

# The analysis of paternity and maternity in the marine hydrozoan *Hydractinia symbiolongicarpus* using randomly amplified polymorphic DNA (RAPD) markers

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## Abstract

For organisms in which direct observation of mating and subsequent dispersal of offspring and relatives is impossible, patterns of reproductive success and genealogical relationship can only be established using genetic markers. The ideal genetic assay would (1) employ highly polymorphic genetic markers for distinguishing among individuals; (2) use little tissue for analysing early life-history stages; and (3) require minimal investment in time and money for population level studies. From this perspective, DNA polymorphisms revealed by PCR amplification using random ten-base primers [Randomly Amplified Polymorphic DNA (PCR-RAPD) or Arbitrarily Primed DNA (AP-PCR)] have great potential. However, the evidence that RAPD/AP markers are both heritable and can be repeatably amplified remains controversial. This study characterizes patterns of inheritance and polymorphism of RAPD markers in the free-spawning, colonial marine hydrozoan *Hydractinia symbiolongicarpus*. In all cases, the amplification products were identical among extractions from the same clone. Of 56 primers screened, 13 had sufficient polymorphism and scoreability for an analysis of parentage and higher-order genetic relationships in three matings. These primers generated 156 unique amplification products (putative loci), of which 133 were polymorphic. All but four of these loci were inherited as dominant mendelian markers. Our study suggests that the presence of a marker represents a single allele at a locus; however, what appear to be single null alleles may actually comprise several segregating alleles. When the identity of neither parent was known a priori, inclusion (unique markers present in offspring and only one of the potential parents) proved to be more efficient than exclusion for assigning offspring to parents. The most powerful approach, however, was cluster analysis of all presence/absence information for the marker bands. Clustering avoided the pitfalls caused by the appearance of occasional nonparental bands, and constructed a hierarchical framework that correctly reflected all genealogical relationships.

*Keywords:* genealogy reconstruction, hydroid, *Hydractinia*, kinship, maternity determination, paternity determination, population structure, AP-PCR, PCR-RAPD, reproductive success

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## Introduction

The accurate measurement of individual reproductive success is pivotal to understanding how selection operates on variation in morphological, physiological, demographic, and behavioural traits (Williams 1966; Wade & Arnold 1980; Endler 1986; Clutton-Brock 1988; Roff 1992;

Stearns 1992). A comprehensive empirical analysis of individual reproductive success potentially involves characterizing genetic relationships, or pedigrees, at several levels of population structure. The most basic level concerns relationships between parents and their offspring. For species in which relatives are likely to interact nonrandomly either in a mating or social context, it may also be important to document higher level genetic relationships among members of a deme, and among demes (Hamilton 1964; Krebs *et al.* 1980; Michod 1980; Wade & Breden 1981, 1987).

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Even under the best of circumstances (e.g. when there is protracted association between parents and their offspring), the reconstruction of pedigrees by direct observation has proven notoriously elusive (reviewed in Westneat *et al.* 1987, 1990). The problem of direct analysis of genealogical relationships and the determinants of reproductive success is still more daunting in the many plants and aquatic animals in which one, or both, sexes sheds its gametes into the air or water. In this case, the temporal association between parents, their offspring, and other relatives is so brief that genetic methods must be used to reconstruct pedigrees. In principle, these data, to the extent that the analysed offspring and parents represent a true statistical sample of the entire population, can be used to make inferences about patterns of reproductive success.

Genetically based analyses of individual reproductive success and higher-order genetic relationships present numerous empirical, as well as theoretical, obstacles. In particular, the number of polymorphic genetic markers that can be analysed, and the frequencies of those markers in a population, potentially limit the power to reconstruct genealogies (Westneat *et al.* 1987; Burke 1989; Lynch 1988; reviewed in Reeve *et al.* 1992). This problem is especially acute for deducing parent-offspring relationships: with a limited number of polymorphic genetic markers, some percentage of potential parents can be excluded, but only probabilities of parentage can be assigned to the remaining, nonexcluded individuals (Meagher 1986; Thompson 1986; Meagher & Thompson 1987; Devlin *et al.* 1988). Moreover, for a given number of genetic markers, as the degree of relatedness among potential mates increases (as would be the case in small, inbred populations) the power of exclusion decreases (Cohen 1990). It is still more difficult to infer parentage when neither parent of an individual is known. In this case, exclusion techniques are virtually useless, because in order that a marker be useful, it must be absent in all members of one sex before it can be used to exclude potential parents of the other sex.

The only escape from the problem of uncertainty in assigning paternity and maternity – especially when both are in question – is to use markers that are so polymorphic that at least some are unique to each possible parent, and hence to their offspring (i.e. inclusion). Multilocus DNA fingerprinting, which uses a combination of restriction endonucleases and probes that hybridize to core sequences of variable number of tandem repeat (VNTR) loci, offers one solution to the problem of marker polymorphism (reviewed in Burke *et al.* 1991). However, the method is laborious, expensive, and requires substantial amounts of undegraded DNA (Weatherhead & Montgomery 1991).

The use of randomly constructed ten-base sequences as arbitrary primers to amplify template DNA via the polymerase chain reaction (PCR) circumvents many of the

technical problems of conventional, multilocus fingerprinting. This technique, known as PCR RAPD (for randomly amplified polymorphic DNA; Williams *et al.* 1990) or AP-PCR (for arbitrarily primed; Welsh & McClelland 1990) amplifies DNA sequences between inverted priming sites (i.e. loci) on complementary strands of the same DNA molecule (Williams *et al.* 1990; also see Welsh & McClelland 1990). Variation among individuals in the distances between these inverted priming sites, likely produced by the deletion and addition of priming sites, as well as insertions and deletions between priming sites, yields amplification products with variable lengths. Because of the very large number of loci that can be assayed using such primers, the overall degree of polymorphism rivals that revealed by multilocus fingerprinting. Moreover, because only small amounts of DNA are required, offspring can be analysed at a very early stage in ontogeny, thus reducing the likelihood that selection on juveniles can affect estimates of reproductive success.

