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Outcrossing in field populations of two species of self-fertile ascidians

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Abstract: Self-compatible gametes and short-lived larvae in the sessile solitary ascidians *Corella inflata* Huntsman and *Chelyosoma productum* Stimpson suggest that field populations might be characterized by limited dispersal and self-fertilization. Because *C. inflata* in the laboratory spawns gametes into its atrium and retains offspring until they are competent to settle, it was expected to show an almost complete lack of outcrossing in the field. *C. productum*, in the laboratory, spawns streams of gametes, sometimes eggs and sperm simultaneously, into the water and is self-compatible. Embryos develop for an additional day in the plankton before hatching. Thus, some selfing was also expected for *C. productum*, though it appears to have a far greater potential for gene flow with both outcrossing and more extended larval dispersal possible. Genetic population structure in these species was examined using protein electrophoresis to determine frequencies of heterozygotes at the polymorphic locus glucosephosphate isomerase (GPI). Heterozygotes were found in both species indicating that outcrossing occurs. At three locations, *C. inflata* genotype frequencies did not differ significantly from Hardy–Weinberg expected frequencies and indicated from 41 to 100% outcrossing. Another measure of breeding system (S), calculated from the same data, gave a pooled location outcrossing estimate of 82%. Also, the three locations did not differ significantly from each other in allele frequencies, indicating a lack of genetic differentiation on a scale ≈ 100 km. Brooded embryos and short-lived larvae do not appear to be limiting gene flow on this scale in this species.

Key words: Ascidian; Brooding; *Corella inflata*; Gene flow; Mixed mating system; Self-fertilization

INTRODUCTION

A short planktonic dispersal phase and gametic self-compatibility are traits that may be associated with high levels of self-fertilization and local genetic differentiation (Shields, 1982; Strathmann et al., 1984, 1986; Jackson, 1985; Jackson & Coates, 1986; Grosberg, 1987; Stoddart, 1988). Comparisons between congeneric plants with contrasting breeding systems have shown that self-compatible species have less genetic variation and greater between-population genetic differentiation than their self-incompatible relatives (Layton & Ganders, 1984). Populations of *Impatiens capensis* (jewelweed) with different proportions of obligately, self-fertile and protandrous flowers show varying amounts of heterozygote deficiency (Knight & Waller, 1987). Gametic self-compatibility has been observed in a wide variety of hermaphroditic animals, but

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the effect of the ability to self-fertilize on genetic population structure has been examined in only a few cases. A selfing mating system eventually produces a population of individuals homozygous at nearly all loci, except in cases where selection is extremely strong and acts to maintain heterozygosity or mutation rates are unusually high. Each generation of selfing reduces the number of heterozygotes in the population by one half relative to the previous generation. High levels of self-fertilization have resulted in monomorphism at many genetic markers in cnidarians (Bucklin et al., 1984), a terrestrial snail (Selander & Kaufman, 1973), bivalves (Thomas, 1959; possibly O'Foighil, 1986), fish (Harrington & Kallman, 1968), and possibly chitons (Eernisse, 1984). A coral with self-compatible gametes, *Goniastrea favulus*, showed no detectable level of inbreeding at a single location (Stoddart, 1988).

Limited dispersal of gametes, embryos, and larvae should limit gene flow, resulting in local genetic differentiation either by drift or selection. Some benthic animals without dispersal via a planktonic larval stage show local genetic differentiation (gastropods, Berger, 1973; Grant & Utter, 1988; a holothurian, Hess et al., 1988). Genetic studies on organisms with extremely short-lived planktonic larvae, as opposed to direct development, are fewer. The brooding colonial ascidian *Botryllus schlosseri* shows local genetic differentiation in the Venetian Lagoon, Italy (Sabbadin & Graziani, 1967) on a scale of 20 km but no differentiation between two stations separated by 1300 m. The spirorbid polychaete *Pileolaria pseudomilitaris* shows genotypic differences over distances of a few meters on a floating dock (Beckwitt & Chakraborty, 1980). The bryozoan *Schizoporella unicornis* shows genetic differentiation over short distances along a temperature gradient (Schopf & Gooch, 1971), suggesting that selection may be more important in this case than genetic drift in producing genetic differentiation. Others have found correlations between dispersal characteristics and genetic heterogeneity suggesting that increased dispersal capability causes higher levels of gene flow (chitons, Eernisse, 1984; bivalves, Buroker, 1985; marine shore fish, Waples & Rosenblatt, 1987). However, larval duration in the plankton is not always indicative of a subdivided genetic population structure. For example, organisms that produce short-lived larvae or crawl-away juveniles may achieve gene flow by rafting of adults (Johannesson, 1988).

