyclostome clades was obtained here using molecular techniques. This confirms historical inferences based on microscopy and supports the widely held view that this remarkable reproductive mode characterises this ancient order.

As with much of the existing literature relating to polyembryony in cyclostomes, the contribution of the current research to the understanding of this reproductive mode is restricted largely to the Crisiidae. Evidence for the prolonged production of larvae from a single brood in *Filicrisia geniculata*, combined with the presence of genetic diversity between broods within a colony (as shown in both *F. geniculata* (this study) and *Crisia denticulata* (Hughes *et al.*, 2005)), makes polyembryony more akin to regular sexual reproduction. This testing of multiple genotypes at a given time and also against varying environmental conditions over time may enable crisiid cyclostomes, at least, to circumvent the paradoxical nature of polyembryony. However, colonies do not always possess multiple broods; large colonies of *C. denticulata* with only one or very few broods have been recorded (Pemberton *et al.*, 2011). In this instance, even if inter-brood diversity is not attained, temporal sampling of varying environmental conditions remains (Pemberton *et al.*, 2011) and a colony is able to capitalise on a low frequency of fertilisation (Ryland, 1996).

Observations of gonozooid development in *Filicrisia geniculata* may provide an explanation for the low frequency of brooding documented in some crisiids. Gonozooid development commences prior to receipt of allosperm (in reproductive isolation) in this gender specialist (Chapter 3). Although allosperm is required for the completion of gonozooid development and brooding, whatever controls initial gonozooid formation ultimately controls the frequency of brooding and variation in the number of gonozooids present (as the number of gonozooids varies between genotypes —Chapter 4), along with external constraints such as food and sperm supply. It is also possible that other crisiids, such as *Crisia denticulata*, produce hermaphroditic colonies and that there may be greater investment in male function (sperm production) relative to female when few broods are produced.

The potential for polyembryony to compensate for infrequent fertilisations is likely to extend to cyclostome families other than the crisiids but other aspects of the mating system may have some effect. For instance, mating trials undertaken here with *Tubulipora plumosa* revealed some evidence of 'emergency selfing' when in reproductive isolation (Chapter 3). In the absence of allosperm, fewer gonozooids were formed, suggesting some degree of inbreeding regulation. Selfing thus appears to provide some degree of reproductive assurance through production of offspring. This advantage may be enhanced by polyembryony, although it does not appear immune to the effects of inbreeding depression (IBD) as fewer progeny were produced per gonozooid compared to outcrossed progeny. However, the production of fewer larvae may not be the result of IBD but inbreeding regulation. It is possible that primary embryos produced through self-fertilisation, may themselves receive reduced female investment from the maternal colony, resulting in the production of fewer offspring and the redirection of resources to sperm production. There were, however, a few cases where gonozooids produced in reproductive isolation did not release offspring – an observation consistent with genetic variation in inbreeding regulation.

Investigating inbreeding was not the focus of the mating trials undertaken here with *Tubulipora plumosa* but the results obtained highlight areas for future study. There was an original intention to study inbreeding regulation and IBD in *Filicrisia geniculata* during this PhD, using a pedigree of individuals of known relatedness. However, the identification of colonies of separate sexes and the settlement of mostly male progeny from controlled crosses precluded this.

The present research makes little contribution to other arguments concerning the occurrence of polyembryony in cyclostomes. Thus, this study provides little direct evidence for sperm limitation, sperm storage or the mechanism of sperm uptake. However, in *Filicrisia geniculata* at least, exposing 'female' colonies with incipient gonozooids to a single 'dose' of allosperm suggested that allosperm is not stored. Thus, colonies resumed production of incomplete gonozooids after a transient period when one gonozooid completed after exposure to allosperm (Chapter 3). Silén (1972) proposed the involvement of the transitory lophophore in these incipient gonozooids in sperm uptake, but this requires further investigation. This is in contrast to sperm uptake and storage by non-reproductive zooids, with subsequent transfer to female zooids, in the cheilostome bryozoan *Celleporella hyalina* (Hughes *et al.*, 2002). Furthermore, sperm limitation is regarded as unlikely, given the circumstances proposed to favour the apparent transition to gender specialisation in *F. geniculata* (see following section

6.2 for details). However, data on sperm production and fertilisation dynamics among cyclostomes are still required to resolve many of these questions.

Phylogenetic constraint has been suggested previously to account for the ubiquity of polyembryony in cyclostomes (Ström, 1977; Hughes *et al.*, 2005; Pemberton *et al.*, 2011) and the evidence presented here does not rule this out. Polyembryony may have evolved as an adaptation in a common ancestor or from a "spontaneous aberration of embryogenesis" (i.e. the budding of a primary embryo) (Hughes *et al.*, 2005) coupled with development of mechanisms for placental nourishment and enlargement of brood chambers. This may have led to further development and eventual genetic fixation. However, it would seem a relatively simple evolutionary step from polyembryony back to conventional development (i.e. the production of single embryos in brood chambers), especially in crisiids where gonozooid development occurs on a zooid-by-zooid basis. The loss of polyembryony would seem possible in this group (given sufficient sperm supply), with the production of many gonozooids each producing fewer and fewer larvae, resulting in eventually just one larva per gonozooid (Pemberton *et al.*, 2011). In order to accept polyembryony as a phylogenetic constraint, a difficulty in its loss needs to be identified.

6.2 Gender specialisation in *Filicrisia geniculata* – a transition to gonochorism?

Evidence from mating trials and estimates of sperm production are consistent with the existence of separate sexes, or at least very pronounced gender specialisation, in the crisiid *Filicrisia geniculata*.

Prior suggestion of separate sex colonies in crisiids has been made, but this has been questioned in favour of sequential hermaphroditism — specifically colonial protandry (Harmer, 1893; Robertson, 1903; Borg, 1926; Beauchamp, 1984). However, in the absence of sperm production data, these inferences remained speculative. Observations of *Filicrisia geniculata* here rule out colonial protandry. In *F. geniculata* there is evidence for fixed gender specialisation including: 1) observations of large, old male colonies (Chapters 3 & 4) and, 2) the persistence of gender during clonal propagation in the laboratory (Chapter 3).

Colonies of *Filicrisia geniculata* exhibit extreme biased sex allocation, resulting in effectively male and female colonies. This specialisation remains fixed within an individual colony over time, and is at odds with the view that flexibility (phenotypic plasticity) in sex allocation is a

major advantage afforded to simultaneous hermaphrodites (Michiels, 1998). This pattern of male OR female as definitive colony states differs from the pattern of continuous variation in gonozooid production found amongst wild colonies of *Crisia denticulata*. The latter has been described as a continuum of female investment in a hermaphroditic population, with colonies without gonozooids at one extreme (Pemberton *et al.*, 2011). Furthermore, empirical studies of sex allocation in the bryozoan *Celleporella hyalina* from the Order Cheilostomata, have shown wide intraspecific variation in sex allocation between colonies. While variation may approach nearly pure male and pure female extremes, colonies never function exclusively as one gender at the expense of the other (Hunter & Hughes, 1995; McCartney, 1997; Hughes *et al.*, 2009). However, in *F. geniculata* the existence of colonies that function exclusively as one gender is clear. Although the lack of anatomical evidence precludes confirmation of gonochorism in *F. geniculata* and the use of 'gender specialisation' here is favoured as a more flexible term (see Chapter 4), colonies are effectively separate sexes as they function exclusively as male or female. This pattern observed in *F. geniculata* is the first report of its kind and counters the paradigm that separate sexes are unknown (Ryland & Bishop, 1993).

The mechanism underlying gender specialisation (or effectively, gender determination) in *Filicrisia geniculata* is at present unclear. The founding of both male and female colonies under the same controlled laboratory conditions, from larvae released by wild populations (2010 & 2011), suggests that gender was determined prior to settlement and growth. However, the question of whether this is attributable to a genetic or environmental cause, such as a response to population sex ratios, remains. Settlement and rearing of progeny from single isolated gonozooids produced from controlled crosses in the laboratory may enable some insights. Thus, most broods from laboratory crosses were male, suggesting an environmental basis to sex allocation related to culture conditions. Identifying a male bias and a rarity of females in 'stable' or controlled conditions, but the production of broods of both sexes from wild populations (where variable environmental conditions would be predicted) further suggests an underlying environmental control of gender specialisation in this species. However, until the developmental stage at which gender is determined is identified, this remains speculative particularly in light of the observed 'switch' in gender of progeny from the same brood (Chapter 5: Brood IV). Limited observations preclude any firm inferences about gender determination in *F. geniculata*. Further work, combining evidence from both wild populations and laboratory cultures, is needed before a more informed conclusion can be reached.

Hermaphroditism is considered the ancestral reproductive condition in the Bryozoa. This is a more parsimonious explanation for its apparent ubiquity across the phylum than extant clades having evolved the condition independently (Hughes *et al.*, 2009). Bryozoans first appear in the fossil record in the Ordovician, suggesting the persistence of hermaphroditism since this time (Taylor & Ernst, 2004). In accordance with sex allocation theory, hermaphroditism in this group is expected to have evolved during conditions favouring selfing (Hughes *et al.*, 2009). Circumstances creating low population densities, such as periods of changing environmental conditions, changes in larval dispersal capabilities and habitat fragmentation, are likely to favour hermaphroditism as an adaptation for reproductive assurance, favouring bias to female function and economy in male function (Ryland, 1976; Charlesworth & Charlesworth, 1981; Hughes *et al.*, 2009). Conversely, changes in the balance between the advantages of selfing and the cost of inbreeding depression may stand to promote the evolution of gonochorism (Charlesworth & Charlesworth, 1981). However, despite evidence for obligate outcrossing (Hughes *et al.*, 2002), genotypic variation in gender allocation resulting in near separate-sex colonies (Hunter & Hughes, 1995; McCartney, 1997), and the formation of dense clusters of colonies, *Celleporella hyalina* has not attained gonochorism. This has led to the suggestion that the continued prevalence of hermaphroditism is not adaptive in all bryozoan clades and that a phylogenetic constraint may prevent its replacement by gonochorism in any bryozoan population (Hughes *et al.*, 2009).

The suggestion that phylogenetic constraint contributes to the prevalence of hermaphroditism in bryozoans is countered by the frequent reversions to gonochorism observed in other taxa. Shifts from simultaneous hermaphroditism to gonochorism (and vice versa) have occurred in both plants and invertebrates. Two routes to gonochorism have been proposed in plants, one involving the spread of sterility mutations (either male or female) and the other involving the gradual divergence of two classes of individual within a population, each specialising in a particular gender (Charlesworth, 1999; Barrett, 2002; see Pannell & Verdu (2006) and the references therein). Among invertebrates, reef-building corals of the order Scleractinia exhibit both hermaphroditism and gonochorism within the same genus, in addition to differences at sub-ordinal level (Carlon, 1999; Kerr *et al.*, 2011). Thoracican barnacles also demonstrate great diversity in reproductive mode among species, with males evolving numerous times from a hermaphroditic ancestor (resulting in either gonochorism or androdioecy) (Kelly & Sanford, 2010). However, shifts in reproductive mode may be prevented in other taxa due to phylogenetic constraints (Shärer, 2009), as proposed in bryozoans (Hughes *et al.*, 2009).

Colony density and its link to sperm competition and outcrossing opportunities may provide an explanation for the gender specialisation observed among colonies of *Filicrisia geniculata*. Crisiids are often found at high densities in the wild (*Crisia denticulata*: Pemberton *et al.*, 2011) and *F. geniculata* is no exception (author pers. obs.). Dense *F. geniculata* stands are observed and allosperm competition, as a consequence of high density, may favour increased investment in male function (Charnov, 1982). This may promote specialisation in this gender. In accordance with sex ratio theory, this may in turn favour specialisation in the sex in limited supply i.e. females (Fisher, 1930; Charnov, 1982), leading to the gradual divergence of colonies functioning effectively as separate sexes. Furthermore, gonochorism is likely to be favoured when mating is no longer limited i.e. finding a mate is easy or energy efficient, as would be the case at high densities (Charnov, 1982; Avise, 2011). Finally, the maintenance of hermaphroditism for reproductive assurance does not seem relevant here, as gender specialisation forces obligate outcrossing. Notably, there was no evidence of selfing in *F. geniculata* here.

Overall, evidence from observations across the Crisiidae suggests a transition towards gonochorism, with *Crisia denticulata* at an intermediate stage and *Filicrisia geniculata* more advanced. Mating trials undertaken here revealed differences in reproductive mode between cyclostome species. *Tubulipora plumosa* demonstrated mating 'behaviour' consistent with simultaneous hermaphroditism, confirming previous inferences from the literature (Harmer, 1898; Borg, 1926) with colonies exhibiting continuous variation in female investment related to allosperm availability. Other non-crisiid cyclostome families similarly exhibit simultaneous hermaphroditism (Harmer 1896, 1898; Borg, 1926) and, perhaps in accordance with this, are found at lower densities than crisiids (Hayward & Ryland, 1985; Ryland pers. com. in Pemberton *et al.*, 2011). Furthermore, evidence of some degree of facultative selfing in reproductive isolation in *T. plumosa* (Chapter 3) demonstrates that simultaneous hermaphroditism may be adaptive, with the maintenance of this reproductive mode enabling reproductive assurance and possibly flexible resource allocation.

6.3 Variation in gonozooids and female investment within cyclostome species.

Wide variation in gonozooid number, and therefore female investment, among cyclostome colonies of the same species has frequently been reported in the literature. For instance Harmer (1896) remarked that, in most cyclostomes, a large proportion of colonies are found without gonozooids. More recent investigations describe a wide range of female investment

among colonies of *Crisia denticulata* (Pemberton *et al*., 2011). Other reports of variation in gonozooid number in Recent bryozoans include those of Robertson (1903) and Beauchamp (1984) and, for bryozoans across geological time, McKinney & Taylor (1997). There are a number of possible reasons for this variation in female investment within species, which will be discussed in the following section. Many of these factors are inter-related.

(A) Developmental processes controlling gonozooid formation.