Several problems counter the ease with which RAPD markers could be used to characterize first- (and higher-) order genetic relationships. First, RAPD markers are expressed as dominant alleles, such that when a marker band is present (i.e. the primer anneals to a complementary priming site and downstream amplification ensues), it is impossible to distinguish whether it is in the homozygous or heterozygous state. Consequently, in contrast to genetic assays involving codominant alleles (e.g. allozyme electrophoresis), RAPD markers provide less information per amplifiable site. Nevertheless, because literally hundreds of primers are commercially available, and single primers often amplify multiple sites, the number of polymorphic loci (i.e. amplifiable sites) that can be assayed is potentially enormous. A second class of problem concerns the repeatability of amplifications (Hadrys *et al.* 1992): at least one study indicates that different amplification products can be produced from different runs using the same primers and template DNA. Finally, although several studies show that RAPD markers are inherited as mendelian loci (Williams *et al.* 1990; Carlson *et al.* 1991; Martin *et al.* 1991; Welsh *et al.* 1991; Hunt & Page 1992), one frequently cited study suggests that RAPD markers may not be inherited as mendelian markers, and that nonparental marker bands can appear in offspring with appreciable frequency (Riedy *et al.* 1992).

In this study, we characterize patterns of inheritance and polymorphism of RAPD markers in a free-spawning, colonial marine hydrozoan, *Hydractinia symbiolongicarpus*. We chose to focus on *H. symbiolongicarpus* for two reasons. First, its reproductive biology renders impossible any direct analysis of parent-offspring and higher-order relationships. Second, *H. symbiolongicarpus* (Buss *et al.* 1984; Yund *et al.* 1987; Buss 1990; Buss & Grosberg 1990), like many other colonial invertebrates (Buss 1990), competes

intensively with conspecifics for habitable space. The nature and outcomes of these contests depend on the relatedness of contestants (Hauenschield 1954; Müller 1969; Ivker 1972; reviewed in Grosberg 1988). Thus, understanding the evolution of such conditional competitive behaviours necessarily involves the establishment of both parent-offspring and higher-order relationships under natural conditions (Grosberg 1988). This paper shows how RAPD markers can be used to analyse these evolutionarily critical relationships.

## Materials and methods

### *Reproductive Biology of Hydractinia symbiolongicarpus*

*Hydractinia symbiolongicarpus* belongs to a group of hydractiniid hydrozoans that colonize various species of gastropod shells inhabited by hermit crabs (Buss and Yund 1989). Befitting its name, *H. symbiolongicarpus* associates primarily with the hermit crab *Pagurus longicarpus* (Buss & Yund 1989). The gonochoric colonies spawn shortly after dawn, releasing both eggs and sperm into the water column (Bunting 1894; Ballard 1942), and fertilization presumably occurs externally. After 48–96 hours of benthic development, demersal planula larvae form (Müller 1969; Weiss & Buss 1987; Yund *et al.* 1987; Shenk & Buss 1991). When the larvae contact the shell of a passing hermit crab, they metamorphose into a small, polypoid form (Schijfsma 1935; Cazaux 1958; Müller 1969; Chia & Bickell 1978, Weis *et al.* 1985, Weis & Buss 1987; Yund *et al.* 1987). The combination of external fertilization, a crawling larval stage, and the motility of the substrata upon which *H. symbiolongicarpus* live makes direct observation of mating patterns and reproductive success virtually impossible.

### *Collection, culturing and mating techniques*

We collected *Pagurus longicarpus* encrusted with colonies of *Hydractinia symbiolongicarpus* from Barnstable Harbor, Massachusetts. We discarded crabs carrying more than a single *H. symbiolongicarpus* colony. To ensure that the hydroid colonies remained sexually isolated, we maintained the remaining crabs in two aerated seawater aquaria at 16°C in two groups, one with only male colonies, and the other with only female colonies. We haphazardly chose three reproductively mature male, and three female colonies, and established three male–female pairs in separate aerated aquaria. We induced the colonies to spawn by holding the mated pairs in complete darkness for 48 hours, and then directing a bright light at each aquarium for 2–3 h (Bunting 1894; Ballard 1942). Forty-eight hours later, we gathered with a pipette 50–200

planula larvae from the bottoms of each of the mating aquaria.

To induce metamorphosis of the larvae, we then transferred them to a solution of 53 mM CsCl, mixed 1:1 with full-strength seawater, for 2–4 h until the larvae began to contract (Müller 1973). At this point, we transferred the larvae individually to separate 4.5-cm × 6.0-cm glass slides, placed horizontally in dishes of seawater at room temperature. Twenty-four hours later, when the larvae completed metamorphosis and formed a primary feeding polyp, we hand-fed each polyp with a dense suspension of 2-day old *Artemia salina* nauplii. After this brief feeding, we organized all of the slides into vertical slots in plastic racks, and maintained the colonies in 650-l recirculating seawater aquarium at 16°C.

At the same time, we established clonal explants of the parental colonies by cutting a small section of ectodermal mat containing several feeding polyps from the stock colonies on the hermit crabs. We positioned these sections on the slides under a loop of 1.8-kg test nylon monofilament. After 5–7 days, when the explants attached to the slides, we removed the nylon loops. We fed both the offspring and parental explants daily by submerging them in a dense suspension of two-day old brine shrimp nauplii for 2–5 h.

After five months, colonies covered from 2–8 cm<sup>2</sup> of the slides, and consisted of roughly one hundred to well over a thousand feeding polyps. We then starved the colonies for three days, and removed two 9-mm<sup>2</sup> sections of ectodermal mat, bearing 10–25 polyps, from the six parents and 30 offspring (10 from each mating). Table 1 lists the identification codes for the three pairs of parents and the 10 offspring from each mating that we genetically screened.

