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Paradoxical polyembryony? Embryonic cloning in an ancient order of marine bryozoans

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Prolific polyembryony is reported in few major taxa, but its occurrence has generated theoretical debate on potential conflict between sexual and asexual reproduction. It is, therefore, important to genetically confirm a widely cited inference, based on microscopy, that polyembryony characterizes marine bryozoans of the order Cyclostomata. Microsatellite genotyping of brooded embryos and maternal colonies conclusively demonstrated polyembryony, while genetic variation among broods within colonies indicated outcrossing via water-borne sperm, in the rocky-shore species *Crisia denticulata*. The characteristically voluminous brood chamber of cyclostomes is judged to be an adaptation linked to larval cloning and hence an indicator of polyembryony. We speculate that although the almost universal occurrence of polyembryony among crown-group Cyclostomata is probably attributable to phylogenetic constraint, adaptive consequences are likely to be significant.

Keywords: clonal reproduction; cross-fertilization; larvae; microsatellites; phylogenetic constraint

1. INTRODUCTION

Monozygotic polyembryony is a form of asexual reproduction that proceeds by division or budding during post-zygotic or early embryonic stages of the life history (Hughes 1989). Only monozygotic polyembryony occurs in animals, whereas in certain higher plants, especially among angiosperms, a second form of 'polyembryony' involves somatic embryogenesis and perhaps represents a maternal strategy, without equivalents in animals, to counteract brood reduction resulting from sibling rivalry among sexual embryos (Shaanker & Ganeshaiah 1996). Hereafter, polyembryony refers to the monozygotic process. The persistence of polyembryony in certain taxa has puzzled evolutionary biologists, because it can appear to combine the contrasted fitness disadvantages of cloning and sexual reproduction, while compromising the respective benefits (Craig *et al.* 1995, 1997; but see Hardy 1995*a*).

Extensive polyembryony, generating copious clonemates per zygote, is characteristic of rust fungi (Alexopoulos 1952), red algae (Searles 1980), encyrtid wasps (Hardy 1995*b*) and cyclostome bryozoans (Ryland 1996). Some authors also include cases where cloning occurs in post-embryonic or larval stages of the life history, as in endoparasitic hydrozoans, flatworms and barnacles and in a few starfish and brittle stars (reviewed in Craig *et al.* (1997); see also Cable & Harris (2002) on gyrodactylid flatworms). Limited polyembryony, or 'twinning', forms an integral part of the life history in many gymnosperms (Filonova *et al.* 2002), certain angiosperms (Carman 1997) and armadillos (Prodohl *et al.* 1996). Twinning also occurs aberrantly in diverse mammalian species (Gleeson *et al.* 1994).

Polyembryony in cyclostome bryozoans was first reported by Harmer (1892, 1898), and although amended in certain detail, his interpretation of embryogenesis in *Crisia* species, based on microscopy, was corroborated by Borg (1926) for a range of genera. A syncytial reticulum nourishes the budding embryos, all enclosed within an expanded chamber. Although this brood chamber varies considerably in size among cyclostome families, it is almost always relatively much larger than the ovicells of cheilostome bryozoans, which typically house single embryos (Ryland 1970). We may, therefore, reasonably infer that the brood chamber, recorded in all living families of cyclostomes except the Cinctiporidae (Boardman *et al.* 1992), is associated with embryonic budding (Borg 1926; Ström 1977). Moreover, because most post-Triassic fossil species, again apart from the Cinctiporidae, also possess similar brood chambers (McKinney & Taylor 1997) it is probable that embryonic budding is a plesiomorphic character of the crown-group Cyclostomata (Taylor 2000; Taylor & Weedon 2000). In particular, microscopical evidence of polyembryony in crisiids (above), which have primitive status within cyclostome phylogeny, suggests that polyembryony arose basally in the crown group, while similar evidence for lichenoporids (Borg 1926), which are advanced, suggests that polyembryony was subsequently retained, becoming a general characteristic of cyclostomes (Taylor & Weedon 2000; P. D. Taylor, personal communication). It would be desirable, nevertheless, to obtain independent confirmation of this inference for a more comprehensive range of examples.