Observations of *Filicrisia geniculata* in laboratory culture revealed that the initial formation of gonozooids begins even when colonies are in reproductive isolation. Complete gonozooid formation occurs only in the presence of 'male' colonies and therefore is presumed to be mediated by allosperm. This forms the basis of the argument for the existence of gender specialisation or separate sexes in this species. The initial provisioning for female investment occurs before reproduction is assured and appears to suggest that development of gonozooids is 'pre-destined' – perhaps controlled by some developmental mechanism. These observations may contribute to our understanding of gonozooid development in crisiids, a subject that has been raised previously in the literature. Borg (1926, p. 414) proposed that the position of gonozooids within crisiid internodes is fixed, and reflects "the general laws of the development of the zoarium (colony), in each species" and is not related to fertilisation alone. He also records the occurrence of "stunted" or "suppressed" gonozooids in *Crisiella producta* and *Crisia eburnea*. These were not illustrated but could correspond to unfertilised incipient gonozooids, i.e. aborted in development, as seen here in *F. geniculata* in the absence of allosperm. He took these observations as further evidence that the origin of a gonozooid lies in the developmental programme of the colony in each species (1926, p. 424). Ryland (2000) however proposed that gonozooid development is a product of fertilisation and that its position within an internode is not highly regulated. This inference would seem plausible if not for the observations of *F. geniculata* in culture. Ryland has not accounted for Borg's observations of "suppressed" gonozooids.

Studies of *Filicrisia geniculata* described here suggest an explanation lies somewhere between Borg's and Ryland's suggestions. In *F. geniculata*, the *completion* of gonozooid development (leading to brooding) relates to fertilisation. However, gonozooid initiation appears to be controlled by some endogenous developmental mechanism. It is still unclear what controls the initial development of gonozooids in crisiids. Understanding this may

provide an explanation for the observed variation in gonozooid number and female investment among colonies, and among cyclostomes in general.

A further question related to gonozooid development is why all cyclostome colonies initially develop more ova than will eventually be incorporated into gonozooids, since the majority produced in the colony growing margin degenerate (Harmer, 1898; Borg, 1926; Ryland 2000). Despite this being an issue fundamental to our understanding of cyclostome development, the mechanism is not yet understood (Reed, 1991). Again, it suggests an endogenous developmental programme that strongly couples initiation of gonozooid development/female investment with zooid development in general.

Additional insights into gonozooid development may be illustrated by the Crisiidae. In response to Robertson's (1903) question "Why does not every internode possess an ovicell (gonozooid)?", Borg (1926) suggested there was a resource constraint on the degree of nutrition available to nourish multiple gonozooids which precluded the development of gonozooids on all internodes. Ryland (2000) (as noted by Hughes *et al*., 2005) also proposed a similar explanation for the observation that gonozooids are not found close to the proximal end of internodes and, why typically, only a single gonozooid per internode is observed. These suggestions seem most relevant to crisiids that have several zooids per internode, unlike *Filicrisia geniculata*. Although energy availability for brooding is likely to have some influence on the relative frequencies of gonozooids among cyclostomes in general (see (C) below), how this relates to initial 'over-production' of ova remains unclear.

Gonozooid development in *Filicrisia geniculata* appears to contrast with patterns observed in non-crisiid cyclostomes. In *F. geniculata* this development involves a morphologically distinct incipient gonozooid stage. *Tubulipora plumosa* colonies in culture show no sign of a distinct incipient gonozooid stage, as also reported by Harmer (1898) and Borg (1926). In the former, these morphologically distinguishable gonozooids indicate the proportion of female zooids with the potential to become gonozooids. In *T. plumosa* however, it is not possible to infer this proportion. Histological examination of colony growing margins may reveal which polypides are initially associated with ova but not those that may actually become gonozooids. Fertilisation leads to gonozooid formation in this species (particularly in the presence of allosperm) and presumably, any incipient gonozooids not fertilised become (presumably functional) autozooids. In *F. geniculata* unfertilised incipient gonozooids that do not complete development are aborted. These aborted gonozooids appear non-functional.

Furthermore, *T. plumosa* does not produce aborted or incomplete gonozooids in reproductive isolation (unlike *F. geniculata*), although the number of gonozooids formed is reduced.

The developmental pathway of cyclostomes involves the incorporation of germ cells with the developing polypide bud (Borg, 1926). Thus, in both species studied here, the number of potential gonozooids (polypides with ova) is allocated before fertilisation. The control mechanism determining this is unknown (Reed, 1991; Ryland, 2000) but may be common to both species. It may, for example, relate to energy availability at the colony level. The adaptive explanation as to why *F. geniculata* should develop morphologically distinct incipient gonozooids and *T. plumosa* does not is unclear. Perhaps they allow the transitory lophophore in *F. geniculata* to capture sperm more efficiently. In the simultaneous hermaphrodite *T. plumosa*, perhaps the number of potential gonozooids relates to resource allocation trade-offs within the colony or a greater reliance on self-fertilisation (i.e. when in reproductive isolation). Elucidation of the mechanisms of fertilisation and gonozooid development among cyclostomes requires further investigation.

(B) Allosperm availability.

The flexibility in resource allocation afforded by a modular organisation enables colonies to regulate maternal investment in relation to outcrossing opportunities. Colonies are able to conserve or re-direct resources from female function to some extent until receipt of conspecific allosperm, which is an advantage when sperm are limited. This is in contrast to other, often unitary, sessile marine invertebrates, where external fertilisation via broadcast spawning risks wastage of relatively more energy-expensive eggs if fertilisation fails (Levitan, 1995; Levitan & Petersen, 1995).

Evidence from mating trials indicated that, when given an outcrossing opportunity, there was a greater degree of gonozooid formation (and therefore female investment) in *Tubulipora plumosa*, and completion of gonozooids and brooding of offspring in *Filicrisia geniculata*. This suggests some role of fertilisation in the frequency of gonozooids and brooding in both species.

The ability to conserve female investment until outcrossing is assured, with presumed resource reallocation to sperm production, is facilitated by the simultaneous hermaphroditic nature of *Tubulipora plumosa*. Flexibility in resource allocation to sex function is thought to be

a major advantage of simultaneous hermaphroditism over gonochorism (Michiels, 1998). While no sex allocation data were collected in the present study on *T. plumosa*, resource allocation trade-offs between growth and reproduction in general, and also between male and female function, are likely (Charnov, 1982). In *Filicrisia geniculata* a large proportion of energy investment for brooding is delayed until fertilisation.

Some evidence for self-fertilisation in *Tubulipora plumosa* in the absence of allosperm was obtained, although this was not investigated in detail. Where self-fertilisation was present, some degree of inbreeding regulation (in terms of fewer gonozooids) was recorded, with reduced progeny produced per gonozooid due to either further inbreeding regulation or inbreeding depression. Inbreeding regulatory mechanisms could explain the absence of gonozooids in some colonies. This requires further detailed investigation, particularly in relation to estimates of sperm production and availability, and in terms of the survival of selfed progeny.

(C) Resource constraints on the frequency of brooding.

As a result of polyembryony, cyclostomes must nourish multiple embryos within their enlarged brood chambers. Nutrients are supplied by the surrounding feeding autozooids, as the gonozooid has no polypide or lophophore at this stage. Therefore, energy availability within a colony is likely to limit the number of gonozooids that can be supported (Borg, 1926; Ryland, 2000; Hughes *et al.*, 2005). This could be reflected in the wide range of relative gonozooid frequencies observed among colonies.

(D) Physical constraints on brooding.

Hermaphroditism has been linked to brooding (Strathmann & Strathmann, 1982), evolving when constraints on brood space limits the resources that can be allocated to female function (Heath, 1977, 1979). However, these arguments are based on allometry in relation to unitary animals (with variable area-to-volume ratios), and do not seem relevant to modular colonial organisms of similar-sized units in which ratios remain fixed. Among corals, for example, brooders are not disproportionately more likely to be hermaphroditic than gonochoristic (Carlon, 1999; Avise, 2011).

Brood-space limitation in cyclostomes may to relate to morphology. Species forming erect or encrusting growth forms are found throughout the order. Encrusting species are likely to experience more overgrowth and space competition in epifaunal assemblages compared to erect forms. This may lead to constraints on space available in which to form relatively large gonozooids (compared to regular autozooids), which are often extensive. However, phenotypic plasticity shown by some encrusting species may circumvent this limitation to some extent. For example, species of *Tubulipora* may form partially or wholly erect sections of colonies where gonozooids may develop (Hayward & Ryland, 1985; author per. obs). Other factors, such as energy resource availability, may be of more importance than availability of brood-space (or morphology) in determining frequency of gonozooids and brooding in cyclostomes.

The two cyclostome species investigated here differ in morphology and reproductive mode, and may suggest an apparent link between the two factors. *Filicrisia geniculata* is a gender specialist that forms erect, branching colonies, whereas the simultaneous hermaphrodite *Tubulipora plumosa* forms encrusting colonies. However, any possible link between colony morphology and reproductive mode that may be inferred here seems coincidental. Furthermore, a relationship is unlikely as cheilostome bryozoans show similar morphological range and are all simultaneous hermaphrodites.

6.4 Concluding remarks – the need for a broad phylogenetic perspective.

This thesis and results from previous research highlight potential genus- and family-level differences in reproductive mode among cyclostomes:

1) Within the Crisiidae, gender specialisation (or effective gonochorism) has been identified in *Filicrisia geniculata*. This situation differs from that described in *Crisia denticulata* (Pemberton *et al*., 2011), suggesting differences in reproductive mode at genus-level. Future research here should focus on uncovering the genetic and/or environmental basis of gender specialisation (effectively gender determination) in *F. geniculata*, using a combination of laboratory culturing and genetic (namely transcriptomic) techniques. By comparing gene expression patterns in the two colony types (‘male’ and ‘female’) formed by *F. geniculata*, genes involved in gender determination may be identified. Gender determination in response to various environmental conditions and at key (early) developmental stages, e.g. embryonic,

pre-settlement and post-settlement, should be investigated. The production of polyembryonous broods will facilitate this, providing the opportunity to test multiple replicates of the same genotype. Such research may also broaden our understanding of the underlying basis of gender allocation in crisiids and cyclostomes as a whole.

2) Family-level differences in reproductive mode are apparent between the Crisiidae and the Tubuliporidae. Evidence from mating trials with *Tubulipora plumosa* is consistent with simultaneous hermaphroditism. This is in contrast to the attainment of gender specialisation in at least one crisiid (*Filicrisia geniculata*), although *Crisia denticulata* may represent an intermediate stage in the transition from simultaneous hermaphroditism towards gonochorism. Further work should address the more general question of 'why be a hermaphrodite?' and begin by gathering empirical data on differential allocation to sex function in these colonies, which may then be used more widely to investigate theories relating sex allocation and the existence of different reproductive modes. Sperm production data, in particular, is of wider relevance to our understanding of other aspects of cyclostome reproduction, such as polyembryony and its possible association with sperm limitation. In addition to colony-wide sperm production, further investigations should focus on uncovering cyclostome fertilisation dynamics and the route and efficiency of sperm uptake mechanisms. Anatomical studies and observations from the field and laboratory cultures should be utilised.
3) Research in the present study focussed only on two cyclostome families. Of those families not investigated, the reproductive biology of the Lichenoporidae is likely to be of most interest. Lichenoporids differ in a number of ways from other cyclostomes, and it is likely that differences in reproductive mode exist too. Members of this family are understood to be simultaneous hermaphrodites but exhibit zooidal hermaphroditism in addition to zooidal gonochorism (Harmer, 1896). Furthermore, lichenoporid brood chambers are not homologous to gonozooids of other cyclostomes and there are even differences in brood chamber construction within the family (*Lichenopora* – large central brood space; *Disporella* – chamber may be divided internally) (Borg, 1944). There is much potential for further work with this group. Although the family forms a major clade with some Plagioecidae (Waeschenbach *et al.*, 2009) (*Plagioecia patina* was investigated here), historical inferences for both the occurrence of polyembryony and the possible co-occurrence of multiple primary embryos within the large lichenoporid brood cavities, have not yet been confirmed genetically and should be a priority of future study.

4) Understanding of the reproductive biology of the family Cinctiporidae, in which brood chambers are unknown, remains elusive and should be a focus for future investigations.
5) The apparent persistence of polyembryony throughout an entire order of bryozoans (the Cyclostomata) is exceptional among metazoans and further investigations are required in order to understand this. Future studies should investigate the genetic basis of polyembryony among cyclostomes. By using a transcriptomic approach, the patterns of gene expression throughout colony growth/development and at different stages of polyembryony may be uncovered and genes involved in these processes identified. Comparisons between species may reveal the degree to which the genetic mechanisms of polyembryony are conserved within the group. Future studies involving laboratory cultures should focus on the influence of environmental factors on polyembryony, for example, the effect of food supply on brood size. Brood size flexibility in relation to the extent of 'colony-wide' brooding is also of interest, especially in terms of the potential conflict between maternal (colony) genotype and offspring (brood) genotype(s). Such investigations could incorporate genetic methods in order to reveal whether (or at what stage) brood size is under maternal or offspring control.

To conclude, most of our current understanding of cyclostome reproduction is based on *Crisia denticulata* or the other crisiids. However, findings reported here in this thesis suggest that generalisations about the reproductive biology of cyclostomes should be made with caution. Future investigations should provide a deeper understanding of the evolution and maintenance of polyembryony, and of sex allocation and sex determination in these colonial invertebrates.