**Table 1.** Numbering scheme for the three matings. Capital letters denote parental colonies; numbers denote offspring from each of the matings

	Mating 1	Mating 2	Mating 3
Parents			
Female	A	B	C
Male	F	D	E
Offspring	2	1	5
	4	3	7
	6	8	9
	11	10	12
	13	17	14
	15	19	16
	20	23	18
	22	25	21
	24	27	28
	26	29	30

### Extraction of DNA

From this point on, we independently processed the two replicate scrapings from each colony. We first macerated the tissue samples with a scalpel in a 1.5-ml microcentrifuge tube, and then ground the samples further with a steel pin. We then added 500  $\mu$ l of CTAB extraction buffer (2% hexadecyltrimethyl ammonium-bromide, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, and 100 mM Tris (pH 8.0)), 5  $\mu$ l of Proteinase K (25-mg/ml stock solution), and incubated the samples at 65°C for 90 m. We extracted the samples twice with 500  $\mu$ l of a 25:24:1 solution of phenol:chloroform:isoamyl alcohol (PCl), followed by a single extraction with 500  $\mu$ l of a 24:1 solution of chloroform:isoamyl alcohol (Cl). For each step in the extraction, we added the reagent, mixed it well with the sample, spun the sample for 18 min at 8000 r.p.m. in a microfuge, and then transferred the supernatant. We then precipitated the DNA with 1 ml of 95% ethanol, stored the sample at -20°C for 20 min, spun the sample at 8000 r.p.m. for 20 min, then washed the pellet twice with 70% ethanol. We decanted the 70% ethanol, airdried the pellet overnight, then re-suspended the pellet in 50  $\mu$ l of a solution of 10-mM Tris, 1 mM EDTA (1X TE buffer). Finally, we quantified DNA concentration in each sample with a Hoefer TKO 100 fluorometer, using #33258 Hoechst dye, and adjusted DNA concentration in each sample to 5 ng/ $\mu$ l by adding 1X TE buffer. Twelve of the samples contained 1.5–3.0 ng/ $\mu$ l, and were not further diluted.

### Amplification of samples and effects of template DNA concentration

We modified the PCR RAPD procedure of Williams *et al.* (1990). For each reaction, we used 0.2  $\mu$ l of a 5 unit/ $\mu$ l solution of AmpliTaq DNA Polymerase (i.e. 1 unit total), 0.625  $\mu$ l each of 1-mM dATP, dCTP, dGTP and dTTP, 1.25  $\mu$ l of 10-mM MgCl<sub>2</sub>, 2.5  $\mu$ l of reaction buffer (containing 100-mM Tris-HCl (pH 8.3), 500-mM KCl and 15-mM MgCl<sub>2</sub>), and added glass-distilled water to a volume of 21  $\mu$ l. For primers in each reaction, we used 3  $\mu$ l of 10- $\mu$ M solution of a single, random sequence, ten-nucleotide oligomer (Operon Technologies Inc., Alameda, California, USA). In each of the amplifications, we added 1  $\mu$ l of sample (i.e. template) DNA (previously adjusted to 5 ng/ $\mu$ l) to the entire reaction mixture. Thus, the final volume of the entire reaction mixture was 25  $\mu$ l, and the final concentrations were 100- $\mu$ M dNTP, 2-mM MgCl<sub>2</sub>, 10-mM Tris-HCl and 50-mM KCl. Finally, we topped off the tubes with a drop of mineral oil.

We then placed the samples in a Perkin Elmer Cetus Model 480 thermal cycler. The first cycle consisted of 2.5 min at 94°C, 1 min at 35°C, and 2 min at 72°C. We allowed the amplification process to continue for 44 additional cy-

cles, each comprising 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C. We electrophoretically analysed the amplification products by adding 4  $\mu$ l of Type 2 loading buffer (Maniatis *et al.* 1982, p. 455) to the amplified sample, and transferring this mixture into the well of an agarose gel made of a 0.6% Ultra-Pure agarose (Bio-Rad Laboratories) and 1.0% Synergel (Diversified Biotech, Newton Centre, Massachusetts, USA), in 0.5-TBE buffer (pH 8.0). We also loaded at least two DNA ladders (123 bp and 1 kb) onto each gel. The gels were immersed in 0.5-TBE, and run at 100 V (4 V/cm) for 5 h. We then stained the gels in 2.5-mg/500 ml ethidium bromide for 15 min, destained for 1 h in water, and photographed them under UV light.

In our preliminary screening of the six parental genotypes using 56 primers, we used undiluted DNA samples ranging in concentration from 1 to 30 ng/ $\mu$ l. Over this range of DNA concentrations, we could not detect any influence of concentration on banding patterns, with independently extracted replicate samples from the same parental colony showing identical patterns, even when DNA concentration in the two replicates varied by as much as an order of magnitude.

## Results

### Levels of polymorphism

Of the 56 Operon Technologies, Inc. primers screened, we selected 13 primers to analyse parent-offspring relationships (F-01; F-02; F-05; F-06; F-07; F-15; F-17; F-19; R-06; S-01; Y-02; Y-03; Y-06). We used three criteria to choose these 13 primers: (1) they produced different (i.e. polymorphic) banding patterns in at least some of the parents; (2) the majority of the amplified marker bands were generally sharp and clear; and (3) the number of bands amplified in each individual was small enough to ensure that bands could be resolved ( $\leq 15$ ), but large enough to provide information on parentage ( $\geq 5$ ). Of the 43 unused primers, we eliminated 19 because they revealed no polymorphism, 14 because less than five polymorphic bands amplified, two because  $>15$  bands amplified, and eight because the amplified bands were difficult to resolve.