Some scenarios for the evolution of selfing in animals (Shields, 1982; Strathmann et al., 1984; Charlesworth & Charlesworth, 1987) and plants (reviewed by Jain, 1984) suggest that selfing arose in nondispersing organisms already having high levels of inbreeding. It is hypothesized that selection would already have eliminated recessive deleterious alleles, and further advantages such as reduced male allocation (Strathmann et al., 1984) and locally adapted gene complexes (Shields, 1982) could evolve. In any of these cases, species with self-compatible gametes and limited dispersal would be most expected to have highly inbred, locally adapted and genetically differentiated populations with high selfing rates.

This study focuses on two species of solitary ascidians, *Chelyosoma productum* Stimpson and *Corella inflata* Huntsman, which self-fertilize readily in the laboratory and have short-lived larvae. *C. inflata* releases both eggs and sperm into the atrium where

embryos are brooded until hatching; this spawning pattern suggests virtually obligate self-fertilization (Lambert et al., 1981). Larvae are short-lived and can settle as soon as they leave the parent, so gene flow between populations is expected to be rare. Genetic isolation of populations should lead to local genetic differentiation of populations by genetic drift or selection, if local conditions vary.

C. productum often releases eggs and sperm simultaneously into the plankton where fertilization occurs, and embryos develop and disperse for an additional day before hatching out as competent, swimming larvae. The reproductive biology of *C. productum* suggests that selfing may occur (Young & Braithwaite, 1980), but since this species has an apparently greater opportunity for outcrossing, greater numbers of heterozygous individuals are expected than in *C. inflata*. These predictions of genetic population structure based on laboratory observations on gametic self-compatibility and dispersal potential were tested using starch gel electrophoresis. Allozyme frequencies at the polymorphic locus glucosephosphate isomerase (GPI) were measured at three locations and compared to expected frequencies under Hardy-Weinberg equilibrium conditions to determine whether these species typically self-fertilize in the field. Allele frequencies of *C. inflata* in three locations separated by ≈ 5 and 100 km were compared for evidence of genetic population substructuring.

MATERIALS AND METHODS

COLLECTION

Adult *C. productum* and *C. inflata* were collected from February 1987 to June 1988 at various locations in the San Juan Archipelago north of Puget Sound, Washington. *C. productum* sites (with numbers in parentheses corresponding to numbered sites in Table I) in Upright Channel were midchannel dredges located between Shaw and Blakely Island (1), between Hankin Pt, Shaw Is and Upright Head, Lopez Is (4), and west of Flat Pt, Lopez Is (6). Two *C. productum* sites under floating docks in Friday Harbor were located at the Friday Harbor Laboratory breakwater (2) and Capron's Landing (3). A final *C. productum* site was a subtidal wall in San Juan Channel outside of Friday Harbor 0.6 km north of Cantilever Pier (5). *C. inflata* sites (with letters corresponding to sites listed in Table II) in the San Juan Archipelago were located in Friday Harbor (FH, same dock as *C. productum* Site 3 above) and in Fisherman's Bay, Lopez Is, under a floating dock on the east side of the bay (FB). FH and FB were separated by ≈ 5 km. The third *C. inflata* site (ED) was located ≈ 100 km south of FH and FB in mid-Puget Sound under a covered floating dock at the town of Edmonds.

Animals used for observations of spawning were collected from floats at Roche Harbor, an Juan Island, Washington, and held in the laboratory overnight. *C. inflata* were held in the laboratory up to 1 wk before use. *C. productum* were held in flowing seawater up to 4 months before use. Samples were checked for changes in allozyme frequencies during laboratory storage of frozen tissues.