Appendix I: **ISSR Genotyping Analysis – PAGE gels**

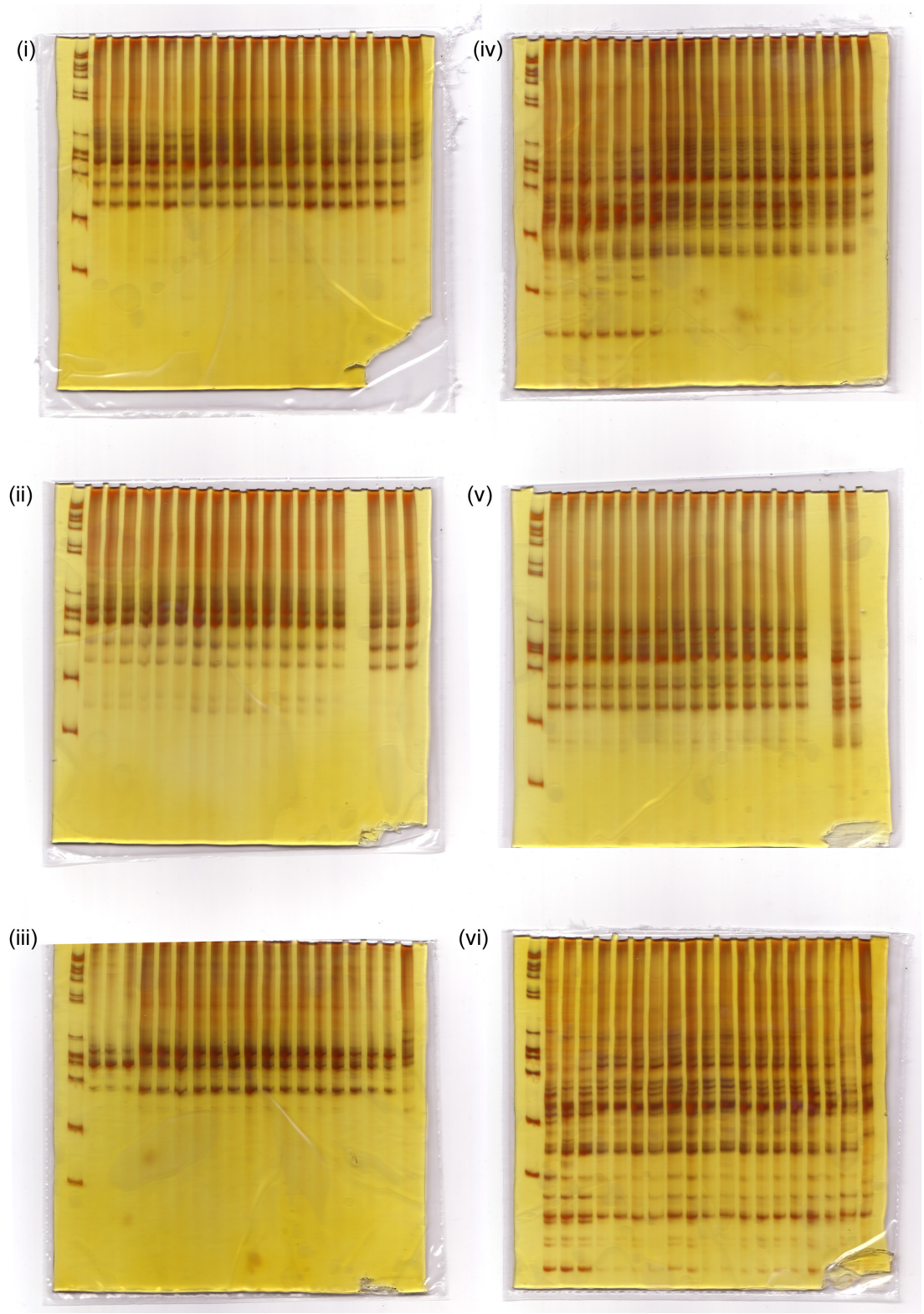


Figure 1: PAGE gels from genotyping analysis of *Crisia denticulata*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primers UBC 827 and UBC 850. UBC 827: (i) Brood A, (ii) Brood B, (iii) Brood C. UBC 850: (iv) Brood A, (v) Brood B, (vi) Brood C. Lane 1 = ladder.

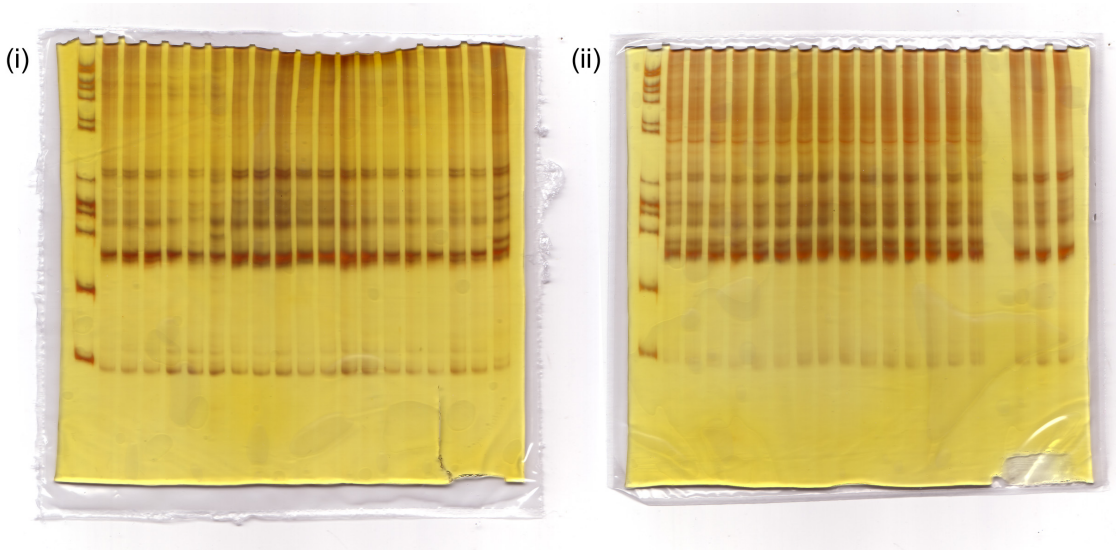


Figure 2: PAGE gels from genotyping analysis of *Crisia denticulata*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primer UBC 884: (i) Brood A, (ii) Brood B. Lane 1 = ladder.

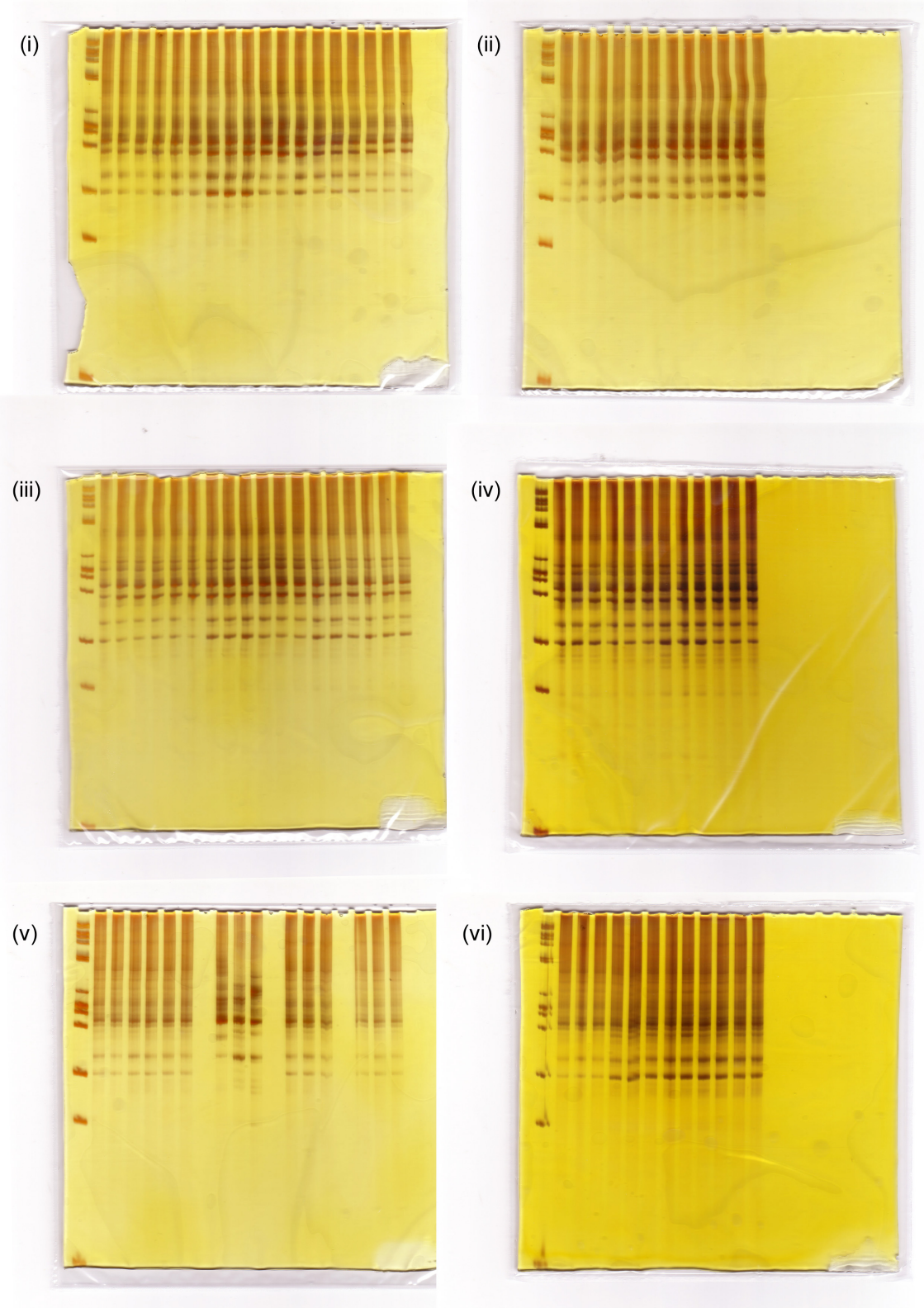


Figure 3: PAGE gels from genotyping analysis of *Hornera robusta*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primer UBC 817: (i-ii) Brood D, (iii-iv) Brood E, (v-vi) Brood F. Lane 1 = ladder.

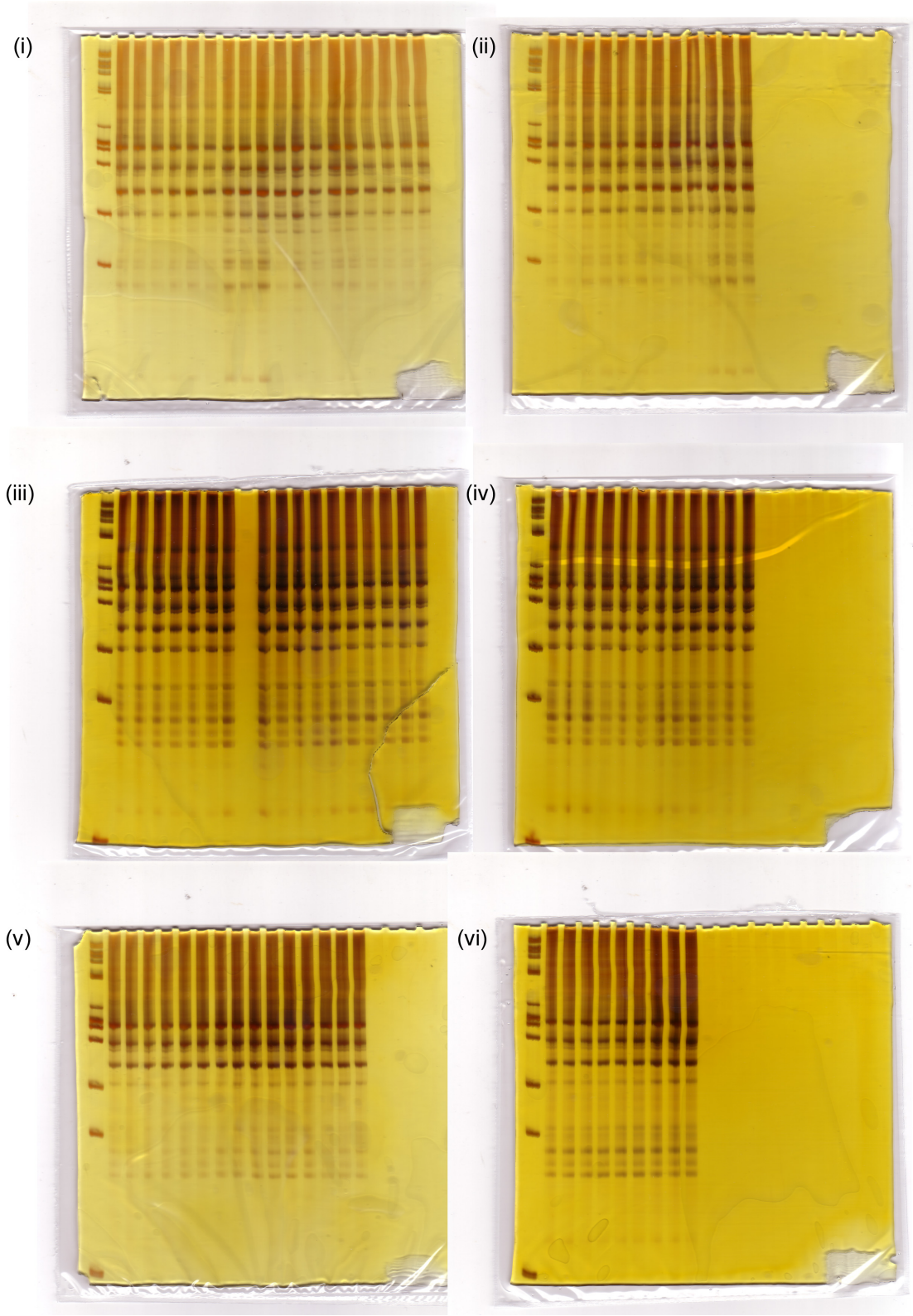


Figure 4: PAGE gels from genotyping analysis of *Hornera robusta*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primer UBC 855: (i-ii) Brood D, (iii-iv) Brood E, (v-vi) Brood F. Lane 1 = ladder.