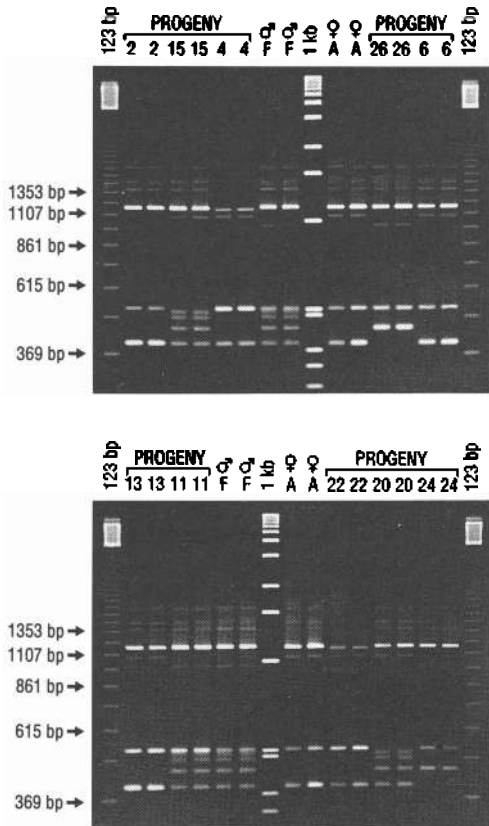
Under the assumptions that every marker band with a different molecular weight represents a unique locus, and that bands amplified by different primers also represent distinct loci, these 13 primers yielded a total of 133 loci polymorphic in both parents and offspring (mean = 10.2 polymorphic bands/primer). By the same criteria, a total of 16 loci did not vary among all individuals (mean = 1.2 invariant bands/primer). We also found a total of six loci present in all of the parents, but not in all of their progeny, and one locus present in all progeny, but absent from some of the parents. This yields a grand total of 156 loci assayed in all 36 individuals. At four of the 156 loci, we identified

bands in offspring that were not amplified from either of the true parents.

Hunt & Page (1992) considered other kinds (i.e. non-presence/absence) of RAPD polymorphisms, and showed that in *Apis mellifera*, band brightness is heritable and polymorphic. In our study of *Hydractinia symbiolongicarpus* (and unreported data for *H. milleri*), brightness patterns were consistent within clones and transmitted to offspring as expected for mendelian loci (Figs 1 and 2). Nevertheless, we found ample variation for band presence/absence and did not score brightness as a character.

*Intraclonal comparisons*

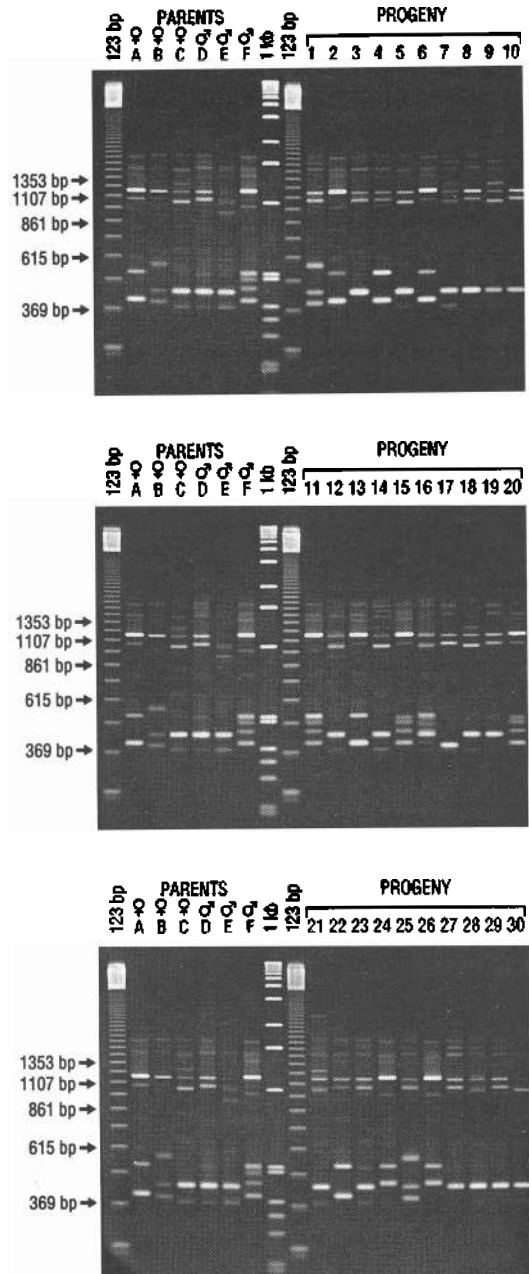
We found no variation either for band presence/absence, or brightness, among clonal replicates of the same colony. The two extractions per clone produced identical banding patterns (Fig. 1). Moreover, amplification products from the same primer-template combinations run on different days did not visibly differ (Figs 1 and 2).



**Fig. 1** Amplification products from RAPD primer OP F-07 applied to two independent tissue extractions of 10 offspring and their parents. DNA ladders (123 bp and 1 kb) and base-pair sizes provide references. The progeny number indicates the code for each offspring and the letter indicates the code for the male and female parent (see Table 1 for key). Each gel contains DNA from five offspring and both parents. These gels were run for 6 h on 21 April 1992.

*Inheritance of bands*

Figure 2 shows the banding patterns revealed by a single primer (F-07) used to amplify the DNA of six parents and their 30 offspring. These marker bands, as well as all but four of those amplified by the other 12 primers, were inherited as mendelian markers. We confirmed this pattern of inheritance by plotting histograms of the cumulative



**Fig. 2** Amplification products from RAPD primer OP F-07 applied to the 6 parents and 30 offspring. Each gel contains DNA from 10 offspring and all six parents. Offspring numbers and parental letters correspond to Fig. 1. Table 1 provides the key to parent-offspring identities. These gels were run for 5 h on 13 August 1992.