Although the histological patterns described by Harmer (1892, 1898) and Borg (1926) seem clearly to indicate polyembryony, the possibility of multiple fertilization within the brood chamber or of parthenogenesis cannot be dismissed without knowing the corresponding genotypic composition of mother and embryos. Accordingly, we used previously isolated microsatellite markers for *Crisia denticulata* (Craig *et al.* 2001) to genotype mothers and their

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brooded embryos. Having thus confirmed polyembryonic cloning in *C. denticulata*, we discuss its evolutionary significance in the crown-group Cyclostomata and examine adaptive theories of polyembryony.

2. MATERIAL AND METHODS

Colonies of *C. denticulata* were collected from the low intertidal and shallow subtidal at Wembury, Devon, UK (National Grid reference SX518482; 48°19′ N 4°5′ W). Independent samples were taken in the summers of 1998 and 2001, the first being analysed at the University of Hull and the second at The Marine Biological Association, Plymouth.

For the 1998 samples, whole branches bearing brood chambers were preserved frozen in 0.1 M ethylenediaminetetraacetic acid (EDTA; pH 8) before the brood chambers were dissected and embryos preserved individually in 8 μl EDTA in microwell plates. Embryos and maternal branches were incubated overnight at 55 °C in 500 μl of a digestion solution containing 0.1 M Tris–HCl pH 8, 1.25% SDS, and 400 μM Proteinase K. After one phenol–chloroform and one chloroform extraction, DNA was precipitated overnight at −20 °C in 0.3 M sodium acetate and two volumes of 99% ethanol. Samples were washed once in 70% ethanol, dried and resuspended in 50 μl of doubly distilled sterile water. Polymerase chain reaction (PCR) reactions were performed with 2 μl DNA extracted from embryos, or 0.5 μl DNA from maternal branches, in a 20 μl volume containing 0.3 mM dNTPs for embryos or 0.2 mM dNTPs for branches, 2 mM $MgCl_2$, and 0.12–0.3 μM of each primer and 1 U BioLine Taq Polymerase. Optimal cycling conditions for the primers CD 4b, CD 5, CD 7-1 and CD 17-3 were described in Craig *et al.* (2001). PCR products of the CD 5 end-labelled primers were screened on an ALF*express* automatic sequencer (Pharmacia) and run along with size markers obtained as in Van Oppen *et al.* (1997). One hundred and fifty-seven individuals were genotyped, representing six colonies, 3–8 chambers per colony and 3–10 embryos per chamber.

Year 2001 samples were kept overnight in 100 ml of aged filtered seawater at 17 °C to ensure gut contents were expelled before being preserved as whole colonies in 95% ethanol at −20 °C. Following washing in water, maternal DNA from approximately 10 feeding zooids was extracted in 35 μl of 6% Chelex resin. Dissected individual embryos were extracted in only 10 ul of the Chelex suspension. Tubes were heated to 100 °C for 10 min, briefly vortexed and spun at 13 000 r.p.m. on a microcentrifuge for 5 min before a final 10 min heating at 100 °C. Tubes were left at 4 °C overnight then spun for 10 min and the supernatant, containing the DNA, transferred to a new tube and stored at −20 °C. PCR amplification was performed on a Biometra T-gradient machine, with 10 μl reactions consisting of 0.5 μl (maternal) or 1 μl (embryo) template DNA, 0.13 mM dNTPs, 2 mM MgCl, 0.15 μM forward and reverse primer and 0.5 U Promega Taq Polymerase. One-sixth of the total forward primer used was 5′-labelled with IRD 700 or IRD 800. PCR conditions and loci were slightly different from those used for the 1998 samples. Loci CD 4b (labelled with IRD 700), CD 5 (IRD 800), CD 8-5 (IRD 700) and CD 15-16 (IRD 800) had initial denaturing at 94 °C for 2 min, followed by six cycles of 94 °C for 40 s, 60 °C for 50 s and 72 °C for 40 s; 10 cycles of 94 °C for 25 s, 55 °C for 50 s and 72 °C for 40 s; 45 cycles of 94 °C for 20 s, 50 °C for 35 s and 72 °C for 30 s; with a final 72 °C extension for 3 min. Locus CD 7-1 (IRD 700) had 94 °C for 2 min then 60 cycles of 94 °C for 20 s, 54 °C for 30 s and 72 °C for 45 s; again with a 72 °C for 3 min final extension. PCR products were screened on a LI-COR automatic sequencer and sized against 20 bp ladders (Lab Scientific, UK). Fifty-two individuals were genotyped, representing four colonies, 1–4 brood chambers per colony and 3–8 embryos per chamber.