TABLE I

Summed genotype frequencies of homozygotes and heterozygotes at GPI locus in *C. productum*. There were approximately seven alleles (see text for explanation).

Location	Genotypes		<i>n</i>
	Homozygotes	Heterozygotes	
(1) Potato Patch (dredge)	27	18	45
(2) Friday Harbor (Dock 1)	11	21	32
(3) Friday Harbor (Dock 2)	4	8	12
(4) Lopez ferry (dredge)	6	4	10
(5) Shady Cove (subtidal wall)	3	3	6
(6) Flat Pt (dredge)	0	5	5
	51	59	110

TABLE II

Allele and genotype frequencies of *C. inflata* at GPI locus in three locations: Edmonds (ED), Friday Harbor (FH), and Fisherman's Bay, Lopez (FB). χ^2 values are calculated by comparison to Hardy-Weinberg expected frequencies. *n*, number of individuals; *MO*, minimum frequency of outcrossing

Location (<i>n</i>)	Allele frequency		Observed genotypes			χ^2	<i>P</i>	<i>MO</i>
	Fast	Slow	FF	FS	SS			
ED (46)	0.71	0.29	24	17	5	0.55	> 0.1	0.44
FH (47)	0.67	0.33	22	19	6	0.31	> 0.5	0.46
FB (32)	0.80	0.20	21	9	2	0.46	> 0.1	0.33

SPAWNING AND SELFING IN THE LABORATORY

Spawning sequences of clumps of aggregated individuals of *C. productum* were observed in flowing seawater in the laboratory on two separate occasions. Periods of release of eggs and sperm were timed. To observe self-fertilization, nine individuals were rinsed and placed in separate bowls near ambient sea temperatures. Gametes collected from individuals which initially spawned a single type of gamete ($n = 7$) were then mixed with the opposite type of gamete in one self- and one cross-fertilization each, producing a total of 14 manipulated fertilizations. Individuals that released both male and female gametes simultaneously ($n = 4$) were used to supplement the data on self-fertilization. Control cultures of eggs from seven individuals that spawned eggs without sperm did

not develop, confirming that self- and cross-fertilizations occurred as planned. A particular mating was judged successful if > 85% of the eggs ($60\text{--}400$ eggs \cdot bowl $^{-1}$) developed into swimming tadpole larvae.

HORIZONTAL STARCH GEL ELECTROPHORESIS

Tissue samples (detested body of *C. inflata* and siphon muscles of *C. productum*) were dissected from live animals, manually ground for 1 min with a glass rod on 0.5 M Tris-HCl, and centrifuged for 15 min at 15000 rpm. Supernatant was either used immediately or stored frozen at -70°C . Tests comparing fresh and frozen samples showed no degradation with freezing over time. A wide variety of enzyme and buffer systems were used in screening for polymorphic loci (*C. productum*: 34 enzymes, 12 buffers; *C. inflata*: 21 enzymes, five buffers). Most enzymes had low activity and failed to form bands. The only reliable polymorphic locus was glucosephosphate isomerase (GPI; EC 5.3.1.9). Samples were electrophoresed with Selander three buffers (Selander et al., 1971) for 4–5 h (short gels) or overnight (long gels). Staining for GPI was done by the method of Tracey et al. (1975).

RESULTS

SPAWNING AND SELFING IN THE LABORATORY

Egg and sperm release in *C. productum* occurred both separately, in alternating bursts, and simultaneously. Spawning sequences of a single type of gamete lasted up to 5 min, with no release of the other type of gamete by that same individual, thus showing a potential for outcrossing. Simultaneous spawning by one individual of both eggs and sperm shows the potential for self-fertilization in this nonbrooding species. Individuals did not consistently spawn in a single pattern. Individuals which released only one type of gamete during one spawning bout might release the other type in their next bout or release both simultaneously.

Eggs were spawned in a stream of 10–25 eggs and remained in a coherent strand, possibly encased loosely in mucus. They sank in both still and slowly moving water. Sperm was released either in a continuous stream or in short streams.

Offspring of all adult individuals were successful to the tadpole stage in both self-fertilization and cross-fertilization with heterologous sperm. A sample of tadpoles from culture, both selfed and crossed, were kept and successfully metamorphosed into feeding juveniles.