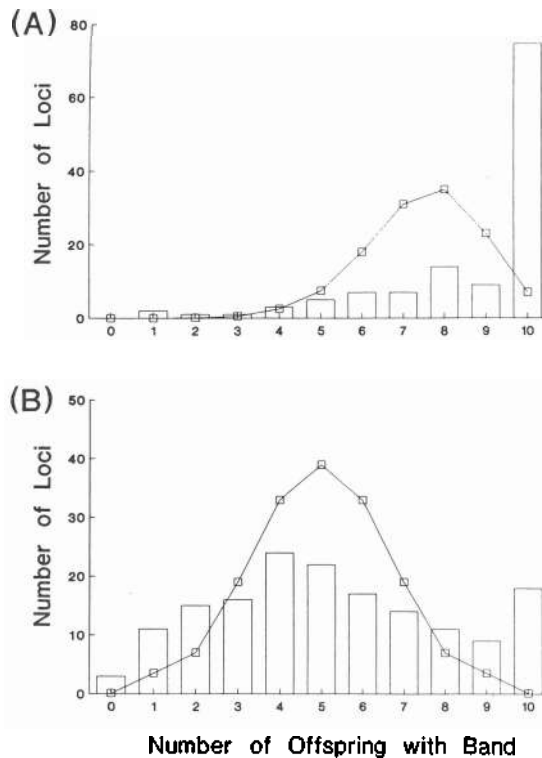


Fig. 3 Frequency distributions of each of the parental markers among the 10 offspring from all three matings, (A) when both parents express the marker and (B) when only one parent expresses the marker. The square symbols connected by the solid lines indicate the binomial distribution with an expected probability of (A) 0.75 and (B) 0.50. In both histograms another peak occurs at 10 offspring. This peak is largely composed of loci at which one, or both, parents is homozygous for a dominant allele at a locus. In this case, the probability of offspring inheriting the band is 1.0.

distribution of each of the parental marker bands among the ten offspring from all three matings. We compare histograms when both parents possessed a band ( $n = 124$  cases; Fig. 3a) with when only one of the parents had a band ( $n = 160$  cases; Fig. 3b). Because RAPD markers are expressed as dominant alleles, an individual with a band may be either homozygous (AA) or heterozygous (Aa) for that allele. Any parent homozygous for the dominant allele will transmit it to all of its progeny, hence both empirically derived histograms should, and do, show a peak where all ten offspring in a sibship carry a parental marker band (corresponding to those loci in which at least one of the parents was homozygous for the dominant marker).

We used a binomial distribution to generate expectations for the other peaks expected from loci at which both parents are heterozygous (i.e.  $Aa \times Aa$ ; Fig. 3a), or one is heterozygous and the other is homozygous for the null allele (i.e.  $Aa \times aa$ ; Fig. 3b). The expected probability of whether an offspring will receive a dominant allele when

both parents are heterozygous at a locus is 0.75; when only one parent is heterozygous for the dominant marker, and the other lacks it, the expected probability of transmission is 0.50. The binomial distribution for 10 offspring therefore gives peaks at 8 and 5 offspring. When both parents express the marker band, we observed a peak at 8 offspring (Fig. 3a). Similarly, Fig. 3(b) shows that when only one of the two parents possesses a given band, there are peaks at 4 and 5 offspring (median = 5). The absence of a peak at 10 offspring in the expected binomial distributions is because the binomial expectations do not include matings involving loci at which one, or both, parents are homozygous for the dominant allele. This is also the source of the discrepancies between the heights of the expected (binomial) and observed peaks.

Table 2 Assignment of parentage by exclusion when neither parent is known a priori. Values in columns beneath each parent indicate the number of loci (i.e. unique marker bands) that excluded a particular parent. Outlined values show incorrect exclusions due to the presence of nonparental bands in offspring

Offspring	Parents					
	Female			Male		
	A	B	C	D	E	F
1	5	0	7	0	11	11
2	0	5	6	10	9	0
3	3	0	5	0	6	6
4	0	3	5	6	6	0
5	6	7	1	1	1	2
6	0	3	6	3	3	0
7	11	10	0	2	0	1
8	4	0	6	0	6	5
9	5	5	0	1	0	1
10	4	0	6	0	7	7
11	0	3	5	6	7	0
12	5	4	1	3	1	4
13	0	6	6	9	9	0
14	7	6	0	2	1	2
15	0	6	8	6	5	0
16	4	4	0	3	0	2
17	5	0	8	0	11	11
18	5	5	0	2	0	3
19	2	0	2	0	4	3
20	0	3	5	5	6	0
21	7	6	0	1	0	3
22	0	4	6	7	8	0
23	4	0	6	0	3	3
24	0	6	7	7	7	0
25	2	0	4	0	5	7
26	0	4	6	6	8	0
27	3	0	3	0	5	3
28	6	5	1	1	0	2
29	2	0	3	0	3	4
30	4	4	1	3	0	2

### Analysis of parentage

We assigned parentage using two approaches. First, we searched for diagnostic marker bands that would allow us to assign parents to their offspring, or *vice versa*. Secondly, to group parents and offspring based on overall similarity of banding patterns, we used a cluster analysis on the presence or absence of markers at all 156 loci.