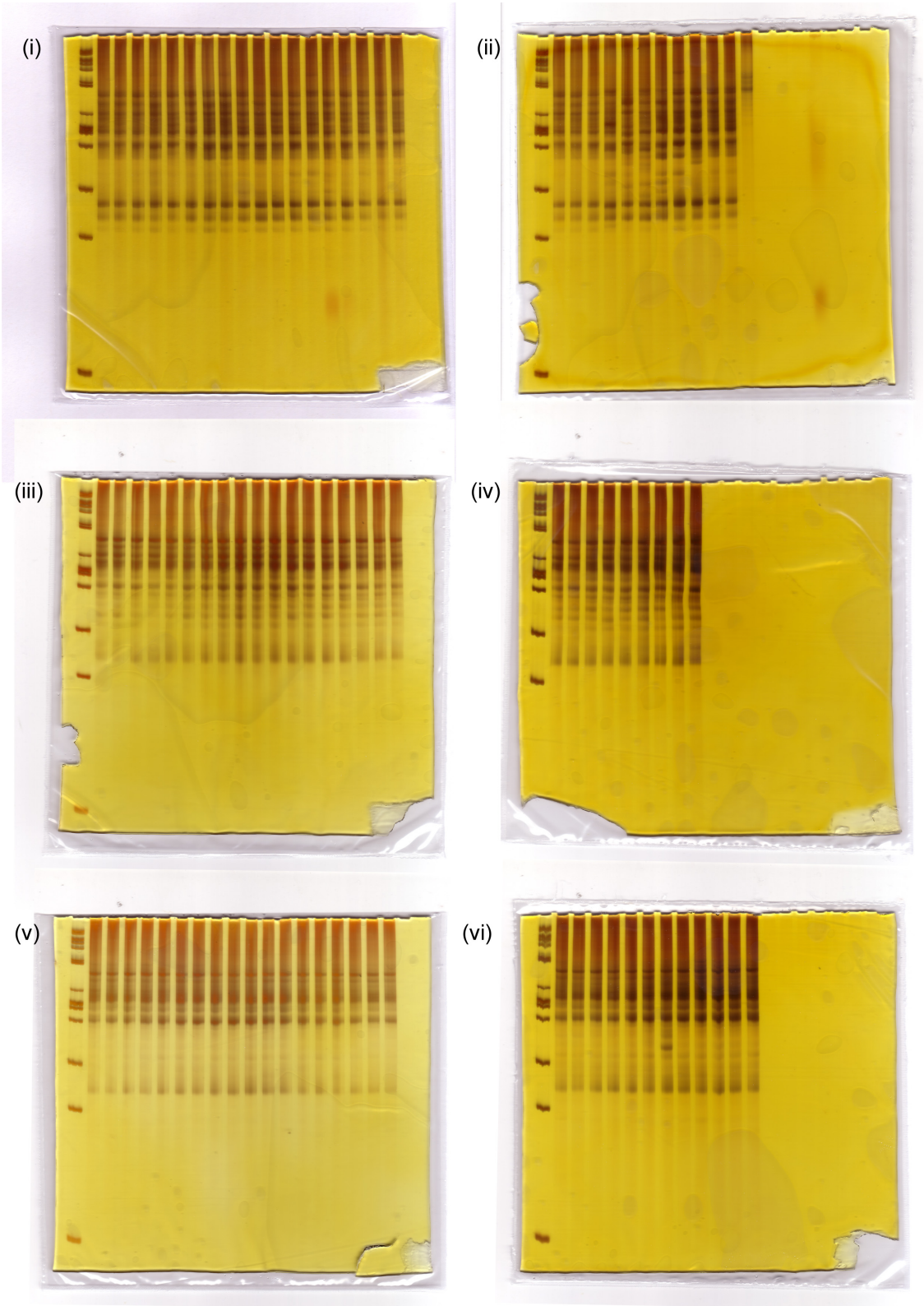


Figure 5: PAGE gels from genotyping analysis of *Plagioecia patina*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primer UBC 827: (i-ii) Brood G, (iii-iv) Brood H, (v-vi) Brood I. Lane 1 = ladder.

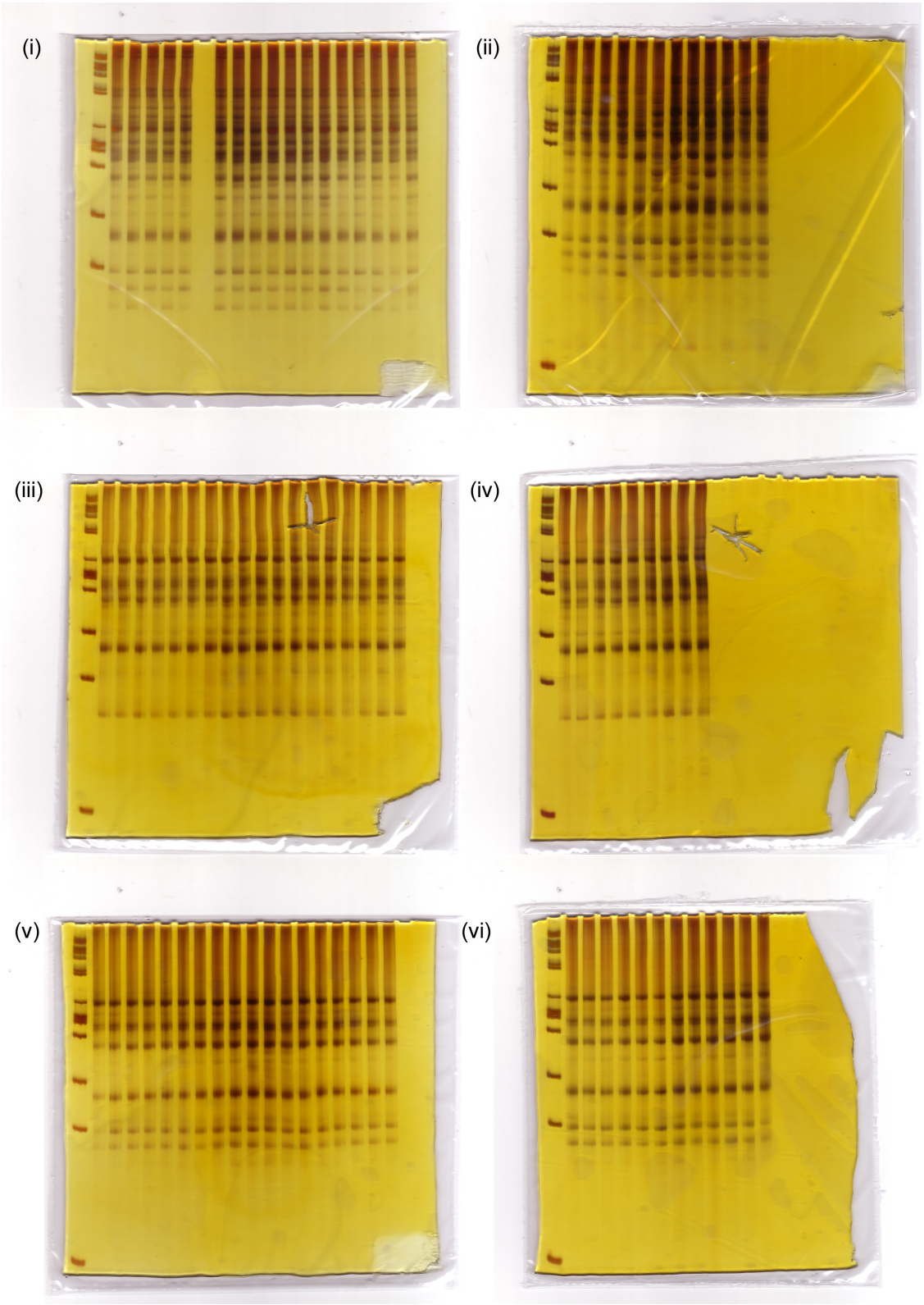


Figure 6: PAGE gels from genotyping analysis of *Plagioecia patina*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primer UBC 850: (i-ii) Brood G, (iii-iv) Brood H, (v-vi) Brood I. Lane 1 = ladder.

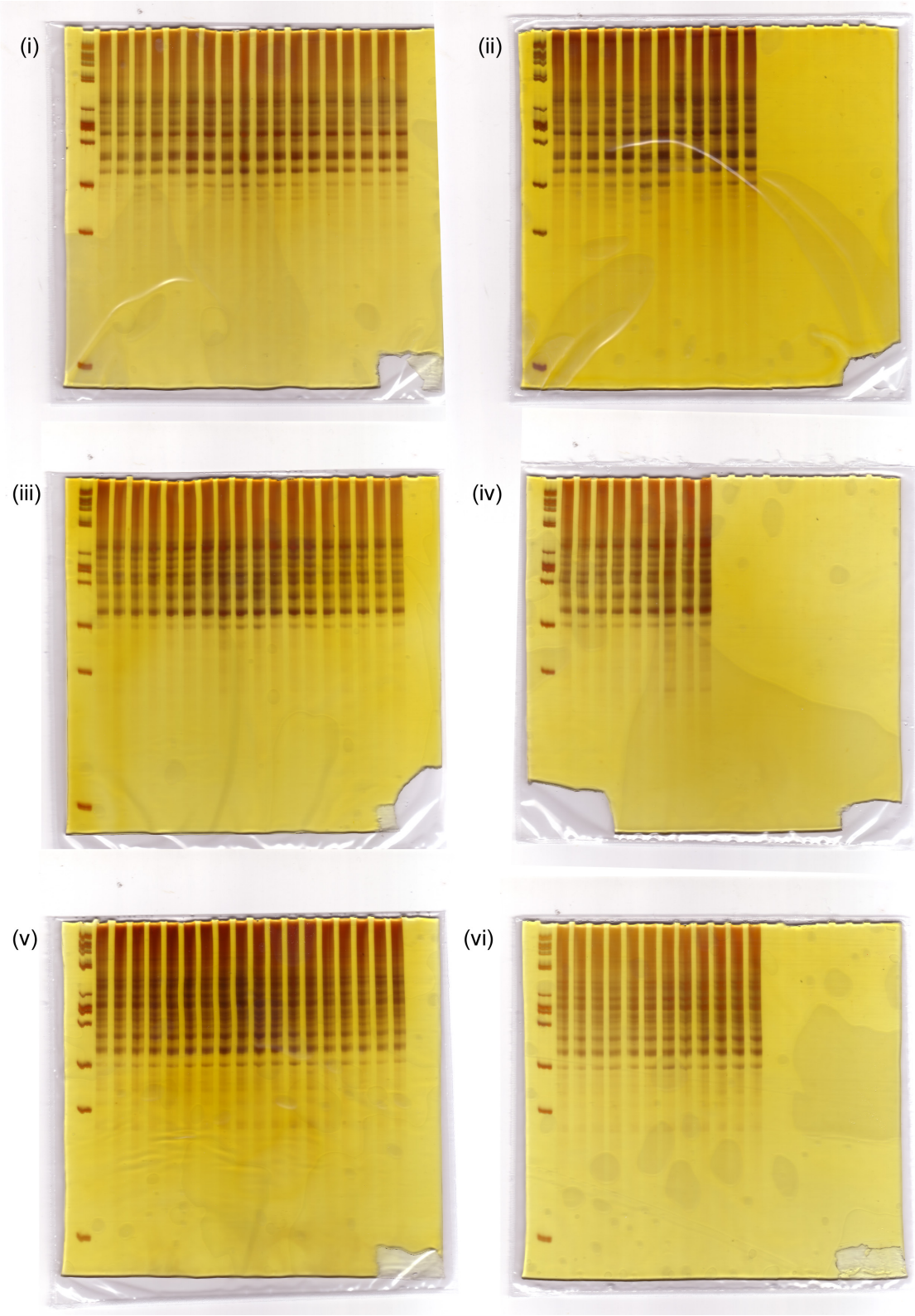


Figure 7: PAGE gels from genotyping analysis of *Plagioecia patina*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primer UBC 855: (i-ii) Brood G, (iii-iv) Brood H, (v-vi) Brood I. Lane 1 = ladder.

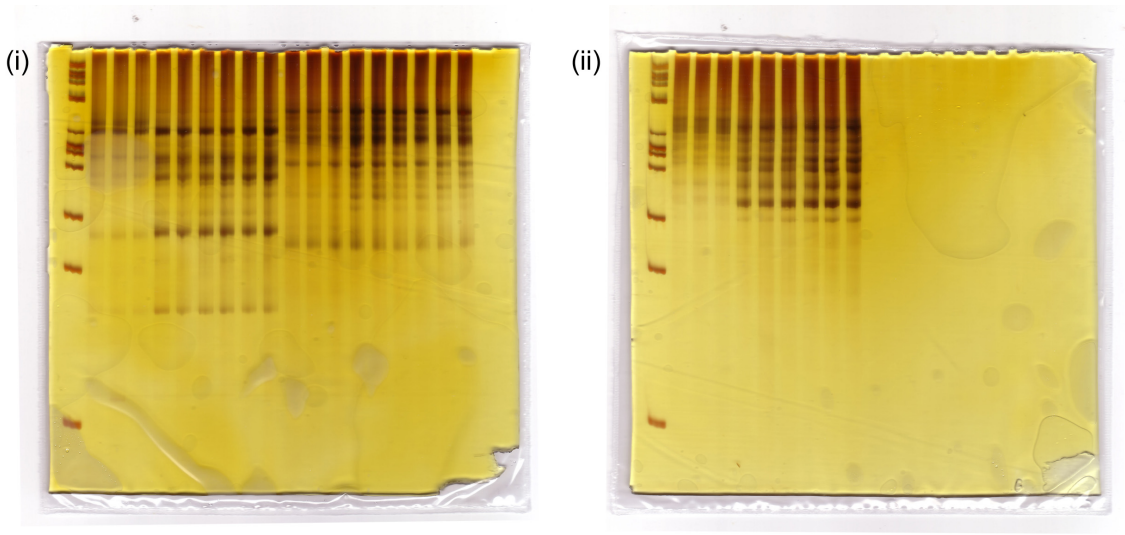


Figure 8: PAGE gels from genotyping analysis of *Plagioecia patina*: within-brood comparison and comparison between broods within the same colony. Gel images show banding profiles of six Brood H larvae and 'whole brood' sample WB 01 (in triplicate) screened with the ISSR primers UBC 827 and UBC 850: (i) UBC 850: WB 01 (lanes 2-4), Brood H (lanes 5-10); UBC 827: WB 01 (lanes 11-13), Brood H (lanes 14-19). (ii) UBC 855: WB 01 (lanes 2-4), Brood H (lanes 5-10). Lane 1 = ladder.