3. RESULTS

Embryos were always genetically identical within broods but genetically distinct among broods and from their mother. Each brood, therefore, resulted from vegetative budding of a primary embryo, itself derived from a zygote resulting from outcrossed mating via water-borne sperm. Genotypes of colonies and embryos are listed in the electronic Appendix.

4. DISCUSSION

Our data conclusively demonstrate polyembryony in *C. denticulata*. Fertilization of multiple ova within a brood chamber can be discounted since brood-mates were always genetically identical. Obligatory parthenogenesis of any type (White 1973; Bell 1982; Hughes 1989) is excluded by the widespread occurrence of paternal alleles that were absent from corresponding maternal genotypes, verifying cross-fertilization. The genetic variation among broods was consistent with open fertilization in a ‘sperm-cast’ mating system (Pemberton *et al.* 2003). It is clear, therefore, that each brood chamber of *C. denticulata* accommodates one clone at a time and that different brood chambers house different clones derived from separately fertilized ova.

The typically low frequency of brooding among zooids within colonies of cyclostome bryozoans led Ryland (1996) to suggest that polyembryony compensates for infrequent fertilization, inferred to be associated with limited sperm production of typically small colonies living at low population density. Contrary to this argument, however, sessile invertebrates that fertilize retained eggs by water-borne sperm seem able to reduce sperm limitation through entrainment in the feeding current, together with prolonged storage of viable allosperm (Pemberton *et al.* 2003). Low frequency of brooding zooids might instead reflect limitation of the total maternal energy allocation that can be supported by a colony. Successful brooding in the cheilostome *C. hyalina* requires translocation of nutrients from neighbouring feeding zooids to each occupied ovicell (Hughes *et al.* 2003) and food restriction reduces colonial allocation to the female function (Hunter & Hughes 1995; Hughes *et al.* 2003). In cyclostomes, the multiply cloned embryos nourished by a syncytial ‘placenta’ (Harmer 1892) within each brood chamber, must require the combined input of many feeding zooids, so restraining the number of such broods that can be supported simultaneously by a colony. Assuming limited transfer of metabolites across the nodes (skeletal joints) of crisiid colonies, the typical presence of only one gonozooid per internode in a range of *Crisia* species, and the absence of gonozooids close to the growth origin of internodes (Ryland 2000), may be consistent with a hypothesis of resource constraint on gonozooid and larval production.

There is little to distinguish cylostomes from other bryozoans in terms of life history, larval behaviour, range of colonial architecture and ecological niche, perhaps suggesting a phylogenetic explanation of polyembryony in the crown group (Ström 1977). Most advantages of clonal reproduction (reviewed in Hughes 1989) seem lost when cloning first requires fertilization to form a zygote, then proceeds through embryogenesis, larval dispersal, settlement and metamorphosis. On the other hand, spreading risk among scattered components of the genet (*sensu* Harper 1977) remains a possible advantage of cloning, while genetic variation persists among broods.

Diverse invertebrate taxa incorporate clonal and sexual phases into their life cycle. The stage at which cloning occurs ranges from adult, as in many colonial invertebrates, larval, as in parasitic flatworms and a few echinoderms, embryonic, as in cyclostome bryozoans and encyrtid wasps, to gametic, as in cyclical parthenogens (Bell 1982; Hughes 1989). All cases could, in principle, reap one or more of the potential benefits of cloning. Moreover, if the relevant unit of selection is the genet, the life cycle becomes equivalent to that of any unitary organism, partitioned between somatic and sexual phases. Viewed from this perspective, polyembryony is not paradoxical, but simply a mode of growth occurring at the most appropriate developmental stage of the genet (see Pearse *et al.* 1989). Phylogeny might account for the absence of polyembryony among non-cyclostome bryozoans. Whereas limited polyembryony could recur through the spontaneous aberration of embryogenesis (Gleeson *et al.* 1994), its further development and genetic fixation in bryozoans would depend on linkage with mechanisms of amplifying placental nourishment and inflating the brood chamber. The required combination of events may simply not have arisen in the appropriate stem groups.

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The supplementary Electronic Appendix is available at http://dx.doi.org/10.1098/rsbl.2004.0259 or via http://www.journals.royalsoc.ac.uk.