The first approach involved searching for marker bands present in offspring that could be used to exclude one, or more, potential parents (exclusion). We also searched for bands unique to a single parent, and then assigned all offspring with those bands to that parent (inclusion). Using exclusion, we could assign all but one off-

**Table 3** Assignment of parentage by inclusion when neither parent is known a priori. Values in columns beneath each parent indicate the number of loci that uniquely identify the parent of a particular offspring. Outlined values denote incorrect inclusions due to the presence of nonparental bands in one, or more, offspring

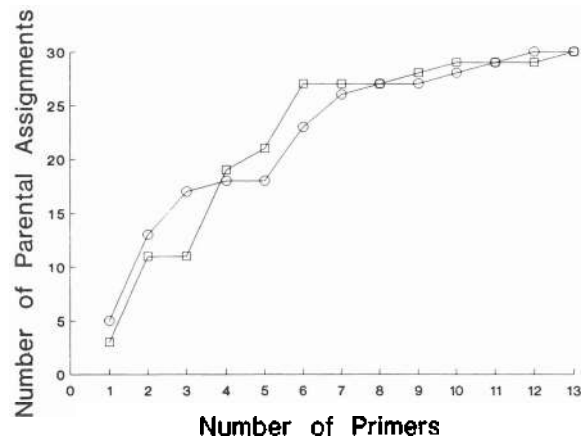
Offspring	Parents					
	Female			Male		
	A	B	C	D	E	F
1		5		9		
2	4					8
3		2		5		
4	3					5
5	1		6		1	
6	3					2
7			10		1	
8		4		4		
9			5		1	
10		4		6		
11	2					5
12	1		4		3	
13	5					8
14			6		1	
15	5					5
16			4		2	
17		4		10		
18		1	5	3	2	
19		1		3		
20	3					4
21			6		1	
22	3					6
23		3		3		
24	5					6
25		2		5		
26	3					5
27		2		3		
28	1		5		1	
29		1		3		
30	1		3		2	

spring to its correct male and female parent (Table 2). For that single individual (offspring 6), we could correctly assign the female parent, but could exclude only one of the potential male parents. We found seven bands (from two loci) present in offspring that did not amplify from any of the true parents. A mean ( $\pm$  SD) of  $4.98 \pm 2.40$  bands correctly excluded nonparents, whereas  $0.12 \pm 0.32$  bands incorrectly excluded the true parents.

With inclusion, we could assign all 30 offspring to both their correct maternal and paternal parents, with  $3.97 \pm 2.19$  bands including each true parent (Table 3). The number of diagnostic bands used to assign each true parent to its offspring therefore decreased only slightly compared to exclusion. In contrast to exclusion, however, only one band found in 4/10 offspring from a single mating produced a contradictory assignment of parentage. In all four of these offspring, between three and six bands correctly diagnosed the true parent. To assign all offspring to both parents required marker bands amplified by all 13 primers; however, 90% of the progeny could be correctly assigned based on just 8 primers (Fig. 4).

The second approach to analysing parentage considered overall patterns of band sharing, rather than a subset of diagnostic bands. We constructed a presence/absence matrix for bands at all marker loci (156 total), and then used cluster analysis (Ward's minimum-variance method, SAS Institute 1988, Release 6.03) to group individuals based on degree of similarity. We performed three separate analyses, one using only the offspring, another using only parents, and the third combining both the parents and offspring.

The 30 offspring formed three distinct clusters of 10 individuals, each of which contained members of only a single sibship (Fig. 5). The analysis revealed no other signifi-



**Fig. 4** Cumulative number of primers needed to assign maternal (□) and paternal (○) parents to the 30 offspring.

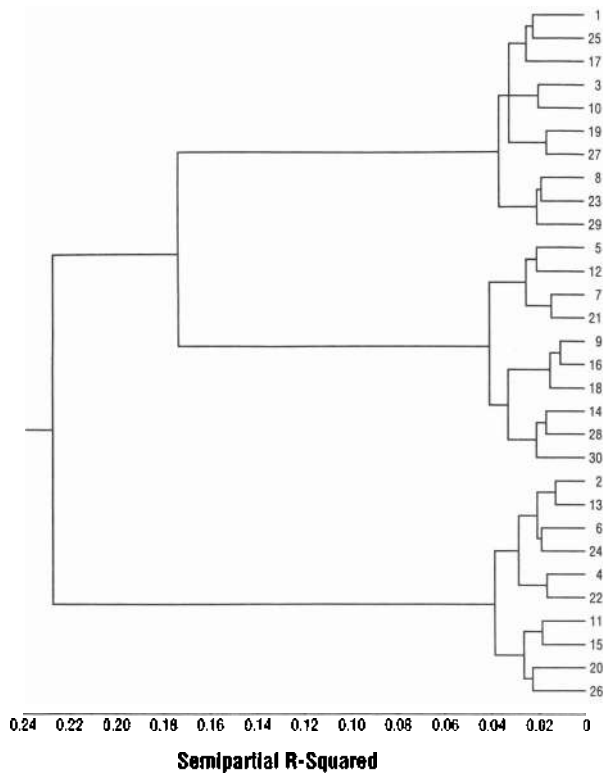


Fig. 5 Cluster analysis of offspring using Ward's minimum-variance method. Table 1 presents key to parent-offspring relationships. All three significant clusters are grouped by sibship.

cant clusters, and the two convergences of the three clusters were highly significant (Student's  $t = 7.6$  and  $8.2$ ; Bonferroni critical  $t = 3.6$  for  $P < 0.05$ ). The mean ( $\pm$  SD) within-cluster semipartial  $R^2$  was  $0.02 \pm 0.01$ , whereas the among-cluster semipartial  $R^2$  was  $0.20 \pm 0.04$ .

When we included only the six parents in the cluster analysis (Fig. 6), no significant clusters emerged (maximum  $t = 1.41$  for all convergences;  $P > 0.5$ ), indicating that all parents were equally related. As would be expected in a

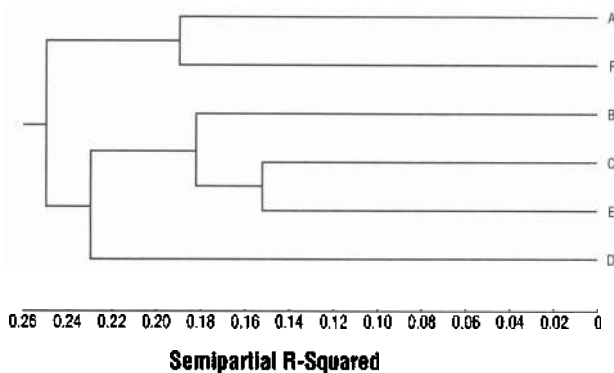


Fig. 6 Cluster analysis of parents alone using Ward's minimum-variance method. There are no significant clusters.