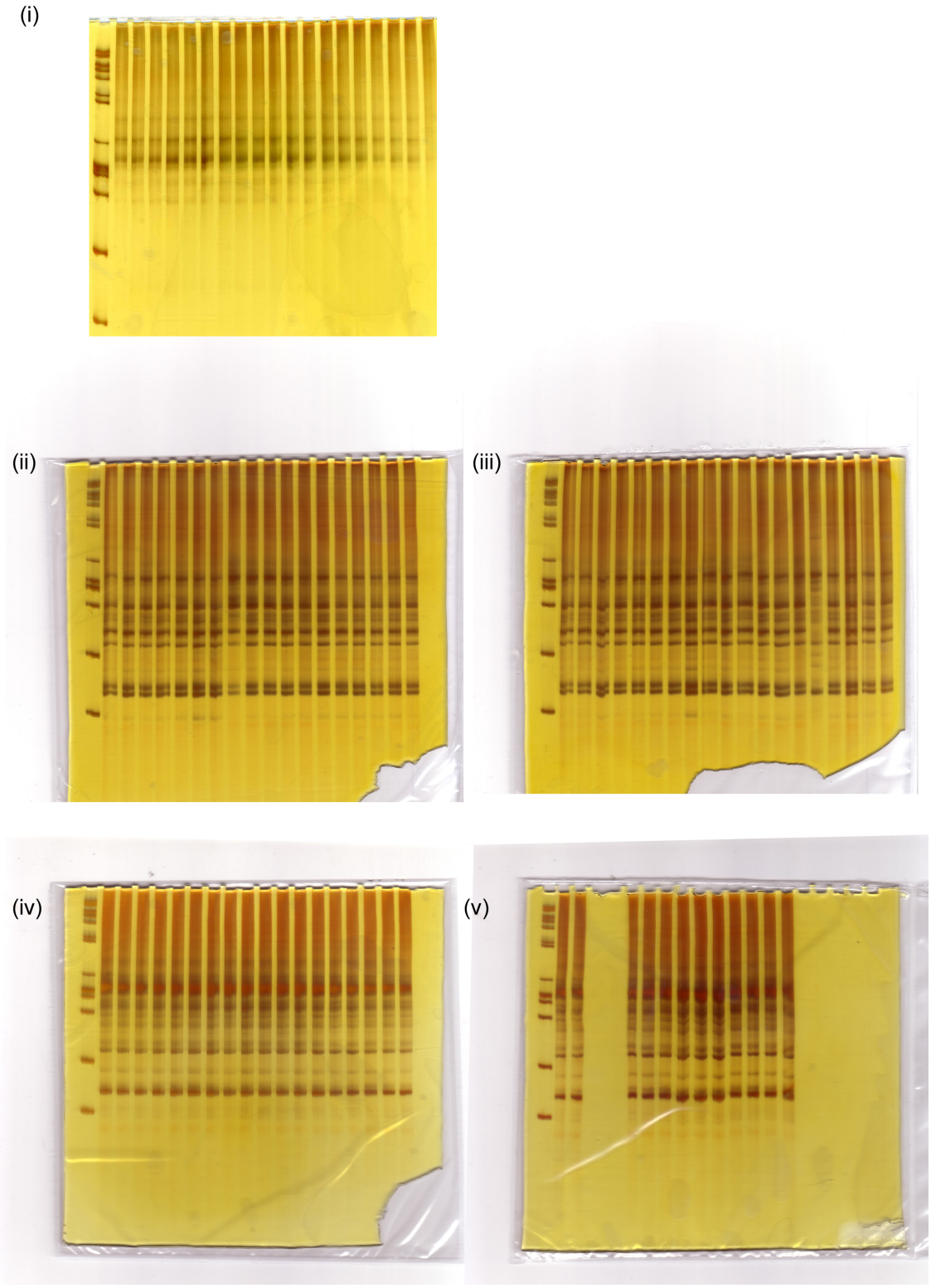


Figure 9: PAGE gels from genotyping analysis of *Tubulipora plumosa*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primer UBC 817: (i) Brood J, (ii-iii) Brood K, (iv-v) Brood L. Lane 1 = ladder.

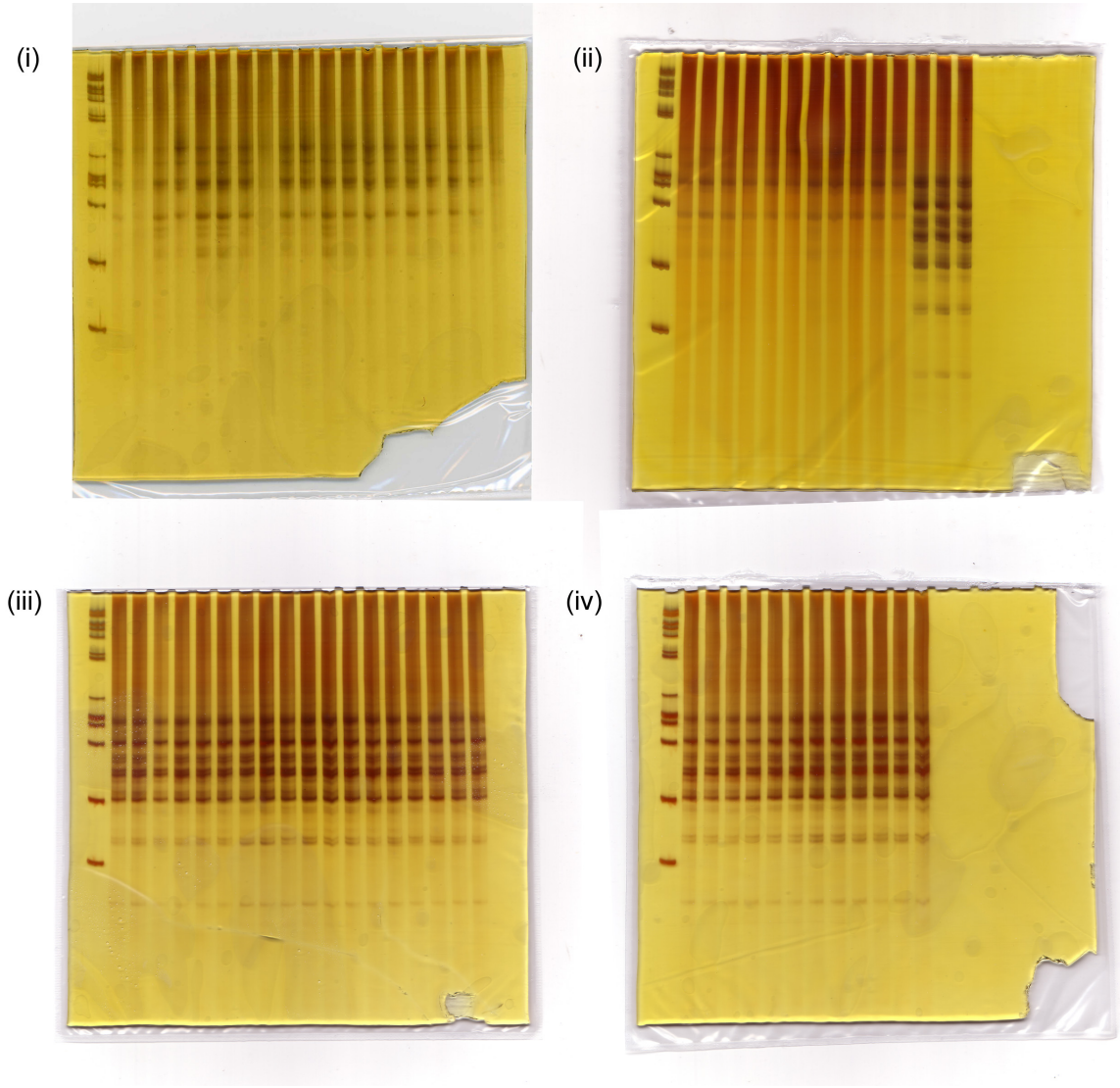


Figure 10: PAGE gels from genotyping analysis of *Tubulipora plumosa*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primer UBC 850: (i) Brood J, (ii) Broods J & L (lanes 2-12 & 13-15, respectively), (iii-iv) Brood L. Lane 1 = ladder.

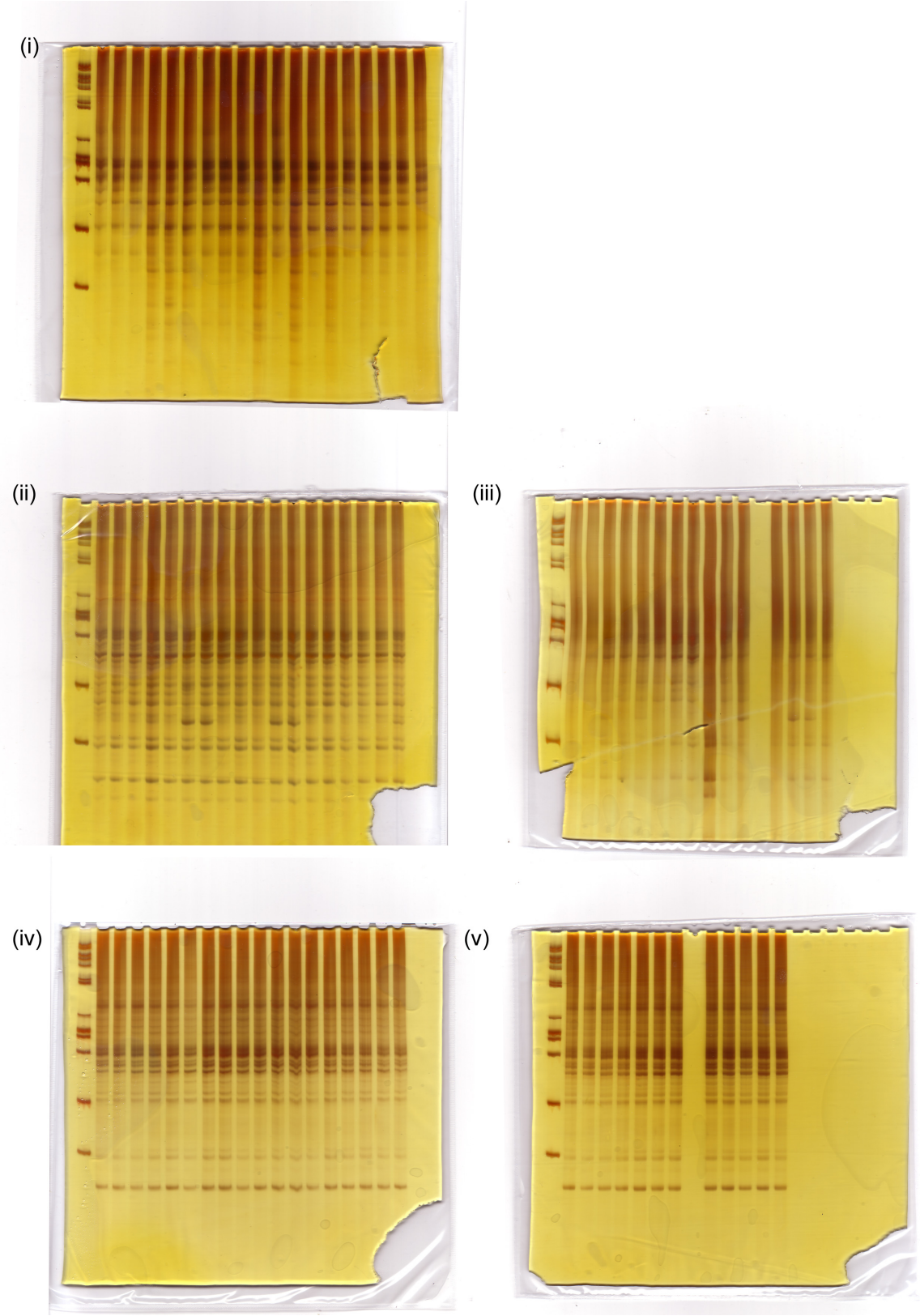


Figure 11: PAGE gels from genotyping analysis of *Tubulipora plumosa*: within-brood comparison. Gel images show banding profiles of larvae (in triplicate) from each brood screened with the ISSR primer UBC 855: (i) Brood J, (ii-iii) Brood K, (iv-v) Brood L. Lane 1 = ladder.