ELECTROPHORETIC EVIDENCE ON BREEDING SYSTEM AND GENETIC POPULATION STRUCTURE

In both species, GPI appeared on gels in patterns indicative of a dimeric enzyme; individuals showed either one band of activity (homozygotes) or three (heterozygotes).

C. inflata had two alleles and *C. productum* showed approximately seven. *C. productum* alleles migrated very similar distances making classification difficult, even on long gels. Results were summarized as total numbers of heterozygotes and homozygotes. Because of these separation problems, the numbers of heterozygotes is probably underestimated for *C. productum*.

In *C. inflata*, frequencies of the three possible genotypes did not differ significantly from Hardy-Weinberg predicted equilibrium frequencies (Table II) at three separate locations. Thus, in the absence of selection, mutation, and migration, the Hardy-Weinberg assumption of random mating is not rejected. Selfing and breeding with close relatives are not occurring to a detectable degree. Outcrossing is occurring at a minimum level of 41% (mean for three locations). Minimum outcrossing (*MO*) (Table II) was obtained by using the observed allele frequencies to calculate the rate of selfing which would generate genotype frequencies significantly different from observed genotype frequencies using a χ^2 test (1 df). Observed allele frequencies were used to calculate genotype frequencies expected under successively increasing frequencies of selfing. Using Hedrick's (1983) model of selfing mixed with random mating, where P_e , H_e , and Q_e are expected genotype frequencies, S is the rate of self-fertilization and p and q are observed allele frequencies:

$$P_e = p^2 + Spq/2-S \quad (1)$$

$$Q_e = q^2 + Spq/2-S \quad (2)$$

$$H_e = 4pq(1-S)/2-S \quad (3)$$

Thus, *MO* equals 1 minus the maximum undetectable level of selfing. Mean minimum outcrossing rate for the three locations pooled together is at least 41% but may be up to 100% outcrossing. Another way to express level of selfing is to calculate a single value of Hedrick's S by replacing H_e , in Eqn. 3 above, with H_0 the proportion of heterozygous individuals observed in a population. Hedrick's S for the three pooled locations equals 0.18 (range = 0.16-0.22 for the three locations separately). Allele frequencies were similar at three locations separated by as much as 100 km. Allele frequencies did not differ significantly among the three locations (G test; Sokal & Rohlf, 1981; $G_2 = 3.18$, $P > 0.1$, $n = 46, 47, 32$).

In *C. productum*, there is a substantial amount of heterozygosity indicating that outcrossing does occur (Table I).

DISCUSSION

Electrophoretic data suggest outcrossing in both *C. inflata* and *C. productum*. Allozyme frequencies of *C. inflata* indicate between 33% and complete outcrossing at the three locations studied. Single-locus estimates of outcrossing (such as these) are expected to be biased lower than multilocus estimates, presumably because multilocus

estimates can better discriminate selfed mating from inbreeding by mating with relatives (Walter & Knight, 1989). These results contradict evidence from laboratory observations suggesting that selfing in *C. inflata* is the predominant breeding system.

Spawning and feeding behavior in the laboratory (Lambert et al., 1981; pers. obs.) suggested complete selfing in *C. inflata*. Eggs and sperm are released into the atrial brood space within 15 min of each other. A cloud of sperm is occasionally visible in the atrium when eggs are released. Eggs are retained in the atrial space due to their buoyant follicle cells and the usually upsidedown or sideways orientation of adults which generally places the atrial siphon below or level with the atrial brood space. Gametes, and then developing embryos, are found in the atrium at all stages up to hatching, ≈ 24 h later. The adults produce mucus and feed during gamete release, so heterologous sperm would presumably be eaten if they entered the branchial siphon. Entry through the atrial siphon might be against filtering water currents. > 100 individual *C. inflata* isolated in separate dishes in the laboratory produced offspring (Lambert et al., 1981) indicating that selfing must be occurring.