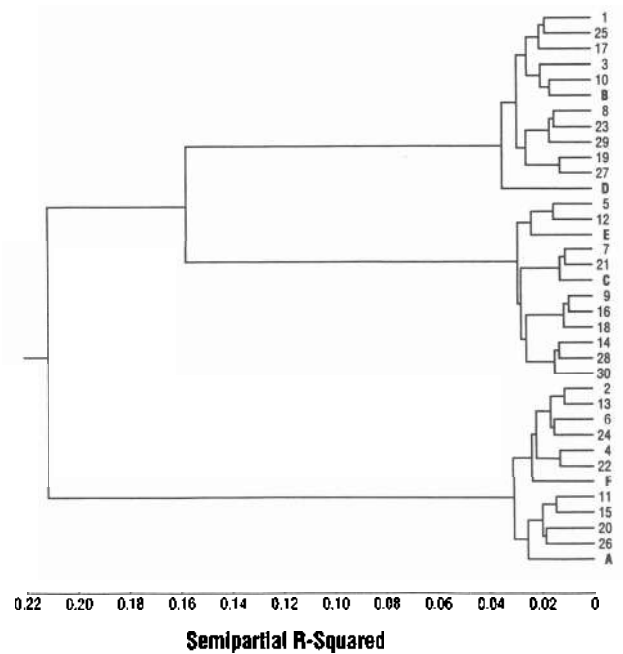


Fig. 7 Cluster analysis of both parents (letters) and offspring (numbers) using Ward's minimum-variance method. All three significant clusters conform to true parent-offspring relationships (see Table 1).

panmictic population, the mean semipartial  $R^2$  among parents was  $0.20 \pm 0.04$ , the same as the among-cluster value for the offspring. Finally, when we combined parents and offspring in the analysis, each parent clustered within the group of its correct offspring (Fig. 7). Thus, each offspring could be identified as genetically unique, each offspring correctly clustered with its siblings, and all offspring clustered with their true parents.

#### Analysis of gene frequencies

RAPD markers are expressed as dominant alleles; therefore, provided that the absence of a marker band at a locus represents a single null allele, individuals that lack a particular band should be homozygous for the null allele at that putative locus. The frequency of that allele can then be calculated from the proportion of individuals that lack a particular marker band. Thus, at Hardy-Weinberg equilibrium, the frequency of the null allele at locus  $i$ ,  $q_i$ , will be the square root of the true proportion,  $x_i$ , of individuals that lack a band at locus  $i$ . If the sample size used to estimate the frequency of the null allele is small, and especially if the null allele is rare (i.e.  $x_i < 0.1$ ), then this estimate of the null allelic frequency could differ substantially from the true frequency (M. Lynch & B. Milligan, personal communication). Our uncorrected estimate of  $\hat{q}(i)$  over all 133 polymorphic loci is 0.809 (lower 95% CI = 0.785; upper



95% CI = 0.831; statistics calculated on arcsine-square-root-transformed data; reported values are backtransformed) in the six parents, and 0.818 (lower 95% CI = 0.789; upper 95% CI = 0.845) in the 30 offspring, suggesting that the population is panmictic. Because of the large number of loci used to estimate  $\hat{q}(i)$ , and the high frequency of null alleles, the estimate should be relatively unbiased.

The high mean frequency of null to amplified alleles suggests either (1) that absence of a band at a locus represents multiple null alleles, rather than a single null allele, or (2) there is some unknown bias such that amplified alleles are less common than null alleles. Under the assumption that RAPDs are selectively neutral markers, there is no obvious reason why, when averaged over many loci, amplified alleles should be less common than unamplified alleles; in other words, in a diallelic system  $\hat{p}(i)$  (the mean frequency of the amplified allele)  $\approx \hat{q}(i) \approx 0.5$ . This suggests that at least some loci carry multiple null alleles, rather than a single null allele.

## Discussion

This study shows that the use of random sequences of ten-nucleotide oligomers as primers in a PCR reaction with DNA from *Hydractinia symbiolongicarpus* yields highly polymorphic markers that are inherited as mendelian alleles. In this species, these RAPD markers appear to be so variable that they offer the potential to resolve genetic identities and relationships at a variety of levels.

To make full use of the power of any genetic marker to resolve genealogical relationships, it is essential to document allelic and genotypic frequencies at each putative locus. There are at least two technical impediments to such an understanding using RAPD markers. First, because RAPD markers are expressed as dominant alleles, calculation of homozygosity (and, of course, heterozygosity) relies on the assumption that the absence of a band in an individual represents a homozygous, null genotype at a locus. It is, however, possible that when a band is absent at a locus, it constitutes not a single allelic class, but rather multiple null alleles. Our study suggests that this is probably the case, at least in the sample of *H. symbiolongicarpus* that we analysed. To the extent that the null allele class at a locus comprises >1 alleles, estimates of heterozygosity and other population genetic parameters may be inaccurate.

Second, a single resolvable marker band may actually be composed of the similarly sized, comigrating amplification products of multiple loci. Hadrys *et al.* (1992) propose eluting specific bands from gels, reamplifying such bands to generate radioactive probes, and then hybridizing these probes to a blot of the original gel. In addition, polyacrylamide gel electrophoresis could provide greater size-specific resolution of bands.