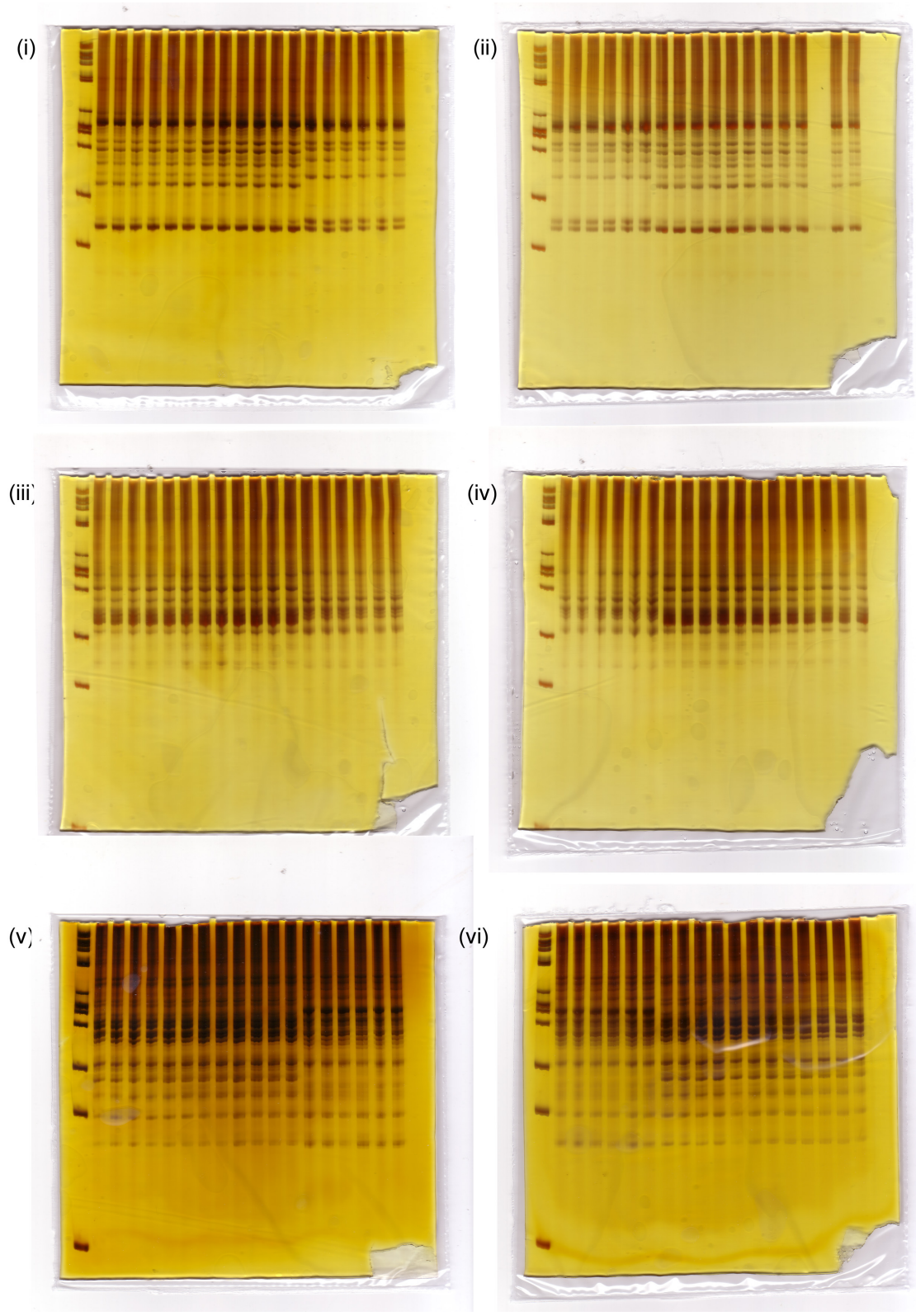


Figure 12: PAGE gels from genotyping analysis of *Tubulipora plumosa*: within-brood comparison and comparison between broods within the same colony. Gel images show banding profiles of eight Brood M larvae and four tissue extracts from 'whole brood' sample WB 02 (all in triplicate) screened with the ISSR primers UBC 817, UBC 850 & UBC 855. UBC 817: (i) Brood M (lanes 2-13), WB 02 (lanes 14-19); (ii) WB 02 (lanes 2-7), Brood M (lanes 8-19). UBC 850: (iii) Brood M (lanes 2-13), WB 02 (lanes 14-19); (iv) WB 02 (lanes 2-7), Brood M (lanes 8-19). UBC 855: (v) Brood M (lanes 2-13), WB 02 (lanes 14-19); (vi) WB 02 (lanes 2-7), Brood M (lanes 8-19). Lane 1 = ladder.

Appendix II: **ISSR Genotyping Analysis – example of virtual gel scoring**

Tubulipora plumosa **UBC 817**

Brood M 3 larvae

	Larval ID and band sizes (bp)			Difference	Average
Band No.	UKL442	UKL445	UKL448	+/-10%?	band size (bp)
1	465	463	461	ok	463
2	683	679	672	ok	678
3	894	880	864	ok	879
4	1147	1120	1096	ok	1121

WB 02 3 extracts

	Tissue extract ID and band sizes (bp)			Difference	Average
Band No.	WB02.A	WB02.B	WB02.D	+/-10%?	band size (bp)
1	462	461	460	ok	461
2	477	477	476	ok	477
3	783	782	778	ok	781
4	911	906	901	ok	906
5	1202	1206	1206	ok	1205

Brood scoring table

UBC 817	Band size/bp	Brood M	WB 02
1	461/463		
2	477		
3	678		
4	781		
5	879		
6	906		
7	1121		
8	1205		

Pair-wise comparison table

	Brood M
WB 02	7

Figure 1: Virtual gel scoring of *Tubulipora plumosa* Brood M larvae and 'whole brood' WB 02 tissue samples analysed using ISSR primer UBC 817. For Brood M and WB 02, three larvae and three different tissue extracts respectively, were selected for automated gel electrophoresis analysis. The corresponding virtual gel showing banding profiles are shown in Chapter 2: Figure 3; brood scoring and pair-wise comparison tables are included here. For each larva/tissue sample, the bands scored and their sizes (bp) are shown. Banding profiles within broods were compared: "Difference +/- 10%?" = for a specific band, the difference in band size between the larvae/tissue samples is checked to be within the sizing accuracy limits specified by the manufacturer; "Average band size (bp)" = band sizes derived from the individual band sizes for each larva/tissue sample, and used in the brood scoring table for pair-wise comparisons between broods. Brood scoring table: green cell = band present. Pair-wise comparison is shown as a matrix of the number of differences based on brooding scoring table.

Appendix III: **ISSR Genotyping Analysis —Level One pair-wise comparisons**

a) *Crisia denticulata*

(i) UBC 827

	Brood C	Brood A
Brood A	2	
Brood B	5	5

(ii) UBC 850

	Brood C	Brood A
Brood A	8	
Brood B	12	8

b) *Hornera robusta*

(i) UBC 855

	Brood D	Brood E
Brood E	6	
Brood F	6	2

c) *Plagioecia patina*

(i) UBC 855

	Brood G	Brood H
Brood H	1	
Brood I	4	3

d) *Tubulipora plumosa*

(i) UBC 817

	Brood J	Brood K
Brood K	6	
Brood L	5	9

(ii) UBC 855

	Brood J	Brood K
Brood K	3	
Brood L	3	6

Figure 1: Level One scoring pair-wise comparisons between broods from ISSR genotyping analysis. Pair-wise comparisons are shown as a matrix of the number of differences in virtual gel banding profiles between broods based on brood scoring tables (see Chapter 2: Table 4).
a) *Crisia denticulata*: (i) UBC 827, (ii) UBC 850; b) *Hornera robusta*: (i) UBC 855; c) *Plagioecia patina*: (i) UBC 855; d) *Tubulipora plumosa*: (i) UBC 817, (ii) UBC 855.

Appendix IV: **ISSR Genotyping Analysis – virtual gels**

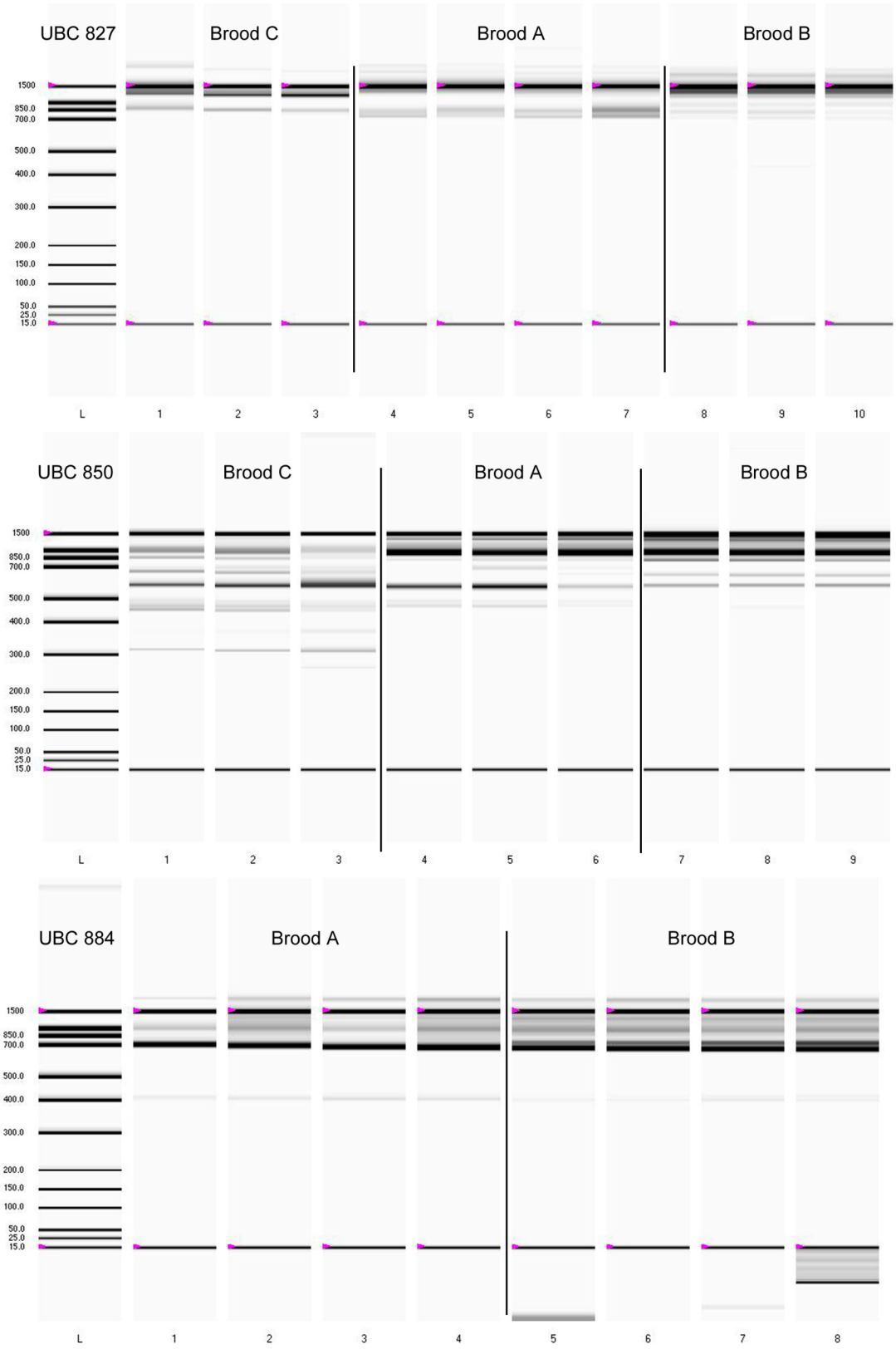


Figure 1: Set of virtual gels from genotyping analysis of *Crisia denticulata*: comparisons between broods from different colonies. Each gel image shows banding profiles, from one ISSR primer, of three/four individuals from two or three different broods. Note: larger bands (> 1.5 Kb) outside of the sizing range not scored. L = ladder. Upper internal size marker = 1500 bp, lower internal size marker = 15 bp.

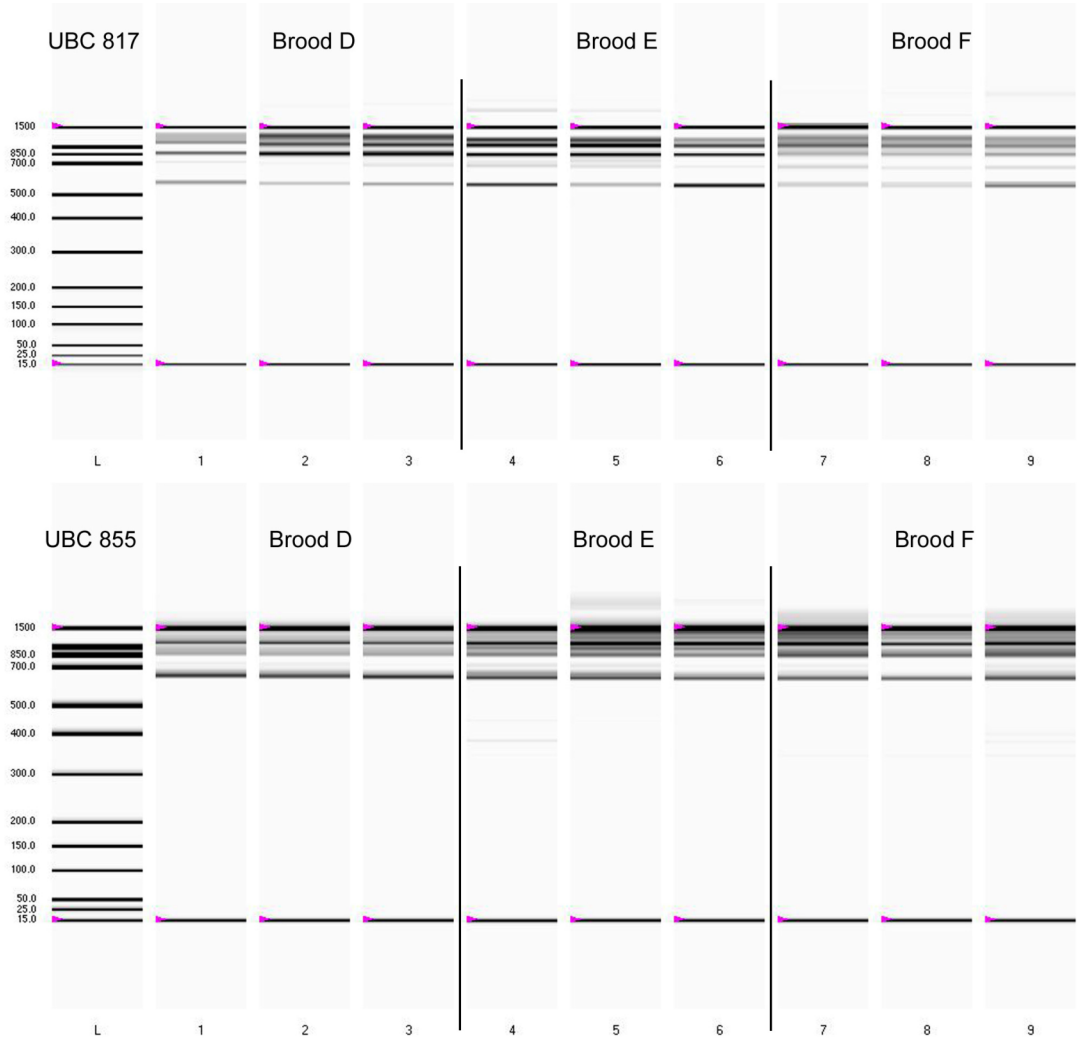


Figure 2: Set of virtual gels from genotyping analysis of *Hornera robusta*: comparisons between broods from different colonies. Each gel image shows banding profiles, from one ISSR primer, of three individuals from three different broods. Note: larger bands (> 1.5 Kb) outside of the sizing range not scored. L = ladder. Upper internal size marker = 1500 bp, lower internal size marker = 15 bp.