Given the above observations, it is initially difficult to imagine how outcrossing is achieved in *C. inflata*. Two possible mechanisms that would allow outcrossing are suggested: (1) eggs may escape the atrium to be fertilized and develop in the plankton or, (2) heterologous sperm may enter through the atrial siphon. In the first mechanism, egg escape could occur in individuals whose siphons are oriented upwards so that the buoyant follicle cells lift the eggs out of the atrium. Individuals oriented upwards in the laboratory may lose eggs as they float out of the atrial siphon (B. Goldstein, pers. comm.). In this case, adult orientation may determine outcrossing rate. Although individual orientation was not noted at the time of collection, general observations for each location indicate that brood loss is likely in some individuals. Individuals used in this study were collected from docks. Fisherman's Bay individuals were collected from settling panels crowded with other organisms which often forced *C. inflata* into more horizontal positions. Edmonds individuals were collected from the vertical sides of docks. Friday Harbor individuals were collected from the valves of scallops attached to the underside of the dock. Their orientation may vary with scallop movement. Thus, while individuals held upsidedown in the laboratory may retain their eggs in the atrium, adult orientation may vary in nature allowing buoyant eggs to float out of the adult. One test of this hypothesis would be rearing of broods collected from the field and comparing offsprings' genotypes with the brooding parent's genotype to confirm that retained embryos were all self-fertilized in populations where the genetic structure indicates outcrossing.

The second possible mechanism for outcrossing is by entry of heterologous sperm into the atrial chamber. This could occur as the atrium refills following atrial muscular contractions which force feces out. Or, sperm could enter during nonfeeding periods and be stored for later use. Also, feeding patterns observed in the laboratory may be different due to differing food concentrations and external flow differences.

Outcrossing could also be promoted by a period of latency in gamete activation giving

eggs or sperm time to escape homologous gamete clouds before fertilization. Gametes from self-fertile *Ciona intestinalis* show a delay of 10–50 min in self-fertilization rates relative to outcrossing (Kawamura et al., 1987). Such a period was not noticed in *C. inflata* or *C. productum*, but it could be very short and still be effective given the high density of adult aggregations in both species.

Another potential explanation for the discrepancy between selfing in the laboratory and outcrossing in the field could be a change in gametic self-compatibility in the laboratory with time. Adults of another solitary ascidian *C. intestinalis* may become self-fertile after being held in the laboratory for days although the mechanism for this is unknown (Kawamura et al., 1987).

Results for *C. productum* are compatible with laboratory observations that suggest that outcrossing should be a regular occurrence. Observations of selfed and crossed progeny in the laboratory showed equal viability of offspring to the swimming larval stage. Relative timing of egg and sperm release and rate of dispersion of gametes in moving water indicate that both selfing and outcrossing could occur in nature. Further, varying the release pattern of gametes could produce varying outcrossing rates. Observed allozyme frequencies could not be tested against Hardy–Weinberg predicted frequencies for random mating as alleles with similar mobilities were not sufficiently separated. *C. productum* may have a mixed mating system or may outcross exclusively.

Genetic differences among sites, another prediction based on both gametic self-compatibility and short duration of dispersing larvae, were not found in *C. inflata* on a scale of 100 km. Allele frequencies among the three locations were not significantly different. There was no suggestion of a cline, with the more distant location (ED) actually having allele frequencies intermediate between the two adjacent locations (FH and FB) which are separated by < 10 km. Apparently, separate locations represent sessile adult patches within a broader population with enough gene flow between locations to equalize gene frequencies between locations. An alternative hypothesis is that selection is maintaining similar allele frequencies at all populations in the absence of gene flow.

Genetic homogeneity between distant locations could be maintained by dispersal of gametes, larvae, or adults. The amount of migration required to maintain the same alleles in populations over evolutionary periods of time is quite low; a few successful migrants per generation is theoretically sufficient to prevent divergence between populations (Speith, 1974; Slatkin, 1987; Waples & Rosenblatt, 1987) when migrants are drawn randomly from a large, panmictic source (Wright, 1948; Maruyama, 1970, 1972). Given the logarithmic decay of larval settlement of other ascidian larvae over time in the laboratory (Cloney, 1959), the very few long-lived larvae could be significant to the maintenance of gene flow. However, the maintenance of statistically equivalent gene frequencies requires higher levels of migration (Allendorf & Phelps, 1981). Larval behavior and swimming ability may interact with local hydrographic processes to maintain dispersal between distant locations (Burton & Feldman, 1982; Scheltema, 1986). Larvae of the colonial ascidian *Didemnum molle* are transported hundreds of

meters across uninhabitable areas between patch reefs by currents (Olson, 1985). Rafting of adults is probably important to the distributions of many benthic marine invertebrates (Jokiel, 1984; Carlton, 1985; Highsmith, 1985; Johannesson, 1988). It is particularly interesting that these ascidian populations appear to be panmictic given that some marine organisms with longer larval periods have locally genetically differentiated populations (reviewed in Burton, 1983; Hedgecock, 1986) through force of selection or larval behavior.