These procedures have the potential to reveal whether a single band is actually composed of amplification products from two (or more) loci, but would clearly be impractical for a large number of marker bands. Our alternative statistical approach sacrifices the locus-by-locus accuracy of Southern blot analysis, but over many loci should reveal whether marker bands represent more than a single comigrating amplification product (i.e. multiple loci). For example, if more than a single locus produces electrophoretically indistinguishable amplification products, then the number of offspring exhibiting that size marker band will be higher than if only a single locus produced such an amplification product. Therefore, if many markers constitute multiple unlinked loci, then we would expect the observed peaks in the histograms depicting band-sharing frequencies in offspring of defined matings (Fig. 3) to be shifted to the right. The coincidence of the peaks for observed and expected values suggests that, at least for *H. symbiolongicarpus*, marker bands generally represent the amplification products of a single locus.

Beyond these technical limitations, RAPD markers – because they are expressed as dominant alleles – carry less information per locus than codominant markers. Lewis and Snow (1992) predict that >50 polymorphic loci with dominant alleles are needed to resolve paternity by exclusion. From this perspective, RAPD markers have considerable advantages over other genetic assays because of (1) the large number of commercially available RAPD primers, (2) the presence of numerous polymorphic loci per primer, and (3) the ability to amplify nanogram quantities of DNA. For example, with just 13 primers, we were able to assay 133 polymorphic loci, which could be used to assign both paternity and maternity to 30 offspring.

With a fixed number of polymorphic markers, the power to assign parentage decreases as the number of potential parents increases. We investigated only three male and three female parents, and so assignments were obviously easier than if the population of prospective parents were considerably larger. However, Lewis & Snow (1992) demonstrate that error in parent-offspring assignments is surprisingly insensitive to the number of potential parents, particularly if the frequency of the null allele is high. In their simulations, the confidence in paternity assignments is greatest with a null allele frequency between 0.8 and 0.9 (Lewis & Snow 1992, figure 4). Our empirically derived estimates for parents (mean = 0.809; lower 95% CI = 0.785; upper 95% CI = 0.831) and offspring (mean = 0.818; lower 95% CI = 0.789; upper 95% CI = 0.845) fall within this range. In this range of allelic frequencies, unambiguous paternity can be assigned to over 95% of offspring, from a simulated paternal pool of 100 individuals with 90 independent, polymorphic loci (Lewis & Snow 1992, figure 1). We were able to assign 90% of offspring to both parents using only 8 primers (~ 80 polymorphic loci).

Given the redundancy of the inclusion analysis based on all 13 primers that yielded 133 polymorphic markers (giving an average of four diagnostic bands per offspring), and the robustness of the cluster analysis, there is substantial scope for an increase in the size of the parental pool, without sacrificing much in the way of precision of parent-offspring assignments.

Predictions of the efficiency of parental exclusion using the kinds of simulations developed by Lewis & Snow (1992) rely on two assumptions: (1) the entire pool of parents is included in the analysis and (2) all markers are inherited as mendelian alleles. In field studies, the first assumption can be problematic unless the population is reproductively isolated from all other populations, and all prospective parents are sampled. The second assumption is violated to various degrees in this, and other, studies. For example, in the baboon *Papio cynocephalus* and humans, Riedy *et al.* (1992) found an average of 4.4 and 2.7 bands/primer, respectively, that were present in offspring, but absent in their parents. In the honeybee *Apis mellifera*, four of 432 markers were inherited as non-dominant 'heteroduplex' markers (Hunt & Page 1992). In another study of honeybees, Fondrk *et al.* (in press) could not discern how six of 20 markers were inherited; nevertheless, they could assign paternity and subfamily membership unambiguously. Finally, in *Neurospora crassa* four out of 88 RAPD markers were inherited as codominant, rather than dominant alleles (Williams *et al.* 1990).

In this study of *Hydractinia symbiolongicarpus*, we found a total of four amplification products (out of 156) present in at least several offspring within a sibship, but not amplified from their true parents. Even a low frequency of such novel bands could easily confound assignment of parentage by exclusion. However, the presence of novel bands, whatever their source, should have little effect on assignments based on inclusion. Indeed, only one out of 156 loci presented a pattern of band-sharing that was inconsistent with known parent-offspring relationships.

Cluster analysis circumvents many of the problems associated with exclusion, assuming numerous polymorphic loci can be assayed. Cluster analysis uses all of the information available on patterns of band sharing, rather than the all-or-nothing procedures of exclusion. In so doing, it correctly assigned all offspring to their true parents and sibships. For example, marker bands present in multiple offspring gave useful information on sibling affinities and parentage, even if that marker could not independently exclude a potential parent.

Our study suggests that provided large numbers of polymorphisms are examined (thereby minimizing the intrusion of mutation, non-mendelian inheritance, and PCR artifacts), RAPD markers offer unprecedented potential for the analysis of parent-offspring relationships in organisms with brief or multiple mating encounters. These

markers can also be used to establish such relationships at a very early stage in offspring development, before substantial selective mortality is likely to have occurred. At more inclusive levels of population structure, cluster analysis of RAPD polymorphisms promises to reveal patterns of relationship among individuals, families, and demes (also see Fondrk *et al.* in press), and thereby provide detailed insights into patterns of mating and the attributes of population structure that could promote or oppose the evolution of social behaviors (Michod 1980; Wade 1985; Wade & Breden 1987). From this perspective, RAPD markers could also furnish the detailed data necessary to unravel the selective processes that affect the evolution of conditional agonistic and cooperative behaviours in cnidarians, and a variety of other social organisms.

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This work represents the initial stages of an analysis of population structure and the determinants of gender-specific variation in reproductive success in the clonal hydrozoan *Hydractinia*. Rick Grosberg and Don Levitan share interests in the evolution of life-history traits, sexual allocation strategies, clonal demography, and self/nonsel self recognition systems. Don Levitan is currently a postdoctoral fellow in the Cnidarian Reproductive Analysis Project in Rick Grosberg's laboratory at the University of California, Davis.

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