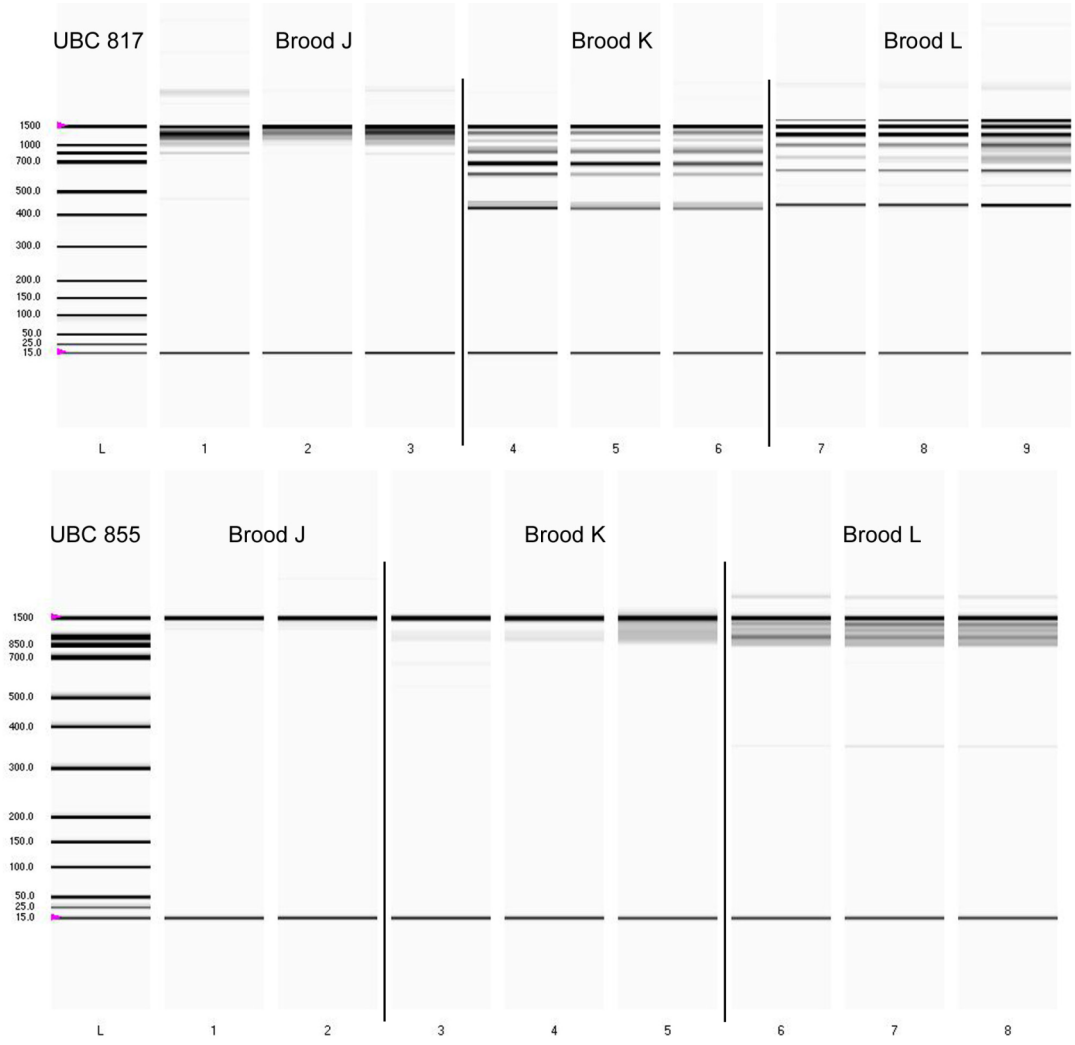


Figure 3: Set of virtual gels from genotyping analysis of *Tubulipora plumosa*: comparisons between broods from different colonies. Each gel image shows banding profiles, from one ISSR primer, of two/three individuals from three different broods. Note: larger bands (> 1.5 Kb) outside of the sizing range not scored. L = ladder. Upper internal size marker = 1500 bp, lower internal size marker = 15 bp.

Appendix V: **Gonozooid production in *Tubulipora plumosa***

Table 1: The number of gonozooids produced per ramet by *Tubulipora plumosa* clones in each experimental treatment (ramet denoted in **bold** in 'Cross' column). All replicates of crosses are shown in the 'Cross' column (mixed-clone treatment: 'b' = slide at back of tank; 'f' = slide at front of tank). Note: 'GZ' = gonozooid.

Treatment	Clone	Cross	No. of GZ
SINGLE	1	**1A** x 1A	0
SINGLE	1	1A x **1A**	0
SINGLE	1	**1B** x 1B	0
SINGLE	1	1B x **1B**	0
SINGLE	1	**1C** x 1C	2
SINGLE	1	1C x **1C**	1
SINGLE	1	**1D** x 1D	3
SINGLE	1	1D x **1D**	2
SINGLE	2	**2A** x 2A	2
SINGLE	2	2A x **2A**	5
SINGLE	2	**2B** x 2B	1
SINGLE	2	2B x **2B**	1
SINGLE	2	**2C** x 2C	0
SINGLE	2	2C x **2C**	2
SINGLE	2	**2D** x 2D	4
SINGLE	2	2D x **2D**	3
SINGLE	3	**3A** x 3A	1
SINGLE	3	3A x **3A**	0
SINGLE	3	**3B** x 3B	1
SINGLE	3	3B x **3B**	1
SINGLE	3	**3C** x 3C	0
SINGLE	3	3C x **3C**	0
SINGLE	3	**3D** x 3D	0
SINGLE	3	3D x **3D**	0
SINGLE	4	**4A** x 4A	0
SINGLE	4	4A x **4A**	0
SINGLE	4	**4B** x 4B	0
SINGLE	4	4B x **4B**	0
SINGLE	4	**4C** x 4C	0
SINGLE	4	4C x **4C**	0
SINGLE	4	**4D** x 4D	0
SINGLE	4	4D x **4D**	0
SINGLE	5	**5A** x 5A	0
SINGLE	5	5A x **5A**	0
SINGLE	5	**5B** x 5B	0
SINGLE	5	5B x **5B**	0
SINGLE	5	**5C** x 5C	0
SINGLE	5	5C x **5C**	0
SINGLE	5	**5D** x 5D	1
SINGLE	5	5D x **5D**	1

Treatment	Clone	Cross	No. of GZ
MIXED	1	**1b** x 2f	12
MIXED	1	**1f** x 2b	12
MIXED	1	**1b** x 3f	7
MIXED	1	**1f** x 3b	13
MIXED	1	**1f** x 4b	19
MIXED	1	**1b** x 4f	10
MIXED	1	**1b** x 5f	0
MIXED	1	**1f** x 5b	2
MIXED	2	1b x **2f**	15
MIXED	2	1f x **2b**	19
MIXED	2	**2b** x 3f	10
MIXED	2	**2f** x 3b	10
MIXED	2	**2b** x 4f	3
MIXED	2	**2f** x 4b	10
MIXED	2	**2b** x 5f	13
MIXED	2	**2f** x 5b	13
MIXED	3	1b x **3f**	7
MIXED	3	1f x **3b**	9
MIXED	3	2b x **3f**	12
MIXED	3	2f x **3b**	11
MIXED	3	**3b** x 4f	4
MIXED	3	**3f** x 4b	13
MIXED	3	**3b** x 5f	0
MIXED	3	**3f** x 5b	1
MIXED	4	1b x **4f**	0
MIXED	4	1f x **4b**	1
MIXED	4	2b x **4f**	0
MIXED	4	2f x **4b**	0
MIXED	4	3b x **4f**	0
MIXED	4	3f x **4b**	0
MIXED	4	**4b** x 5f	0
MIXED	4	**4f** x 5b	0
MIXED	5	1b x **5f**	9
MIXED	5	1f x **5b**	0
MIXED	5	2b x **5f**	4
MIXED	5	2f x **5b**	16
MIXED	5	3b x **5f**	2
MIXED	5	3f x **5b**	1
MIXED	5	4b x **5f**	4
MIXED	5	4f x **5b**	3

Appendix VI: **Sperm production data for *Filicrisia geniculata* colonies**

Table 1: The number of sperm counted from two transects (one horizontal, one vertical) of each filter for three water samples ('Count') taken from tanks containing individual *Filicrisia geniculata* colonies.

Sample	Colony	Colony Type	Transect		Average
			Horizontal	Vertical	
Count 1	July 6	Type 1	8	6	7
	107x104 B	Type 1	575	356	465.5
	Clone D	Type 1	953	878	915.5
	Aug 2A	Type 1	845	731	788
	Aug 4B	Type 1	19	15	17
	104x4 A	Type 1	2063	2096	2079.5
	Aug 7B	Type 2	0	0	0
	Aug 4A	Type 2	0	0	0
	Clone C	Type 2	0	0	0
	107x104 C	Type 2	0	0	0
	Aug 6A	Type 2	0	0	0
	July 4D	Type 2	0	0	0
	Aug 10A	Type 2	0	0	0
	Aug 12A	Type 2	0	0	0
Count 2	July 6	Type 1	74	78	76
	107x104 B	Type 1	984	846	915
	Clone D	Type 1	737	776	756.5
	Aug 2A	Type 1	665	465	565
	Aug 4B	Type 1	55	51	53
	104x4 A	Type 1	2485	2105	2295
	Aug 7B	Type 2	0	0	0
	Aug 4A	Type 2	0	0	0
	Clone C	Type 2	0	0	0
	107x104 C	Type 2	0	0	0
	Aug 6A	Type 2	0	0	0
	July 4D	Type 2	0	0	0
	Aug 10A	Type 2	0	0	0
	Aug 12A	Type 2	0	0	0
Count 3	July 6	Type 1	53	48	50.5
	107x104 B	Type 1	1135	885	1010
	Clone D	Type 1	780	710	745
	Aug 2A	Type 1	625	713	669
	Aug 4B	Type 1	44	41	42.5
	104x4 A	Type 1	1976	1733	1854.5
	Aug 7B	Type 2	0	0	0
	Aug 4A	Type 2	0	0	0
	Clone C	Type 2	0	0	0
	107x104 C	Type 2	0	0	0
	Aug 6A	Type 2	0	0	0
	July 4D	Type 2	0	0	0
	Aug 10A	Type 2	0	0	0
	Aug 12A	Type 2	0	0	0

Appendix VII: **Zooid count data for Type 2 colonies of *Filicrisia geniculata***

Table 1: The number of autozooids and incomplete gonozooids present in each of 10 randomly sampled branches from each Type 2 colony of *Filicrisia geniculata*.

Colony	Branch	Autozooids	Incomplete gonozooids	TOTAL
August 4A	1	42	3	45
August 4A	2	89	4	93
August 4A	3	59	3	62
August 4A	4	130	18	148
August 4A	5	117	15	132
August 4A	6	65	5	70
August 4A	7	106	23	129
August 4A	8	95	9	104
August 4A	9	194	21	215
August 4A	10	156	20	176
August 7B	1	84	14	98
August 7B	2	67	15	82
August 7B	3	66	12	78
August 7B	4	71	12	83
August 7B	5	118	23	141
August 7B	6	103	15	118
August 7B	7	150	28	178
August 7B	8	56	13	69
August 7B	9	52	11	63
August 7B	10	50	10	60
August 6A	1	69	5	74
August 6A	2	90	7	97
August 6A	3	77	2	79
August 6A	4	40	9	49
August 6A	5	88	9	97
August 6A	6	172	15	187
August 6A	7	103	8	111
August 6A	8	115	0	115
August 6A	9	61	2	63
August 6A	10	53	1	54
Clone C	1	136	19	155
Clone C	2	92	18	110
Clone C	3	95	22	117
Clone C	4	110	18	128
Clone C	5	97	18	115
Clone C	6	136	25	161
Clone C	7	106	26	132
Clone C	8	111	11	122
Clone C	9	148	23	171
Clone C	10	260	61	321

Colony	Branch	Autozooids	Incomplete gonozooids	TOTAL
107 x 104 C	1	119	4	123
107 x 104 C	2	170	6	176
107 x 104 C	3	137	6	143
107 x 104 C	4	210	3	213
107 x 104 C	5	107	2	109
107 x 104 C	6	56	1	57
107 x 104 C	7	158	6	164
107 x 104 C	8	222	4	226
107 x 104 C	9	73	2	75
107 x 104 C	10	48	2	50
August 12A	1	109	23	132
August 12A	2	74	15	89
August 12A	3	178	27	205
August 12A	4	145	27	172
August 12A	5	126	21	147
August 12A	6	87	15	102
August 12A	7	170	34	204
August 12A	8	107	18	125
August 12A	9	146	25	171
August 12A	10	123	21	144
July 4D	1	49	5	54
July 4D	2	83	12	95
July 4D	3	94	4	98
July 4D	4	84	2	86
July 4D	5	148	10	158
July 4D	6	92	7	99
July 4D	7	120	4	124
July 4D	8	156	6	162
July 4D	9	81	2	83
July 4D	10	72	3	75
August 10A	1	135	5	140
August 10A	2	127	4	131
August 10A	3	126	6	132
August 10A	4	122	5	127
August 10A	5	109	4	113
August 10A	6	140	7	147
August 10A	7	102	6	108
August 10A	8	123	6	129
August 10A	9	166	5	171
August 10A	10	60	3	63

Appendix VIII: *Filicrisia geniculata* microsatellite primers and preliminary screening information

Table 1: Details of the initial set of 34 microsatellite primer pairs tested in the genotyping analysis of *Filicrisia geniculata*. T_a (ºC), annealing temperature; N_{cycl} , no. of PCR cycles at T_a (ºC). The level of analysis is indicated in 'Amplification and profile test' column: all PCR products were screened on agarose gels, some were then visualised on acrylamide gels for greater resolution. Banding profiles (single-locus or multi-locus) obtained relate to the gel type specified ('Amplification and profile test' column). PCR product sizes were obtained from the primer analysis conducted by Genoscreen. Loci FG08, FG12, FG13 & FG17 were used in fragment analysis and for further details see Chapter 5: Table 1.