Normally outcrossing species that experience a high cost of inbreeding are predicted to evolve self-incompatibility mechanisms. Is the reason that *C. inflata* and *C. productum* are self-compatible simply due to a constraint on the evolution of self-incompatibility? Variation in the phylogenetic distribution of gametic self-compatibility in ascidians suggests that there is no phylogenetic constraint to the evolution of self-incompatibility. Variation in self-compatibility has been reported within all three orders of ascidians, as well as within families, and genera (Morgan, 1942a,b; Kume & Dan, 1968; Reverberi, 1971; Rosati & De Santis, 1978; Lambert et al., 1981; Fuke, 1983; Honegger, 1986; Kawamura et al., 1987). In the laboratory, differences in ability to self-fertilize have been found between individual *C. intestinalis* from a single population (Rosati & DeSantis, 1978; Kawamura et al., 1987) and between conspecific populations (Morgan, 1945).

Self-compatibility of gametes in *C. inflata* and *C. productum* and apparent viability of selfed progeny suggest that a mixed mating system, rather than complete outcrossing, may be the breeding system for both species. Why would a mixed mating system evolve and persist in these species?

Self-fertilization has often been suggested as a breeding strategy for species colonizing new habitats where access to a mate may be limiting (review in Wells, 1979; Jain, 1984). Lambert (1968) suggested that *C. inflata* is a "fugitive" species. As a primary colonizer on floating docks such as those sampled in this study, individuals grow quickly in the summer potentially reaching sexual maturity in < 3 months. In the winter, growth is slower, and individuals may persist for several more months but may eventually disappear from docks. Thus, as a continual reinvader of habitats *C. inflata* may be able to self when conspecifics are rare. *C. productum*, another primary colonizer, although with a life span of at least 3 yr and growth to sexual maturity in 1 yr or less (Huntsman, 1912; pers. obs.), may be a similar case.

Although the exact level of outcrossing has not been determined, it is possible to speculate on the plausibility of a mixed mating system in these species based on a colonizing strategy. Stable intermediate selfing rates depend on additional costs of selfing to counter the 50% cost of outcrossing. Potential disadvantages to selfing include decreased genetic variability in offspring, expression of deleterious recessive alleles, and loss of superior heterozygous genotypes. Additional advantages to selfing beyond the increased probability of finding a mate include increased efficiency of fertilization and the possibility of local adaptation through reduced gene flow.

A scenario for *C. inflata* based on factors producing stable intermediate outcrossing rates (reviewed in Charlesworth & Charlesworth, 1987) follows:

Advantages of selfing may be greater when adults are rare and advantages of outcrossing may be greater when adults are abundant. When adults are rare, selfing is favored as colonizing individuals are assured of a mate. At higher adult densities as space becomes limiting, there is an outcrossing advantage to dispersing genetically variable offspring to a new habitat. Additional outcrossing advantages such as heterosis may be important depending on the relative frequencies of selfing and outcrossing in the population.

This scenario is compatible with allozyme data indicating lack of genetic differentiation between distant locations in this study. Initially, 100 km seems very far in relation to the scale of potential dispersal by brooded larvae competent to settle upon release. However, many potential habitats or stepping stones exist between the most distant locations. Availability of intermediate habitat combined with a colonizing strategy make genetic differentiation seem less likely. If egg escape is the mechanism of outcrossing, gene flow is further enhanced as eggs develop for 1 day in the plankton before hatching. This scenario explains the persistence of gametic self-compatibility in *C. inflata* through the occasional advantage of self-fertilization during colonization, and it explains the high levels of outcrossing in well-established dock populations such as those sampled in this study.

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