Appendix VIII: ***Filicrisia geniculata*** **microsatellite primers and preliminary screening information**

Locus	Repeat motif	Primer sequence	T_a (°C)	N_{cycl}	Amplification and profile test	Banding profile	PCR product size
FG08	$(CAAA)_{11}$	F: TCACCTCCTCATACACGCCT R: TCTGTGCTGTATTGTGAGCG	52	35	fragment analysis	single	102
FG12	$(GTAT)_{11}$	F: TGTATGCATGTATGTATGGAATGG R: TACGATAAGCTCGCAGGACA	52	35	fragment analysis	single	90
FG13	$(AC)_9$	F: ACATTAGACCCGGGATTTCG R: AAGTTGTGAAGTTAAGTTGTTCCAA	51	35	fragment analysis	single	196
FG17	$(TGTA)_{23}$	F: TTTAAAATCCACACTCTATCGCC R: CAGGTACACTTACATGCCAACTACA	52	35	fragment analysis	single	191
FG04	$(AC)_{10}$	F: CAGGCTATGTTTCCATCAGTTC R: GCATTCAGTGATATGATTTTCTATGG	53	35	acrylamide	single	202
FG05	$(ATGT)_9$	F: AGATTGTATCAAAACATGTACAGAAAA R: TCATGTAACTGCCTGCTTGG	51	35	acrylamide	single	141
FG06	$(GT)_{10}$	F: GCATCATCTTGTTTTACTTTCCTC R: GGAGGAGTAAGTGGACAGACAGA	52	35	acrylamide	single	273
FG14	$(CA)_{14}$	F: AGGCTGGCGGTCAGTTATTA R: CGTTGTGCAGTGTGTGTGAG	52	35	acrylamide	multi	98
FG22	$(GT)_{10}$	F: TTTGGGGCTTCTCAAGGTGT R: ACACCTTTGCTAAACGGACC	52	35	acrylamide	single	192
FG24	$(AG)_{11}$	F: ATGCCTCAATGTGCGACAA R: TTGCCTACCAACTCACCCTT	49	35	acrylamide	multi	245
FG26	$(AC)_{10}$	F: TCCATCTTTCAAAATTCATCATAG R: AGGCAGGTTTGCAGAGAAGA	49	35	acrylamide	multi	176
FG28	$(AAC)_{12}$	F: ATGAGGGCAAACATGCAAA R: TGCTCACGACAAAGAAACAGA	47	35	acrylamide	single	135
FG01	$(GT)_{12}$	F: CAAAGTCGAGGTTCCAGCTC R: GGTCTACCTTTGGTGCCTGA	54	35	agarose	single	206
FG03	$(TG)_9$	F: GCTAGCGAAACATGGAAAGC R: CACACACTCACACTCACACACA	52	35	agarose	single	252
FG07	$(AC)_{12}$	F: AGAGCCTTGGCCCTCATATT R: TGTCTGCGTATGACTGACTGC	52	35	agarose	single	195
FG09	$(CT)_{10}$	F: TACATGCAGCAAGGACCCA R: GCAGCCAAGGATGACTTCAG	51	35	agarose	single	240
FG10	$(AAC)_9$	F: TTCCTAAAGGCATACCTTGATAAACT R: TGCGCTGAGCATTAGGAAAT	50	35	agarose	single	116
FG11	$(GT)_{10}$	F: GCGAGTATGCGTGAGCATGT R: AGCCGTTATAGGCTCTGCAC	54	35	agarose	single	91
FG15	$(TGT)_9$	F: AGTCACTCATGCAAAAGCCC R: TTAAGTGGAGCGGGACAATC	52	35	agarose	single	115
FG16	$(AC)_{15}$	F: CCTAACCCTGCATGTACTTACC R: TGAACTATGTACTGTGGACTGGA	54	35	agarose	single	102
FG23	$(TATG)_{10}$	F:ACGCAACGTGTCACCATAGA R: GTCTGACAACGTTCATGCCA	52	35	agarose	single	148
FG25	$(CA)_{10}$	F: TCTATGCGACTCCAGTGTGG R: TAAGCGTGCGGTCATGAATA	50	35	agarose	single	205
FG29	$(CA)_{11}$	F: CAGCTGCTTACCACTAGCCA R: GTGGGTGTGACTGCTGAGAG	54	35	agarose	multi	119
FG30	$(AC)_{13}$	F: ACACAGAGATAGACGTGCACTACA R: CAGTACTTGGCAGTGTTCCG	54	35	agarose	single	95
FG31	$(TG)_{12}$	F: TGTAGGTGGTTTGCGGTACT R: CACGATTTGTTGTATTGCGG	50	35	agarose	single	163
FG33	$(CA)_{10}$	F: TTGGTAGCCAGAGTAGCAGGA R: TCACGAGTATTGAGTCCTGAGGT	54	35	agarose	single	104
FG34	$(CA)_9$	F: CTGGCAGAACCTTAGCCAAT R: GGTTCGCTGAAAATAGGCAG	52	35	agarose	single	113
FG02	$(TG)_{11}$	F: TCATCGTTAACAGCATTCGC R: TGCAGCAGAAGGGTTAGCTT	50	35	non-amplification		126
FG18	$(AC)_9$	F: ATAGCCACCCAAAACATGAG R: GCACAGCTGAATATATGTGCC	50	35	non-amplification		191
FG19	$(TGT)_9$	F: CAAACATCCACAAGTTTCGC R: CTATGAGTCTCAGGGGACGG	50	35	non-amplification		304
FG20	$(CA)_{10}$	F:TTGAGCATGATGGCGATAGA R: TTGCTGTACGGTTTGTACGG	50	35	non-amplification		104
FG21	$(ATGT)_{10}$	F: ATGCAAATGCTTACTGCCAA R: GCTCACCAGCTGCAAAGAC	49	35	non-amplification		140
FG27	$(CA)_{14}$	F: GTGCACAGACGTGAACACAA R: ATGCCAAAGCCATCATGAAC	50	35	non-amplification		120
FG32	$(GT)_9$	F: TGCTCCTTCGGCTTCTTTTA R: ATGTGCACAAGGGCCTACTC	50	35	non-amplification		134

Appendix IX: **Multilocus genotype data for *Filicrisia geniculata* broods**

Table 1: Multilocus genotypes for *Filicrisia geniculata* parents and all Brood I progeny resulting from Cross A x B. All scores are allele sizes at a particular locus. Rows 1 and 2 correspond to mother and father, respectively.

Sample	Batch	FG08		FG13		FG17	
B		110	118	186	238	222	228
A		114	114	194	238	228	228
E01	1st	110	114	186	238	228	228
E02	1st	110	114	186	238	228	228
E03	1st	110	114	186	238	228	228
E04	1st	110	114	186	238	228	228
E05	1st	110	114	186	238	228	228
E06	1st	110	114	186	238	228	228
E07	1st	110	114	186	238	228	228
E08	1st	110	114	186	238	228	228
E09	1st	110	114	186	238	228	228
E10	1st	110	114	186	238	228	228
E11	1st	110	114	186	238	228	228
E12	1st	110	114	186	238	228	228
E13	1st	110	114	186	238	228	228
E14	1st	110	114	186	238	228	228
E15	1st	110	114	186	238	228	228
E16	1st	110	114	186	238	228	228
E17	1st	110	114	186	238	228	228
E18	1st	110	114	186	238	228	228
E19	1st	110	114	186	238	228	228
E20	1st	110	114	186	238	228	228
E21	1st	110	114	186	238	228	228
E22	2nd	110	114	186	238	228	228
E23	2nd	110	114	186	238	228	228
E24	2nd	110	114	186	238	228	228
E25	2nd	110	114	186	238	228	228

Table 2: Multilocus genotypes for *Filicrisia geniculata* parents and all Brood II progeny resulting from Cross A x B. All scores are allele sizes at a particular locus. Rows 1 and 2 correspond to mother and father, respectively.

Sample	Batch	FG08		FG13		FG17	
B		110	118	186	238	222	228
A		114	114	194	238	228	228
F01	1st	110	114	186	194	222	228
F02	1st	110	114	186	194	222	228
F03	1st	110	114	186	194	222	228
F04	1st	110	114	186	194	222	228
F05	1st	110	114	186	194	222	228
F06	1st	110	114	186	194	222	228
F07	1st	110	114	186	194	222	228
F08	1st	110	114	186	194	222	228
F09	1st	110	114	186	194	222	228
F10	1st	110	114	186	194	000	000
F11	1st	110	114	186	194	222	228
F12	1st	110	114	186	194	222	228
F13	1st	110	114	186	194	222	228
F14	2nd	110	114	186	194	222	228
F15	2nd	110	114	186	194	222	228
F16	2nd	110	114	186	194	222	228

Table 3: Multilocus genotypes for *Filicrisia geniculata* parents and all Brood III progeny resulting from Cross A x C. All scores are allele sizes at a particular locus. Rows 1 and 2 correspond to mother and father, respectively.

Sample	Batch	FG08		FG13		FG17	
C		114	114	186	194	192	218
A		114	114	194	238	228	228
B01	1st	114	114	194	238	192	228
B02	1st	114	114	194	238	192	228
B03	1st	114	114	194	238	192	228
B04	1st	114	114	194	238	192	228
B05	1st	114	114	194	238	192	228
B06	1st	114	114	194	238	192	228
B07	1st	114	114	194	238	192	228
B08	1st	114	114	194	238	192	228
B09	1st	114	114	194	238	192	228
B10	1st	114	114	194	238	192	228
B11	1st	114	114	194	238	192	228
B12	1st	114	114	194	238	192	228
B13	1st	114	114	194	238	192	228
B14	1st	114	114	194	238	192	228
B15	1st	114	114	194	238	192	228
B16	1st	114	114	194	238	192	228
B17	2nd	114	114	194	238	000	000
B18	2nd	114	114	194	238	192	228
B19	2nd	114	114	194	238	192	228
B20	2nd	114	114	194	238	192	228
B21	2nd	114	114	194	238	192	228
B22	2nd	114	114	194	238	192	228
B23	2nd	114	114	194	238	192	228
B24	2nd	114	114	194	238	192	228
B25	2nd	114	114	194	238	192	228
B26	2nd	114	114	194	238	192	228
B27	2nd	114	114	194	238	192	228

Table 4: Multilocus genotypes for *Filicrisia geniculata* parents and all Brood IV progeny resulting from Cross A x C. All scores are allele sizes at a particular locus. Rows 1 and 2 correspond to mother and father, respectively.

Sample	Batch	FG08		FG13		FG17	
C		114	114	186	194	192	218
A		114	114	194	238	228	228
C01	1st	114	114	194	238	192	228
C02	1st	114	114	000	000	000	000
C03	1st	114	114	194	238	192	228
C04	1st	114	114	194	238	192	228
C05	1st	114	114	194	238	192	228
C06	1st	114	114	194	238	192	228
C07	1st	114	114	194	238	192	228
C08	1st	114	114	194	238	192	228
C09	1st	114	114	194	238	192	228
C10	1st	114	114	194	238	192	228
C11	1st	114	114	194	238	192	228
C12	1st	114	114	194	238	192	228
C13	1st	114	114	194	238	192	228
C14	1st	114	114	194	238	192	228
C15	1st	114	114	194	238	192	228
C16	1st	114	114	194	238	192	228
C17	1st	114	114	194	238	192	228
C18	1st	114	114	194	238	192	228
C19	1st	114	114	194	238	192	228
C20	1st	114	114	194	238	192	228
C21	1st	114	114	194	238	192	228
C22	1st	114	114	194	238	192	228
C23	1st	114	114	194	238	192	228
C24	1st	114	114	194	238	192	228
C25	1st	114	114	194	238	192	228
C26	1st	114	114	194	238	192	228
C27	1st	114	114	194	238	192	228
C28	1st	114	114	194	238	192	228
C29	1st	114	114	194	238	192	228
C30	2nd	114	114	194	238	192	228
C31	2nd	114	114	194	238	192	228
C32	2nd	114	114	000	000	000	000
C33	2nd	114	114	194	238	192	228
C34	2nd	114	114	194	238	192	228
C35	2nd	114	114	194	238	192	228
C36	2nd	114	114	194	238	192	228
C37	2nd	114	114	194	238	192	228

Table 5: Multilocus genotypes for *Filicrisia geniculata* parents and all Brood V progeny resulting from Cross C x D. This is the 'mixed' brood used to verify microsatellite loci. All scores are allele sizes at a particular locus. Rows 1 and 2 correspond to mother and father, respectively.

Sample	FG08		FG13		FG17	
C	114	114	186	194	192	218
D	110	114	186	194	176	228
G01	114	114	186	194	176	192
G02	114	114	194	194	176	218
G03	114	114	186	194	176	218
G04	114	114	186	186	218	228
G05	114	114	186	194	176	218
G06	114	114	186	194	176	218
G07	114	114	186	194	176	192
G08	114	114	194	194	176	218
G09	110	114	186	194	192	228
G10	110	114	186	194	192	228
G11	114	114	186	194	176	192
G12	114	114	194	194	176	218

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